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ORIGINAL ARTICLE

Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival

M. J. M. Magbanua[1*], L. B. Swigart[1], H.-T. Wu[2], G. L. Hirst[3], C. Yau[3], D. M. Wolf[1], A. Tin[2], R. Salari[2], S. Shchegrova[2], H. Pawar[2], A. L. Delson[4], A. DeMichele[5], M. C. Liu[6], A. J. Chien[7], D. Tripathy[8], S. Asare[9], C.-H. J. Lin[2], P. Billings[2], A. Aleshin[2], H. Sethi[2], M. Louie[2], B. Zimmermann[2], L. J. Esserman[3] & L. J. van 't Veer[1*]

[1]Department of Laboratory Medicine, University of California San Francisco, San Francisco; [2]Natera Inc, San Carlos; [3]Department of Surgery, University of California San Francisco, San Francisco; [4]Breast Science Advocacy Core, Breast Oncology Program, University of California San Francisco, San Francisco; [5]University of Pennsylvania, Philadelphia; [6]Mayo Clinic, Rochester, Minnesota; [7]Division of Hematology/Oncology, University of California San Francisco, San Francisco; [8]Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston; [9]Quantum Leap Health Care Collaborative, San Francisco, USA

Available online 21 November 2020

Background: Pathologic complete response (pCR) to neoadjuvant chemotherapy (NAC) is strongly associated with favorable outcome. We examined the utility of serial circulating tumor DNA (ctDNA) testing for predicting pCR and risk of metastatic recurrence.

Patients and methods: Cell-free DNA (cfDNA) was isolated from 291 plasma samples of 84 high-risk early breast cancer patients treated in the neoadjuvant I-SPY 2 TRIAL with standard NAC alone or combined with MK-2206 (AKT inhibitor) treatment. Blood was collected at pretreatment (T0), 3 weeks after initiation of paclitaxel (T1), between paclitaxel and anthracycline regimens (T2), or prior to surgery (T3). A personalized ctDNA test was designed to detect up to 16 patient-specific mutations (from whole-exome sequencing of pretreatment tumor) in cfDNA by ultra-deep sequencing. The median follow-up time for survival analysis was 4.8 years.

Results: At T0, 61 of 84 (73%) patients were ctDNA positive, which decreased over time (T1: 35%; T2: 14%; and T3: 9%). Patients who remained ctDNA positive at T1 were significantly more likely to have residual disease after NAC (83% non-pCR) compared with those who cleared ctDNA (52% non-pCR; odds ratio 4.33, $P = 0.012$). After NAC, all patients who achieved pCR were ctDNA negative ($n = 17$, 100%). For those who did not achieve pCR ($n = 43$), ctDNA-positive patients (14%) had a significantly increased risk of metastatic recurrence [hazard ratio (HR) 10.4; 95% confidence interval (CI) 2.3-46.6]; interestingly, patients who did not achieve pCR but were ctDNA negative (86%) had excellent outcome, similar to those who achieved pCR (HR 1.4; 95% CI 0.15-13.5).

Conclusions: Lack of ctDNA clearance was a significant predictor of poor response and metastatic recurrence, while clearance was associated with improved survival even in patients who did not achieve pCR. Personalized monitoring of ctDNA during NAC of high-risk early breast cancer may aid in real-time assessment of treatment response and help fine-tune pCR as a surrogate endpoint of survival.

