Title: Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence

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Statement of Translational relevance:

Currently, there are no sensitive and specific clinical tests available to follow breast cancer patients after primary treatment. We developed a patient-specific method to analyze circulating tumor DNA that allows for monitoring of these patients regardless of molecular genotype. In this study, we analyzed 208 blood samples from 49 patients monitored longitudinally for up to 4 years after completion of adjuvant chemotherapy to determine whether personalized ctDNA assays can allow for more effective monitoring than current clinical tests such as CA15-3. Remarkably, for the patients that recurred, our test detected molecular relapse up to 2 years ahead of clinical relapse (median 8.9 months) with 89% sensitivity and 100% specificity. This may provide a critical window of opportunity for additional therapeutic intervention.

ABSTRACT

Purpose: Up to 30% of breast cancer patients relapse after primary treatment. There are no sensitive and reliable tests to monitor these patients and detect distant metastases before overt recurrence. Here we demonstrate the use of personalized ctDNA profiling for detection of recurrence in breast cancer.

Methods: Forty-nine primary breast cancer patients were recruited following surgery and adjuvant therapy. Plasma samples (n=208) were collected every 6 months for up to 4 years. Personalized assays targeting 16 variants selected from primary tumor whole exome data were tested in serial plasma for the presence of ctDNA by ultra-deep sequencing (average >100,000X).

Results: Plasma ctDNA was detected ahead of clinical or radiological relapse in 16 of the 18 relapsed patients (sensitivity of 89%); metastatic relapse was predicted with a lead time of up to 2 years (median=8.9 months; range: 0.5-24.0 months). None of the 31 non-relapsing patients were ctDNA-positive at any time point across 156 plasma samples (specificity of 100%). Of the two relapsed patients who were not detected in the study, the first had only a local recurrence, while the second patient had bone recurrence and had completed chemotherapy just 13 days prior to blood sampling.

Conclusions: This study demonstrates that patient-specific ctDNA analysis can be a sensitive and specific approach for disease surveillance for breast cancer patients. More importantly, earlier detection of up to two years provides a possible window for therapeutic intervention.

INTRODUCTION

Breast cancer is one of the most commonly diagnosed cancers worldwide and the second leading cause of cancer-related deaths in women (1). The current standard of care for women with primary (non-metastatic) breast cancer is surgery, often followed with adjuvant therapy to eliminate microscopic minimal residual disease (MRD) (2,3). Unfortunately, up to 30% of women who present with no evidence of disease following treatment with curative intent, eventually relapse and die of metastatic breast cancer (4). Current guidelines for disease surveillance recommend regular imaging and physical examinations, and additional testing upon presentation of symptoms (5-7). However, imaging tests such as mammography, MRI, and PET/CT often suffer from significant false-negative and false-positive results (8-10). Biochemical methodologies such as serum levels of cancer antigen 15-3 (CA 15-3) have limited sensitivity and accuracy in detecting micrometastases (11-13) and have not been widely incorporated into clinical guidelines (14). Therefore, there is a compelling need to develop more sensitive technologies capable of detecting of pre-clinical metastases and identifying patients with disease recurrence earlier.

Circulating tumor DNA (ctDNA) detectable in blood plasma has been shown to reflect the mutational signatures of the primary tumor and is emerging as a potential non-invasive biomarker for monitoring tumor progression across different cancer types. In breast cancer, previous studies have shown the potential utility of ctDNA to detect pre-clinical metastases and predict relapse following surgery and/or adjuvant therapy in patients with specific hotspot mutations (15,16) chromosomal rearrangement breakpoint junctions (17) and amplifications (18). However, up to 43% of breast cancer patients do not have hotspot mutations and thus cannot be monitored using a driver gene approach. Therefore, a more

comprehensive solution is needed for the surveillance of breast cancer patients after primary treatment (16,19,20).

We previously reported a personalized ctDNA profiling approach for early detection of lung cancer recurrence (21,22). In this study, we applied an improved version of the assay for tracking breast cancer recurrence following surgery and adjuvant therapy. The primary objective was to determine the 'lead interval' between detection of ctDNA in plasma and clinical detection of overt metastatic disease. A secondary objective was to determine whether ctDNA in plasma can detect recurrent disease earlier than current clinical methods, such as imaging or biochemical markers.

METHODS

Patients and samples

EBLIS is a multi-center, prospective cohort study, funded by Cancer Research UK and the National Institute for Health Research. Blood sample collection was conducted in accordance with the Declaration of Helsinki. All the patients provided written informed consent prior to entry into the trial. The trial protocol was approved by the Riverside Research Ethics Committee REC:13/LO/115; IRAS:126462. All research staff were blinded as to patient outcomes.

After removing nine patients who did not fulfill the trial entry criteria, a cohort of 188 patients were followed up with semi-annual blood sampling for cfDNA analysis, along with concomitant clinical examination, and biochemical measurements, including CA 15-3 **(Figure 1)**. Eligible patients were 18 years or older, displayed no clinical evidence of metastatic disease and were therefore, considered free of disease after surgery and adjuvant chemotherapy. All patients had completed adjuvant chemotherapy within 3 years of entering

the study and were considered high risk for breast cancer relapse (risk of mortality greater than 50% at 10 years without therapy, corresponding to a relapse rate of 65% at 10 years without treatment).