Key words: breast cancer, circulating tumor DNA, neoadjuvant chemotherapy, pathologic complete response

INTRODUCTION

Circulating tumor ctDNA (ctDNA) in blood offers a minimally invasive approach for disease monitoring and evaluation of response to therapy.[1-3] Findings from recent clinical studies have shown that ctDNA may play a role in detecting minimal residual disease and emerging therapy resistance, that is, molecular relapse in early stage breast cancers,[4-7] as well as in monitoring of disease progression in patients with advanced breast cancer.[8-10] However, it is not yet known if failure to clear ctDNA during therapy could provide guidance for escalation of treatment to prevent early disease recurrence.[11]

Neoadjuvant chemotherapy (NAC) has become a standard-of-care for patients with locally advanced breast cancer.[12] First, NAC provides a unique opportunity for real-time monitoring of tumor response and evaluation of drug efficacy.[13-15] Second, NAC may downstage tumors and thus improve chances of breast-conserving surgery.[12,16,17] Third,

*Correspondence to: Dr Mark Jesus M. Magbanua, Department of Laboratory Medicine, 2340 Sutter Street, San Francisco, CA 94115, USA. Tel: +1-415-514-3969

E-mail: mark.magbanua@ucsf.edu (M.J.M. Magbanua).

*Prof. Laura van 't Veer, Department of Laboratory Medicine, 2340 Sutter Street, San Francisco, CA 94115, USA. Tel: +1-415-476-6023

E-mail: laura.vantveer@ucsf.edu (L.J. van 't Veer).

response to NAC provides prognostic information which can supplement those derived from standard clinicopathologic characteristics of the primary tumor, such as subtype, nodal status, and grade.[12,16-20]

Pooled analysis by Cortazar and colleagues[21] has shown that patients who achieved a pathologic complete response (pCR, or the absence of residual cancer in the breast and lymph nodes after NAC) have significant survival advantage over those who did not. Standard NAC alone or in combination with other agents has resulted in pCR for 10%-50% of patients depending on subtype.[21] Data from the I-SPY 2 TRIAL, a multicenter phase II trial that evaluates investigational drugs in combination with standard NAC (paclitaxel followed by anthracycline treatment),[22] have shown that pCR in women with molecularly high-risk stage II or III tumors, whether from standard or targeted therapies, unequivocally conferred a survival advantage [hazard ratio (HR) of 0.2].[23]

While pCR accurately identifies patients with low risk of relapse, studies have shown that predicting early metastatic recurrence in those with residual disease (non-pCR) is less robust.[21,23] For example, survival analysis in the I-SPY 2 TRIAL (median follow-up of 3.8 years) showed that the 3-year distant disease-free survival (DRFS) of patients who achieved pCR was 95%.[23] Among non-pCR patients, 22% of experienced metastatic recurrence. In this study, we evaluated the potential role of ctDNA as a biomarker for monitoring of response to NAC and assessed the additive value of ctDNA to further stratify patients with residual disease to predict early metastatic recurrence. We hypothesized that early changes in ctDNA are predictive of response to NAC and that ctDNA dynamics during NAC as well as ctDNA status (positive versus negative) at each time point are associated with patient outcomes. To address these hypotheses, we performed a correlative study in the I-SPY 2 TRIAL to detect ctDNA in serial plasma samples collected before, during, and after NAC.[24] We used a previously analytically validated personalized ctDNA test composed of a panel of up to 16 most clonal somatic variants present in the pretreatment tumor.[10,25-27] The test involves multiplex polymerase chain reaction amplification followed by ultra-deep sequencing to detect tumor-specific mutations (i.e. ctDNA) in cell-free DNA (cfDNA). This approach enables more accurate monitoring of disease burden than prefixed driver mutation panels, as each test reflects tumor heterogeneity at the individual patient level.[5,8,28]

PATIENTS AND METHODS

Patients

We performed a retrospective ancillary ctDNA study on prospectively collected samples from high-risk early breast cancer patients enrolled in the multicenter neoadjuvant I-SPY 2 TRIAL (NCT01042379). Women with $\geq$2.5-cm stage II/III breast cancer were eligible. Patients were screened for metastatic disease by imaging (computed tomography or positron emission tomography) prior to enrollment, and those with *de novo* metastatic disease were excluded. Restaging scans were not performed after NAC prior to surgery. Eligibility was limited to patients with a Mamma Print high score, and thus the trial was enriched for those with increased risk of metastatic recurrence within 5 years after diagnosis. Patients received standard NAC combined with MK-2206 (AKT inhibitor) or standard NAC alone. Detailed descriptions of the design, eligibility, and study assessments in the I-SPY 2 TRIAL have been reported previously.[22,29] Institutional Review Boards at all participating institutions approved the protocol. All patients signed informed consent to allow research on their biospecimen samples.