At the mid-point of the study (2 years) 50% of the predicted events (18 relapses, comprising 1 local recurrence and 17 patients with distant relapse) were observed. Following an interim analysis, the EBLIS Trial Management group recommended an interim laboratory assessment of serial plasma in the first 49 patients. The 49 patients included all 18 relapses, and comprised approximately 1:2 ratio of relapsed to non-relapsed patients. Relapsed and non-relapsed patients were sequential patients recruited over the same time frame. We performed whole exome analysis of the archived primary tumor for each patient for custom assay design. Serial plasma samples were analyzed in a blinded manner. Detailed description of blood sample collection, plasma cell free DNA isolation and quantitation, and whole exome sequencing (WES) are provided in supplementary methods.

Custom Assay design

Patient-specific somatic variants were identified by comparison of paired primary tumor and matched white blood cell DNA WES profiles for all 49 patients. Clonality of variants was inferred based on the estimated proportion of cancer cells harboring the variant as described in McGranahan et al. (23) Note that clonality inference from samples with low tumor cell fraction was limited due to a fairly flat distribution of variant allele frequency. The observed VAF in tumor DNA and sequence context of variants were used to prioritize somatic SNVs and short INDELs identified for each tumor. The bespoke amplicon design pipeline was used to generate PCR primer pairs for the given set of variants. For each patient, 16 highly ranked compatible amplicons were selected for the custom patient-specific panel. The PCR primers

were ordered from Integrated DNA Technologies. Details on steps of the workflow are provided in supplementary methods.

Statistical Analyses

This study was designed to measure the average time between detection of ctDNA and overt metastases. The target sample size for the EBLIS prospective cohort study was 194, using a 20% patient dropout rate and assuming that 20% of patients will relapse within two years. With such a sample size, the mean lead time interval was measured with a precision that extends to ±3 months, with a 95% probability.

All data are presented descriptively as means, medians or proportions. Relapse free survival from the day of surgery was determined using the Kaplan–Meier method. Cox proportional hazard regression was used to model the time to disease relapse. Sensitivity is defined as number of patients with pre-clinical metastasis detected by ctDNA over the total number of patients with clinical relapse. Specificity is defined as the number of patients that were ctDNA negative during the clinical follow up period over the total number of patients who did not relapse. All statistical analyses were performed using Stata, release 12.0 (Stata Corp., College Station, Texas) and survival plots were generated using R version 3.5.1 ("survminer" package version 0.4.2.99).(24,25)

RESULTS

Here, we report the analysis of the first 49 patients that entered into the EBLIS study (**Figure 1**). The cohort comprised 3 main subtypes: 34 patients with estrogen receptor positive, termed herein hormone receptor positive (HR+) and human epidermal growth factor receptor 2 negative (HER2-) tumors, eight patients with HR+ and HER2+ tumors, and seven patients with triple negative breast cancer (TNBC) **(Table 1, Supplementary Table S1)**. Ten patients

received neoadjuvant chemotherapy (NACT) prior to breast cancer resection while 39 received no systemic therapy prior to surgery. All except seven patients received adjuvant or neoadjuvant chemotherapy with an anthracycline/taxane regimen **(Table 1, Supplementary Table S2)**. Thirty-eight patients were receiving adjuvant endocrine therapy throughout the time of blood sampling **(Supplementary Table S2)**. Although repeat scans were not required prior to study entry, all except three patients had radiological imaging performed at diagnosis or at the time of entry into the study, and all were within normal limits **(Supplementary Table S2)**. At the reporting census date (June 30, 2018), 18 of the 49 patients had relapsed and 31 remained disease-free **(Figure 2A)**.

Clinical Validity of Circulating Tumor DNA Detection and Lead Interval

To assess the presence of ctDNA for each patient, assays specific to tumor signatures were designed targeting 16 SNVs and indels identified from the somatic mutation profile of the primary tumor **(Supplementary Figure S2, Supplementary Table S3a and S3b)**. For each patient, we then tested their personalized 16-plex assays across each of their respective plasma samples (range: 1-8 serial samples per patient). Plasma ctDNA was detected in 89% (16 of 18) of the patients who relapsed (**Figure 2**). By subtype, the sensitivity of the assay was 82%, 100%, and 100% in HR+/HER2-, HR+/HER2+ and TNBC, respectively (**Figure 2B**). Two relapsed patients were not detected by ctDNA analysis. The first patient (E009) had three separate foci of primary breast cancer. Exome analysis confirmed a clonal origin with 69 overlapping SNVs between the three tumors. The patient was also recruited at an earlier time point than other patients, with the baseline blood sample drawn just 13 days after the completion of chemotherapy. The 16 SNVs selected from clonal tumor SNVs were undetected in this plasma sample despite sequencing 64ng total cfDNA at >80,000X. Just 4 months after the patient was recruited, a bone scan showed bony metastases in the sternum,

pelvis and vertebrae. The other patient (E010) had a small resectable local recurrence in the sternum **(Supplementary Table S1).** The assay achieved 100% specificity, as ctDNA was not detected in any of the 156 plasma samples collected from the 31 patients who did not relapse (**Figure 2**).

All ctDNA-positive patients relapsed within 50 months after surgery and molecular relapse through ctDNA analysis was detected up to 2 years prior to clinical relapse with a median of 266 days (range: 14-721 days) or 8.9 months (**Figure 2B**). When stratified by subtype, the median lead times for HR+/HER2-, HR+/HER2+ and TNBC were 301, 164, and 258 days, respectively **(Figure 2B-C)**. The presence of ctDNA was significantly associated with poorer prognosis and this is demonstrated by detection of ctDNA in the first post-surgical plasma sample [HR=11.8 (95% CI 4.3-32.5] and in the follow-up plasma samples post-surgery [HR=35.8 (95% CI 8.0-161.3)] **(Figure 3)**.

Detection of driver mutations

Since our method relies upon evolutionary early clonal mutations, we wanted to compare these results to canonical driver genes. Of the 49 patients, 33 (67%) harbored one or more driver genes in the tumor WES, as defined by the genome interpreter (variants identified by WES of tumor DNA were submitted to the cancer genome interpreter (https://www.cancergenomeinterpreter.org/home). For the 33 patients, a second assay pool was designed to track as many driver variants as possible. While results showed concordance between the observed VAF in plasma for patient-specific assays and the driver mutations (**Supplementary Figure S3, Supplementary Table S3c)**, 17% (4/23) ctDNA-positive plasma samples were negative for driver mutations present in the respective tumor exomes. As an example, mutations in *CDH1* and *ERBB3* genes were undetected in a ctDNA-positive

sample E026 time-point 2. The average ctDNA level for this sample was measured at 0.093% VAF by our personalized assay.

Circulating Tumor DNA and other monitoring tests

Concurrent with the plasma ctDNA analyses, additional monitoring tests were also performed, including CT imaging, LFTs (liver function tests) and CA15-3. All CT imaging that was performed prior to clinical relapse was negative **(Supplementary Table S2)**. Interestingly, for seven patients who had scans within 4 months of their first ctDNA positive time point, all scans were negative at this point. Similarly, other tests, such as LFTs were also negative until clinical relapse.

CA 15-3 levels were monitored in 43 of the 49 patients. Of the 18 patients who relapsed, 13 had CA 15-3 measurements; 7 had normal CA 15-3 levels, 6 (30%) had positive CA 15-3 values, but only 2 patients had progressively rising CA 15-3 levels. Six patients (3 relapsed and 3 non-relapsed) had an occasional blood sample with slightly elevated CA15-3, but the levels fluctuated and did not reflect disease progression, whereas all 31 patients who did not relapse were ctDNA negative in 100% of 156 plasma samples. Interestingly, even for the patients that were positive for CA 15-3, ctDNA was detected on average over 200 days ahead of significant CA 15-3 levels **(Supplementary Table S2, Figure 4A, Supplementary Figure S4C)**.

In addition to presence or absence of ctDNA, the levels of ctDNA can also be used to approximate and track disease burden over time as demonstrated by the ctDNA plots for each patient (**Figure 4, Supplementary Figure S4**). In figure 4, we illustrate five patients, representing three cancer subtypes: HR+/HER2- (E017 and E026), HER2+ (E040) and

TNBC (E029 and E033). Three patients were initially ctDNA-negative and became positive over time and subsequently had a rise in the ctDNA ahead of clinical relapse **(Figure 4A-C)**. The other two patients were ctDNA positive at the earliest monitored time point and levels increased corresponding to an expected increased tumor burden **(Figure 4D-E)**. In all cases, ctDNA was detected ahead of clinical relapse **(Figure 4, Supplementary Figure S4)**.

Overall, disease progression can be monitored by both the variant allele frequency (VAF) and the number of detected variants **(Figure 4F)**. The median VAF increased from 0.09% in the first positive time point (range: 0.01–9.22%) to 3.88% (range: 0.05–64.44%) at the last time point, and the median number of variants detected at the first time point was 5 (range: 2–12) compared to 12 variants (range: 5–15) at the last time point. The low number of variants detected at early time points, each present at very low copy numbers **(Supplementary Table S3a)** point to the importance of testing multiple mutations present in a patient's tumor to maximize the probability of early detection.

The individual patient graphs also show the low limit of detection of the assay. For example, patients E017, E029, E033 and E040 had VAFs detected in the range of 0.01 to 0.02% (**Figure 4B-E, Supplementary Figure S4**). The lowest VAF of 0.01% corresponds to the detection of a single mutant molecule in the plasma sample **(Supplementary Figures S5 and S6)**. The average number of mutant molecules per ml of plasma was measured to be less than two in thirteen ctDNA-positive samples **(Supplementary Table S3a)**. A low limit of detection with high specificity was achieved by requiring two or more of the sixteen variants to be measured above the selected confidence threshold **(Supplementary Methods)**, underscored by the fact that all 156 plasma samples from the 31 non-recurring patients were negative.