ctDNA analysis

Detailed description of the clinical samples and the methods for ctDNA analysis[26,27,30] (supplementary Figures S1-S3 and supplementary Tables S1-S3, available at https://doi.org/10.1016/j.annonc.2020.11.007) are found in the supplementary Methods, available at https://doi.org/10.1016/j.annonc.2020.11.007.

Statistical analysis

To determine the cutoff for ctDNA positivity, a large set of negative control samples ($\sim$1000) was preprocessed to build a background error model. For each target variant identified in the plasma, a confidence score was calculated based on the depth of read for mutant and reference alleles.[25] In addition, simulation studies were performed as previously described[10,26,27] to determine limits of detection and quality control thresholds for stringent assessment of ctDNA results (see supplementary Methods, available at https://doi.org/10.1016/j.annonc.2020.11.007 and supplementary Figures S4 and S5, available at https://doi.org/10.1016/j.annonc.2020.11.007). A plasma sample with at least two variants with a confidence score above a predefined threshold (0.97) was defined as ctDNA positive.

Logistic regression was used to assess association between pCR and ctDNA clearance. Survival curves were generated by Kaplan—Meier analysis and compared using log-rank test. Cox regression analysis was used to estimate HR and 95% confidence interval (CI). Survival data were available for 75 of the 84 patients. Detailed description of the study design and the statistical methods can be found in the supplementary Methods, available at https://doi.org/10.1016/j.annonc.2020.11.007.

RESULTS

ctDNA analysis in I-SPY 2 TRIAL patients

This ctDNA study was performed retrospectively on samples collected from I-SPY 2 TRIAL patients who received standard NAC alone or combined with MK-2206 (AKT inhibitor) treatment (Figure 1A). Primary tumor and matched normal samples for whole-exome sequencing were available for 90 patients (Figure 1B and C and supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2020.11.007).