DISCUSSION

Here, we show non-invasive detection of pre-clinical metastases and earlier identification of breast cancer recurrence across breast cancer subtypes through personalized ctDNA analysis. The approach uses tumor exome data to design patient-specific 16-plex assays and ultra-deep sequencing of plasma cfDNA at extremely high depth (average of >100,000 reads per target) to achieve a detection sensitivity down to a single mutant molecule.

In this study, we detected ctDNA in 16 of 17 patients with a lead time of up to 2 years (range:0.5–23.7 months) prior to distant metastatic relapse, demonstrating the ability of the assay to predict breast cancer recurrence earlier than imaging, CA 15-3, clinical examination, and liver function tests. Previously, Olsson and colleagues profiled patient-specific chromosomal rearrangement breakpoint junctions in 20 patients and demonstrated accurate discrimination between patients with and without clinical detection of recurrence (17). In 12 of 14 patients, molecular relapse through ctDNA preceded clinical detection of occult metastasis with an average lead time of 11 months (range: 0–37 months). Similarly, Garcia-Murillas followed patient-specific point mutations using digital droplet PCR, and in 12 of 15 patients ctDNA was detected ahead of metastatic relapse with a median lead time of 7.9 months (range: 0.03–13.6 months) (16). Our data therefore provide further demonstration that ctDNA can be detected in most breast cancer patients several months before clinical relapse and with excellent specificity. This presents a window of opportunity for the introduction of non-cross-resistant therapies to prevent overt metastatic relapse. Importantly, once detected, ctDNA remained positive throughout all subsequent monitoring timepoints. In addition to ctDNA detection, circulating tumor cells (CTCs) have also shown prognostic significance for detection of pre-clinical metastases in patients with breast cancer (26-29). Both ctDNA and CTCs could therefore complement conventional recurrence monitoring tests

that have limited specificity and frequently result in considerable anxiety and expensive follow-up testing.

Using an upfront exome profiling of tumor tissue followed by a personalized targeted multiplex plasma sequencing has distinct advantages. First, this strategy enables detection of ctDNA in all patients regardless of molecular subtype, irrespective of known molecular driver genes. In our study, 100% of all eligible patients were monitored. Previous studies have profiled a small number of genes (i.e. 2–14) to determine the personalized variants for tracking; however, only 57–78% of women have any trackable mutations using such an approach (16,19,20). In our cohort, only 33 of 49 (67%) patients had one or more driver mutations identified in their exome profile, indicating that 16 of 49 patients in this study would not have been able to be monitored using a pre-determined gene panel approach based on driver genes.

Second, the outlined approach enables simultaneous interrogation of 16 somatic variants, whereas technologies such as droplet digital PCR (ddPCR) or BEAMing are usually designed to monitor either one target or a handful of targets (30-33). Increasing evidence suggests that the evolution of cancer is complex, and often results in high levels of inter- and intra-tumor heterogeneity (34). Therefore, measuring 16 tumor-specific variants provides a more robust representation of the tumor, enabling more accurate tracking of disease burden.

Third, sequencing a highly targeted panel at a high depth of read (>100,000X) allows for a very low limit of detection at <0.01%. Larger gene panels used for this purpose rarely reach this depth or limit of detection due to the high cost. However, this low level of detection is critical for assessing pre-clinical metastases as we have shown that variants can be within the

range of 0.01–0.02% VAF. By using a large volume of plasma (up to 5 ml), we were able to achieve a high assay sensitivity and detect down to 1 ctDNA molecule in 5ml plasma with a lead time of up to 2 years prior to distant metastatic relapse.

As with all technologies, there are limitations to the outlined approach. The test is not suitable for detecting a second primary breast cancer unless it recurred from the original tumor; this is exemplified by patient E022, where a second contralateral primary cancer was detected (**Supplementary Table S1**). Second, relying on ctDNA requires that sufficient molecules are present in the plasma at the time of collection, which may not be the case in patients with smaller and less aggressive breast cancers. This is exemplified by patient E010 who relapsed with local resectable disease but was ctDNA negative.

There are some important implications for the future of breast cancer treatment and drug evaluation from our study. In the past, systemic treatment with targeted or cytotoxic therapies have been shown to be curative only when administered in the adjuvant setting; treatment of overt metastatic disease is rarely, if ever, curative (35). The approach described here offers an alternative - that of attempting to salvage patients who are ctDNA-positive with second-line therapies. In conclusion, we present a sensitive and specific clinical test that can be used to identify pre-clinical metastases and follow all breast cancer patients after therapy irrespective of molecular subtype. It out-performs conventional means of monitoring and shows promise as a tool for guiding future precision medicine. Future studies will address the issue of the effects of therapy on ctDNA levels in breast cancer patients.

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Figure Legends

Figure 1: Patient recruitment and collection of clinical samples. For the 49 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 panels were designed to include 16 somatic mutations identified from whole exome sequencing data. Serial plasma samples were analyzed with the corresponding custom 16-plex assay panels using the Signatera^{TM} RUO workflow in a blinded manner. A total of 208 samples were analyzed for ctDNA detection.

Figure 2: Personalized, 16-plex assays accurately detect ctDNA ahead of clinical relapse. A. shows a summary of each patient's (n=49) treatment regimen along with results of serial plasma samples (n=208) analyzed. B. shows a summary table of patients by breast cancer subtype, number of relapsed patients, percentage detected by ctDNA analysis, and the median lead time in days. PPV, Positive Predictive Value [True Positive/(True Positive+False Positive)]; NPV, Negative Predictive Value [True Negative/(False Negative+True Negative)]. C) shows a comparison of molecular and clinical relapse by breast cancer subtype, HR+/HER2- (dark blue), HER2+ (green), TNBC (orange) using the paired Wilcoxon signed-rank test ($p<0.001$).

Figure 3: Personalized ctDNA detection in serial plasma samples predicts recurrence-free survival. A. Relapse-free survival according to the detection of ctDNA in the first post-surgical plasma sample [HR: 11.78 (4.28–32.46), p-value <0.001]. B. Relapse-free survival according to the detection of ctDNA in any follow-up plasma sample post-surgery [HR:35.84 (7.9 –161.32), p-value <0.001]. Data are from n = 49 patients.

Figure 4: Personalized profiling detects rising ctDNA ahead of clinical relapse. A-E. Plasma levels of ctDNA across serial plasma time points for five breast cancer patients (one per panel). Mean VAFs are denoted by dark blue circle and solid lines represent the average VAF profile over time. The lead time is calculated as the time interval between clinical relapse (red triangle) and molecular relapse (blue triangle). CA 15-3 levels are graphed over time (teal circle) and the baseline levels (32 U/ml) are marked in light blue. F. Summary of percent variant allele frequency (VAF) and number of targets detected at molecular and clinical relapse for all ctDNA positive samples. Data are from 13 relapsed patients, excluding 3 patients with only one plasma time point.

Tables

Table 1. Characteristics of the Patients at Baseline

Characteristic	N=49
Median age at diagnosis (range), year	57 (38-81)
Estrogen Receptor status, N (%)	
Positive	37 (76)
Negative	12 (24)
Progesterone Receptor status, N (%)	
Positive	32 (65)
Negative	17 (35)
HER2 status, N (%)‡	
Positive	8 (16)
Negative	41 (84)
Tumour Stage, N (%)	
IA	1 (2)
IIA	1 (2)
IIB	14 (29)
IIIA	17 (35)
IIIB	2 (4)
IIIC	14 (28)
Size of Tumor, Mean (Range), cm	3.8 (0.9-10)
Treatment, N (%)	
NACT	10 (20)
ACT	32 (65)
None	7 (14)

After screening and recruitment patients were followed up with 6 monthly blood samples. ‡HER2 status was determined by immunohistochemical and fluorescence in situ hybridization assays. A patient was considered to have HER2-positive cancer if either assay was positive. NACT; neoadjuvant chemotherapy, ACT; adjuvant chemotherapy.