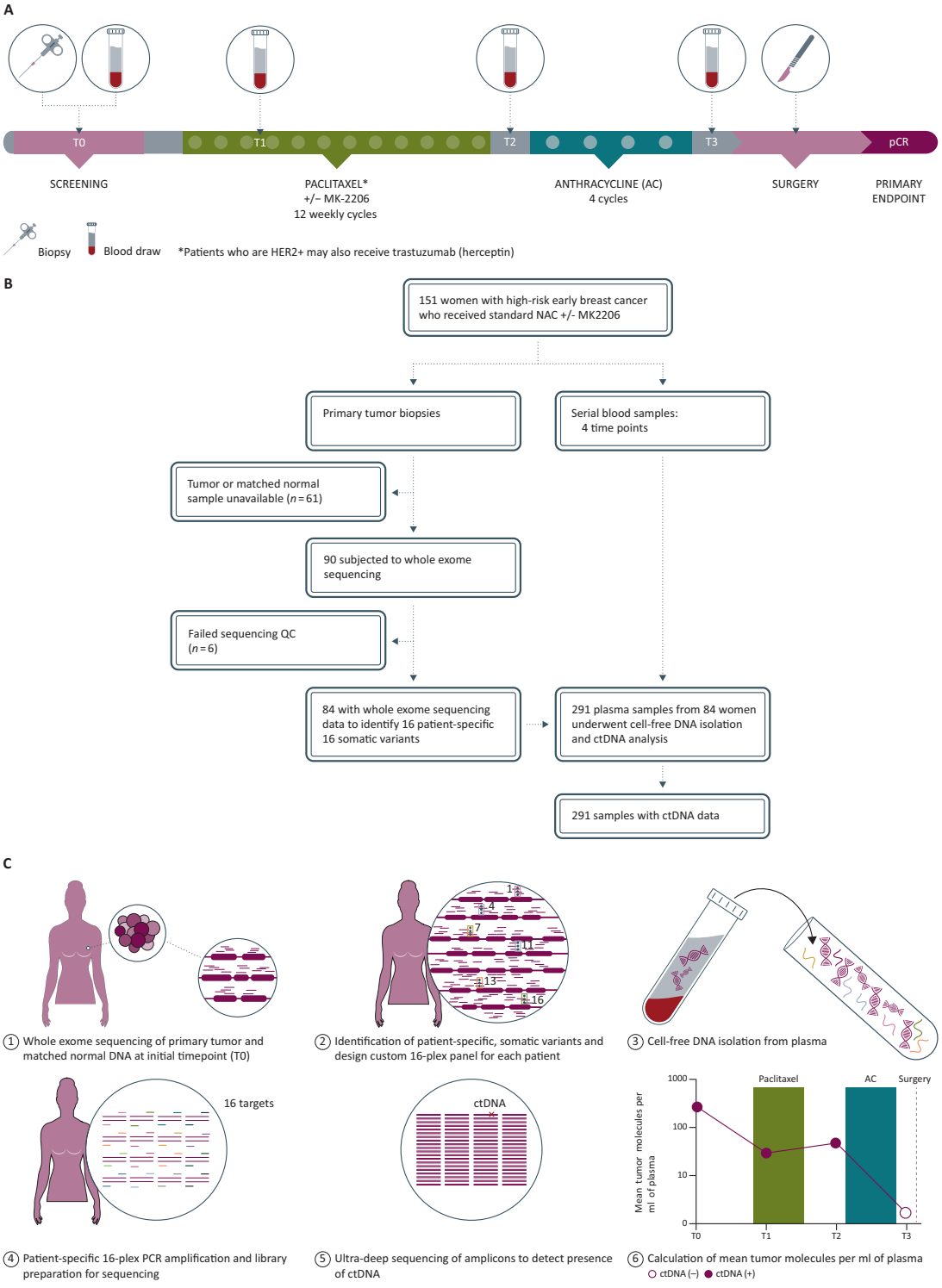

Figure 1. **Study schema, methods for circulating tumor DNA (ctDNA) analysis, patients, and samples.**

https://doi.org/10.1016/j.annonc.2020.11.007

Of these, six were excluded due to poor-quality sequencing data, resulting in an analytic cohort of 84 patients (supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.11.007). Whole-exome sequencing detected a mean of 181 mutations in the 84 untreated primary tumor tissue analyzed (median 159; range 32-772; supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2020.11.007). cfDNA was isolated from plasma samples collected from pretreatment (T0), 3 weeks after initiation of treatment (T1), between paclitaxel and anthracycline regimens (T2), and after NAC prior to surgery (T3) (Figure 1A and supplementary Figure S2, available at https://doi.org/10.1016/j.annonc.2020.11.007). From the list of variants derived from whole-exome sequencing, a unique personalized panel consisting of up to 16 highly ranked somatic mutations were selected (median 16; range 12-16). Multiplex polymerase chain reaction assays were designed and used to interrogate cfDNA for the presence of these mutations (Figure 1C, supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2020.11.007, and supplementary Methods, available at https://doi.org/10.1016/j.annonc.2020.11.007). Amplicons were subjected to ultra-deep sequencing to detect ctDNA (supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2020.11.007). ctDNA analysis was successfully performed on 291 (87%) of the potential 336 total plasma samples (84 patients $\times$ 4 time points). Samples with at least two detectable somatic variants were considered ctDNA positive (supplementary Figures S4 and S5, available at https://doi.org/10.1016/j.annonc.2020.11.007).[10,25-27]

Of the 84 patients, 35% were hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2−), 23% HER2+, and 43% triple-negative breast cancers (TNBCs); 30% had T3 or T4 tumors; 53% were node negative and 61% were considered to be MammaPrint High 2 (ultra-high risk; supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.11.007). There were no significant differences in the clinicopathologic characteristics between patients who were excluded ($n = 67$) and those who were included in the study ($n = 84$).