Figure 1

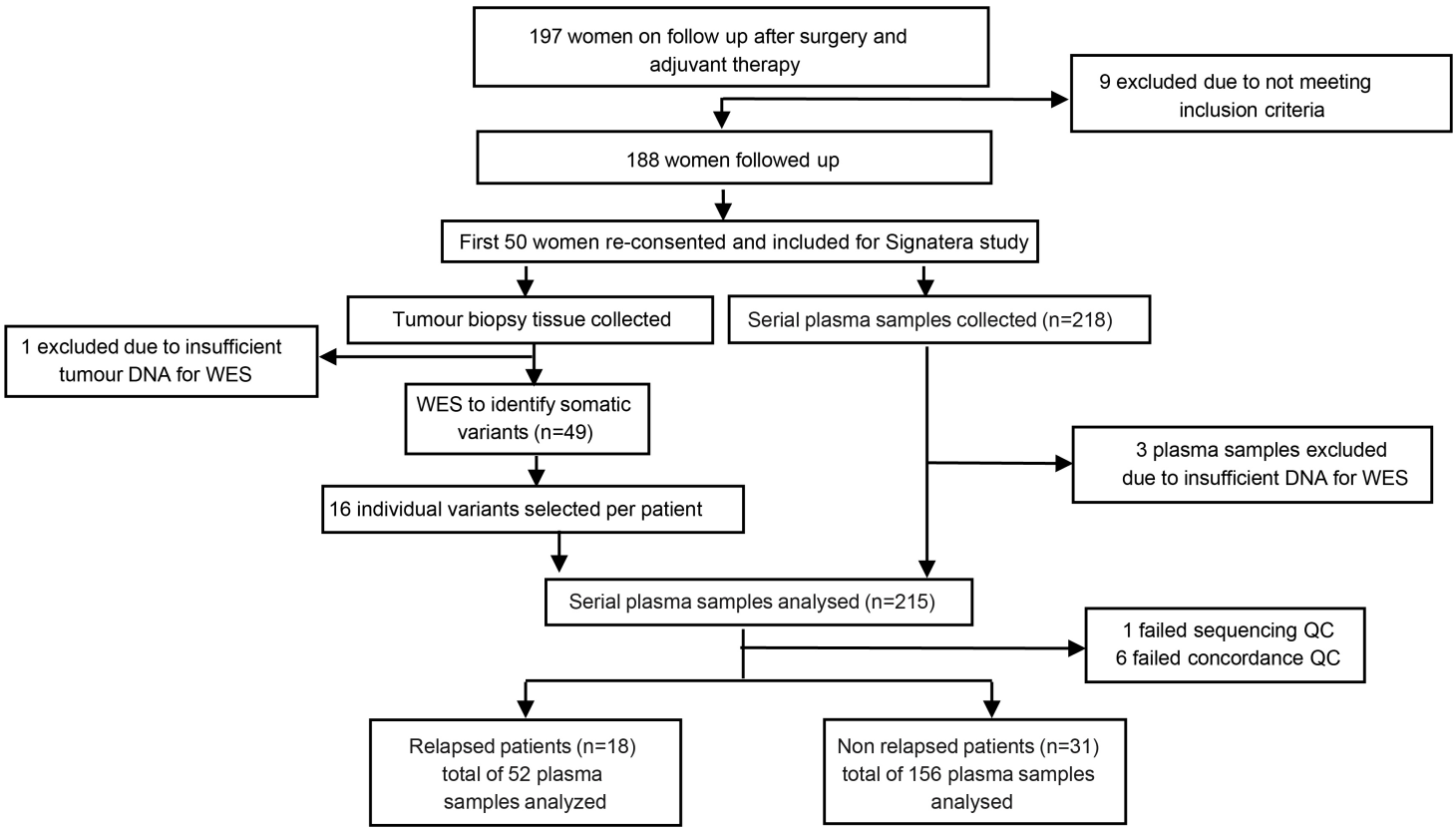
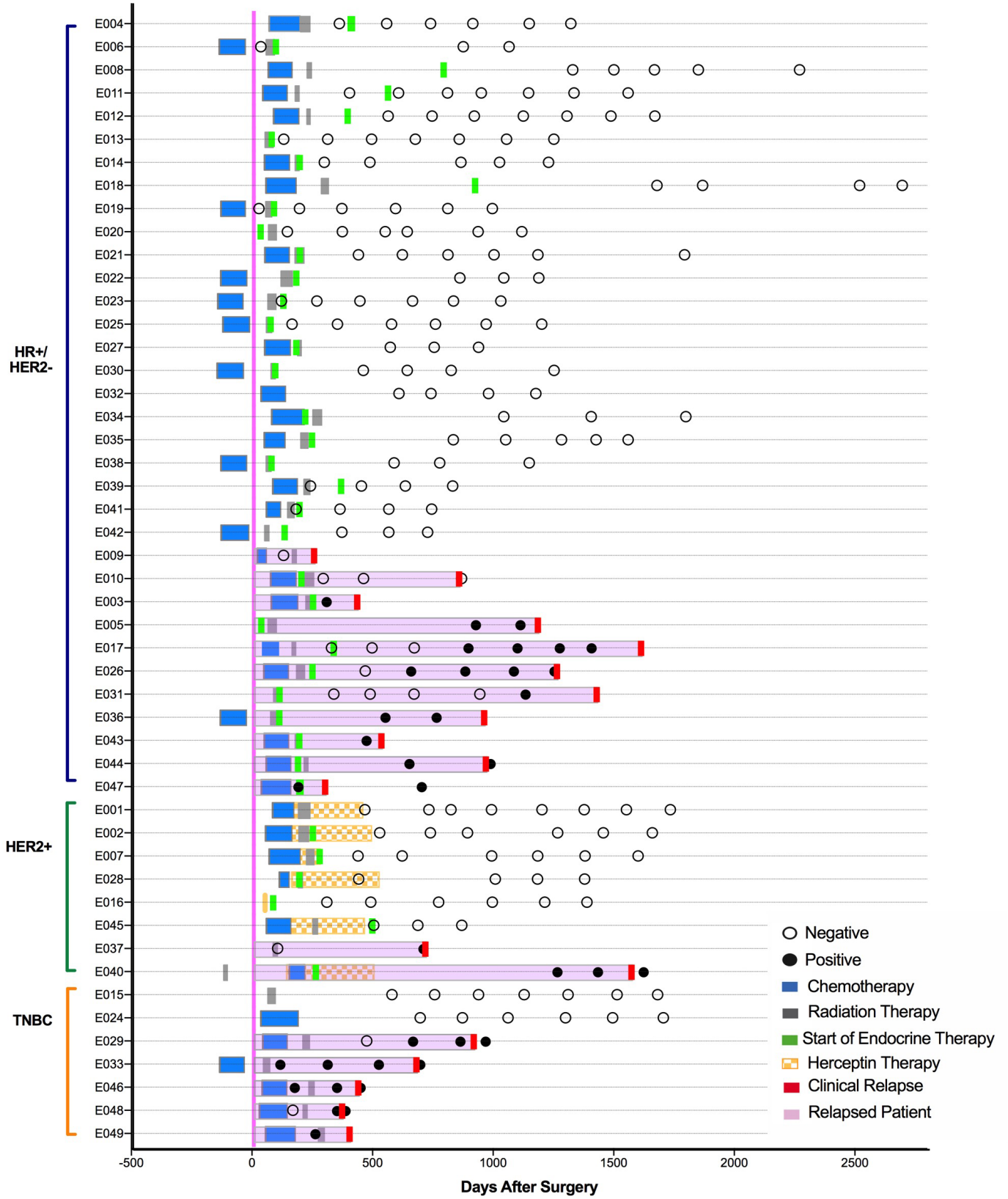


Figure 2



Breast Cancer Subtypes	Total Patients	Relapses	% Detected	PPV	NPV	Median Lead Time (Days)
HR+/HER2-	34	11	82%	100%	92%	301
HER2+	8	2	100%	100%	100%	164
TNBC	7	5	100%	100%	100%	258
TOTAL	49	18	89%	100%	94%	285

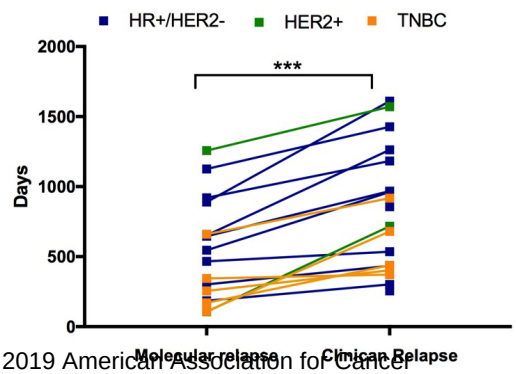
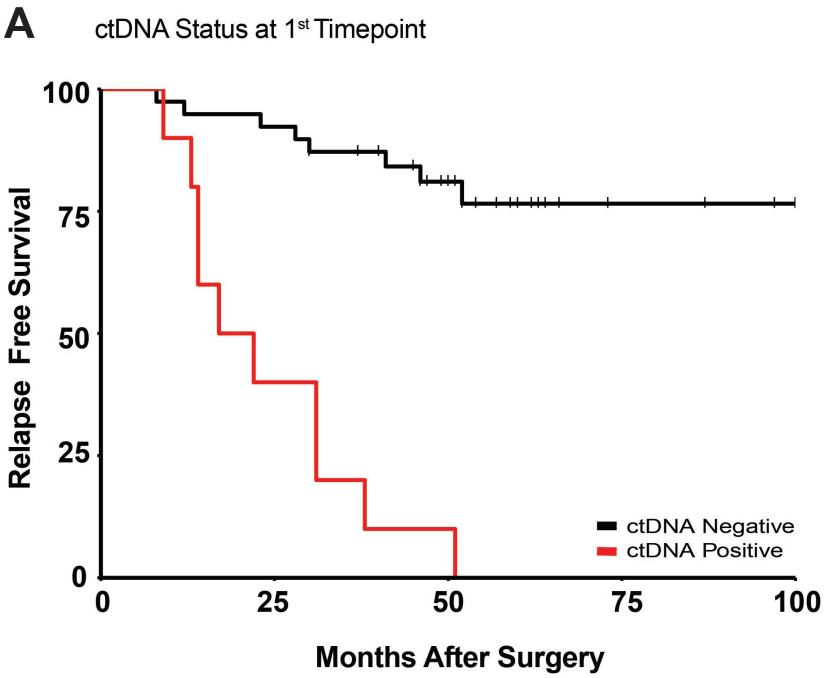