Baseline ctDNA is associated with tumor burden and aggressive phenotype

At pretreatment (T0), 73% of the patients had detectable ctDNA (Figure 2A). ctDNA detection rates in patients who received standard NAC ($n = 27$, 73%) were similar to those who received additional MK-2206 ($n = 57$, 72%; Figure 2A and supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.11.007). The proportion of ctDNA-positive samples was significantly higher among HER2+ (84%) and TNBC (86%) subtypes as compared with the HR+/HER2− (48%) subtype ($P < 0.01$; supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2020.11.007 and Figure 2A and B). ctDNA positivity was also associated with larger tumors (T3/T4, 91%, $P = 0.014$) but not with nodal status at the time of diagnosis. A significantly higher proportion of MammaPrint High 2 patients were ctDNA positive (86%) compared with 52% in MammaPrint High 1 ($P < 0.01$).

We also evaluated the absolute ctDNA levels (i.e. mean tumor molecules per ml of plasma) in the different groups stratified according to these same clinical variables and observed the same trend. The mean tumor molecules per ml in TNBC patients was significantly higher compared with that of HR+/HER2− patients (Figure 2C). Significantly higher levels of ctDNA were also observed for clinical T-stage T3/T4 versus T1/T2 and MammaPrint high 2 versus high 1.

ctDNA positivity decreases with distinct dynamics during NAC

In the population as a whole, ctDNA positivity decreased during the course of NAC, from 73% before treatment (T0), to 35% at 3 weeks (T1), to 14% at the inter-regimen time point (T2), and down to 9% after NAC (T3) (Figure 3A). Similarly, the absolute ctDNA levels decreased over time (Figure 3B). Although, on average the ctDNA positivity decreased with time, at the individual patient level, five main patterns were observed. Figure 3C shows ctDNA positivity as a function of time during treatment for 58 of the 84 patients who had complete serial data available at all four time points: Patients with undetectable ctDNA at T0 who remained undetectable at T3 ($n = 20$, 34.5%); patients who, respectively, cleared at T1 ($n = 20$, 34.5%), at T2 ($n = 9$, 15.5%), or at T3 ($n = 4$, 6.9%); or patients who remained ctDNA positive after NAC (T3) ($n = 5$, 8.6%).

Clearance dynamics of ctDNA is associated with NAC response

We evaluated ctDNA clearance as a predictor of response to NAC. The rates for pCR across subtypes were 13.8%, 47.4%, and 27.8% for HR+/HER2−, HER2+, and TNBC, respectively. As much as 56 patients who were ctDNA positive at T0 had a corresponding T1 plasma measurement (Figure 4A), and of these, 29 (52%) remained ctDNA positive at T1, 3 weeks after the initiation of treatment. As much as 83% of patients who did not clear their ctDNA at T1 had residual disease at surgery (24/29 non-pCR) compared with 52% in patients who cleared ctDNA at T1 (14/27 non-pCR). This association was significant (odds ratio 4.33, $P = 0.012$, adjusted for subtype and treatment received). Among the 39 non-pCR patients who had undetectable ctDNA after NAC, 17 (43%) were ctDNA negative at baseline, 10 (26%) cleared ctDNA by T1, and 12 (31%) cleared ctDNA by

(A) Diagram showing the study schema of the I-SPY 2 TRIAL. Prior to study entry, tumor biopsy from each patient is analyzed to assess hormone receptor and human epidermal growth factor receptor 2 status and MammaPrint scores. Blood samples are collected at the following time points: T0, baseline/pretreatment; T1, 3 weeks after initiation of therapy; T2, between two treatment regimens [paclitaxel ± MK-2206 and anthracycline (AC)]; and T3, after neoadjuvant chemotherapy (NAC) prior to surgery. (B) Flow chart showing patients and samples evaluated in the study and sample performance at different quality control (QC) points. (C) Schema of the methods for ctDNA analysis. pCR, pathological complete response.