Figure 3



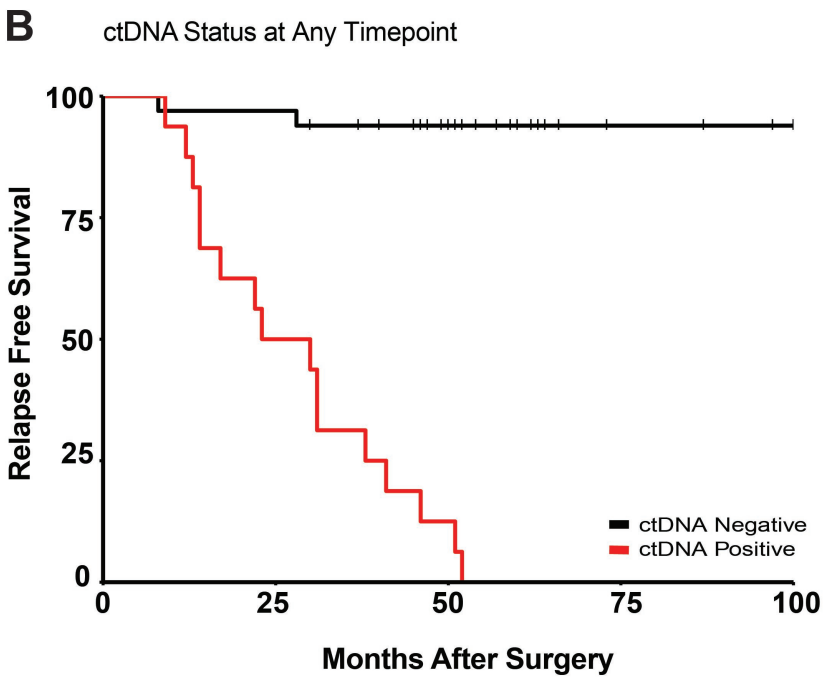
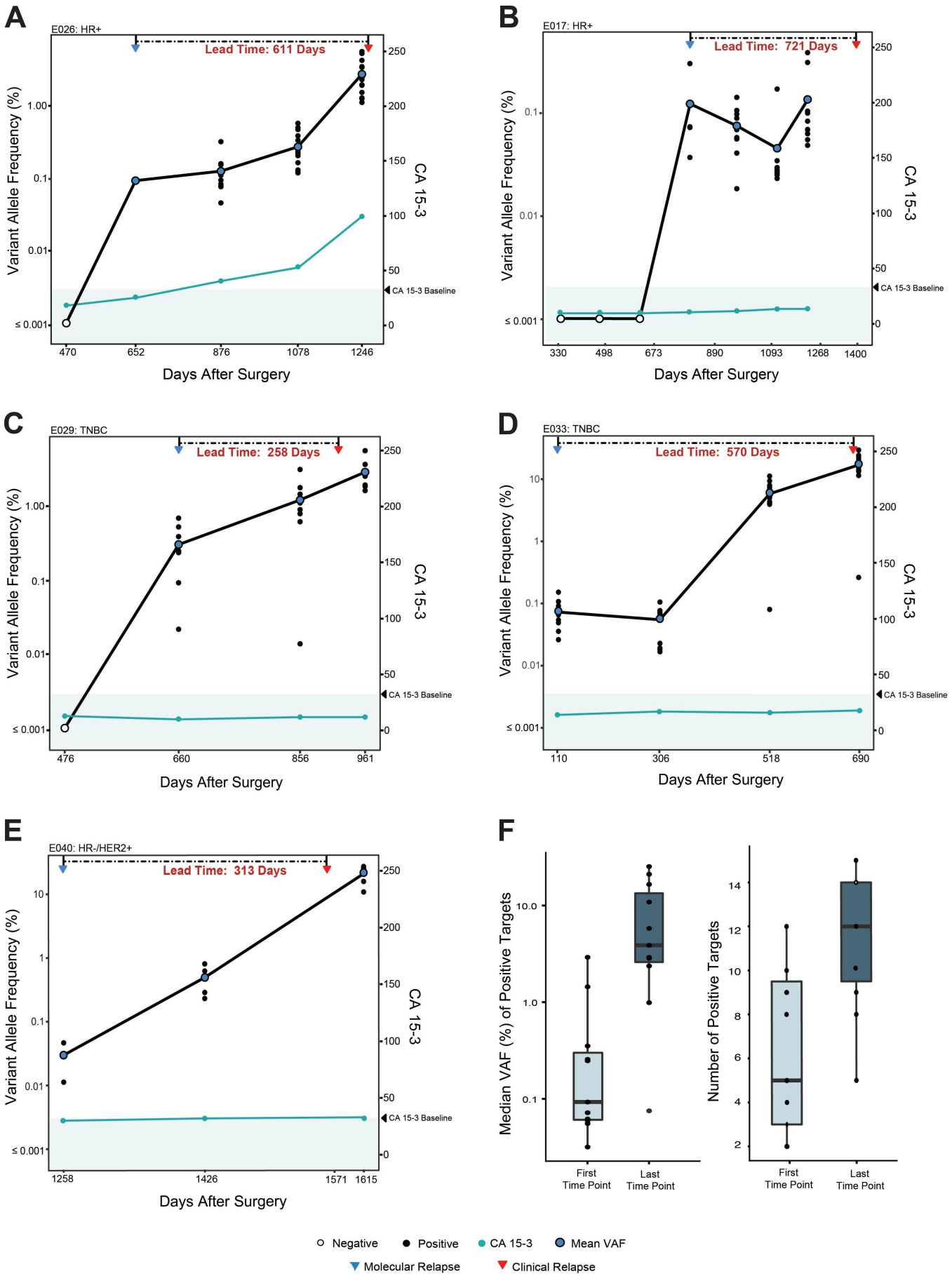


Figure 4



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