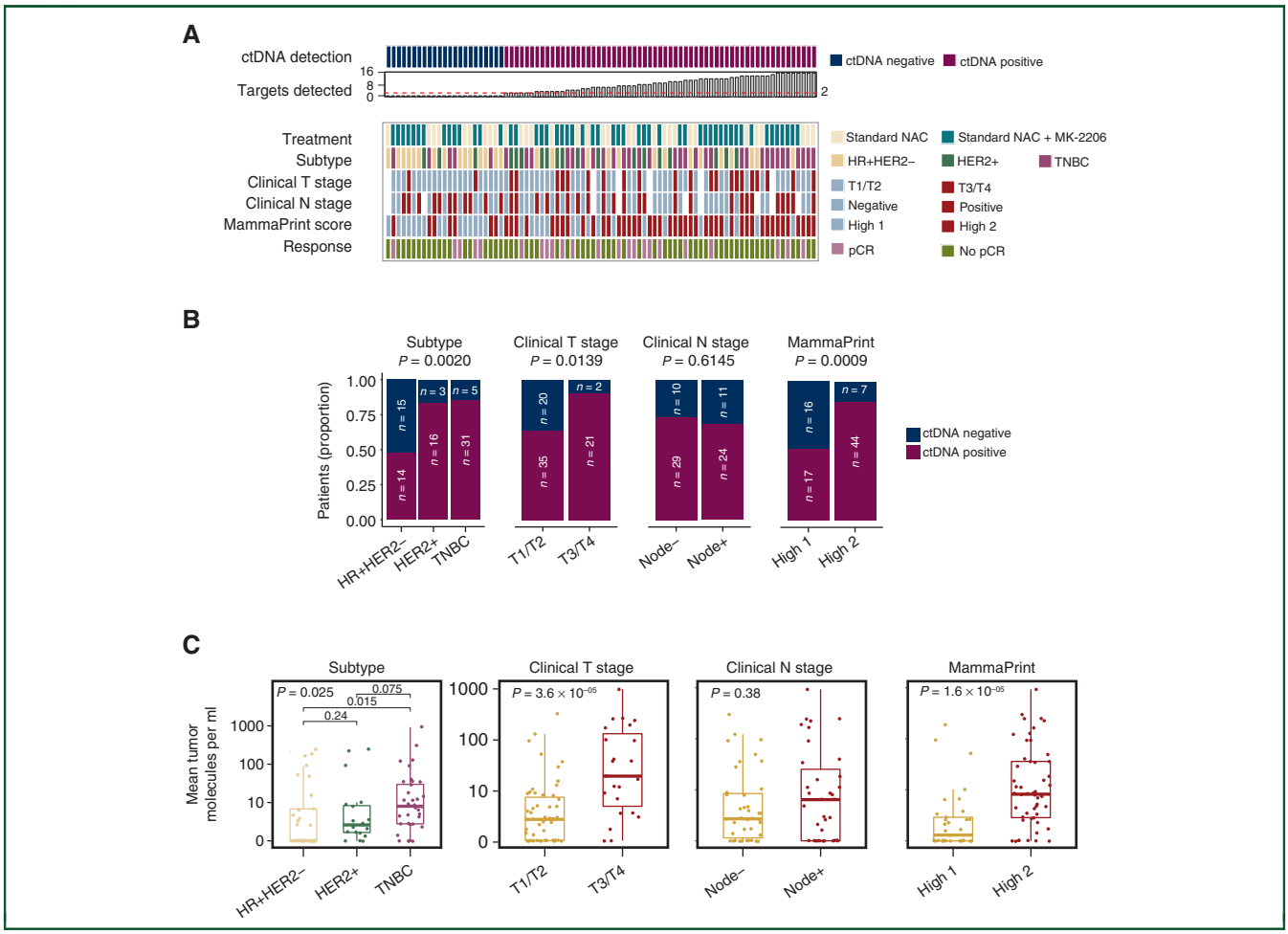

Figure 2. Association between circulating tumor DNA (ctDNA) and clinicopathologic characteristics.
(A) Overview of patient and tumor characteristics according to ctDNA status at baseline (T0). (B) Proportion of ctDNA-positive and ctDNA-negative patients at baseline (T0) according to clinical characteristics. *P* values were calculated using Fisher's exact test. (C) Mean tumor molecules per ml of plasma according to clinical characteristics. Distributions were compared using Wilcoxon rank sum (binary variable) or Kruskal—Wallis (ternary variable) tests. HER2, human epidermal growth factor receptor 2; HR, hormone receptor; NAC, neoadjuvant chemotherapy; pCR, pathological complete response; TNBC, triple-negative breast cancer.

T2 or T3. The positive predictive value (PPV) of the test (for predicting non-pCR) increased with time (Figure 4B).

Clinical events are frequent in patients with detectable ctDNA

Survival data were available for 75 of the 84 patients, with a median follow-up of 4.8 years (range 0.5-6.3 years). In this period, 8 had local recurrences and 10 experienced distant metastases, of whom 8 died (Figure 5A). Detectable ctDNA in at least one time point was observed in 6 of the 8 patients (75%) with local recurrence, 9 of the 10 patients (90%) who had distant recurrence, and in all 8 patients who died (100%). Of note, a patient who experienced brain metastasis did not have detectable ctDNA at all time points.

ctDNA dynamics is significantly associated with metastatic recurrence

We examined whether ctDNA dynamic patterns (Figure 3C) were associated with DRFS, the secondary endpoint of the I-SPY 2 TRIAL. Of 58 patients with ctDNA data at all time points, 54 had follow-up information. Patients who had cleared ctDNA at T1, T2, or T3 ($n = 29$) had similar risk of metastatic recurrence compared with those who were ctDNA negative at T0 ($n = 20$; HR 2.1; 95% CI 0.22-20.2; Figure 3D). Patients who did not clear ctDNA at T3 ($n = 5$) had a significantly higher risk of metastatic recurrence (HR 22.4; 95%, CI 2.5-201, $P < 0.001$).

ctDNA at T1, T2, and T3 but not T0 is associated with increased risk of metastatic recurrence

Next, we examined whether ctDNA status (positive or negative) at different time points was associated with DRFS (supplementary Figure S6, available at https://doi.org/10.1016/j.annonc.2020.11.007). At baseline (T0), ctDNA-positive patients had increased risk of metastatic recurrence, but this association did not reach statistical significance (HR 4.11; 95% CI 0.52-32.4). By contrast, ctDNA positivity at 3 weeks after initiation of therapy (T1; HR 4.5; 95% CI 1.2-17.4), between regimens (T2; HR 5.4; 95% CI 1.3-22.5), and after NAC (T3; HR 11.5; 95% CI 2.9-46.1) was significantly associated with increased risk of metastatic recurrence.

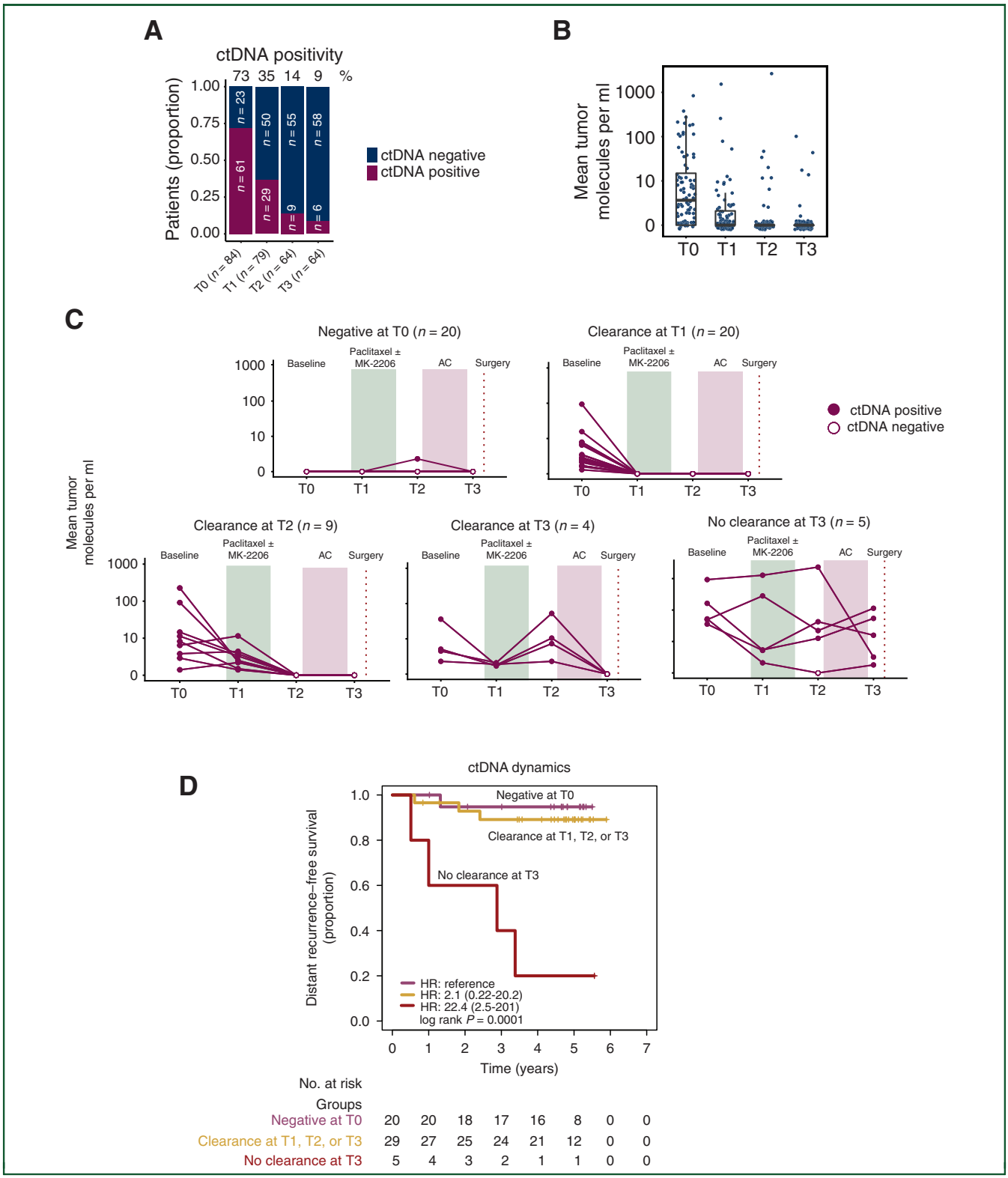

Figure 3. Circulating tumor DNA (ctDNA) dynamics over the course of neoadjuvant chemotherapy.
(A) Proportion of patients according to ctDNA positivity based on number of samples available per time point. (B) Mean tumor molecules per ml of plasma across time points. (C) Patients with complete ctDNA data for four time points ($n = 58$) grouped according to observed patterns of ctDNA clearance or nonclearance. (D) Survival in patients grouped according to ctDNA clearance. Of the 58 patients, 54 had survival data. Patients who cleared ctDNA at T1, T2, or T3 were combined into one group and their survival was compared with that of patients who did not clear ctDNA at T3 and those who were ctDNA negative at T0 (reference group). AC, anthracycline; HR, hazard ratio.

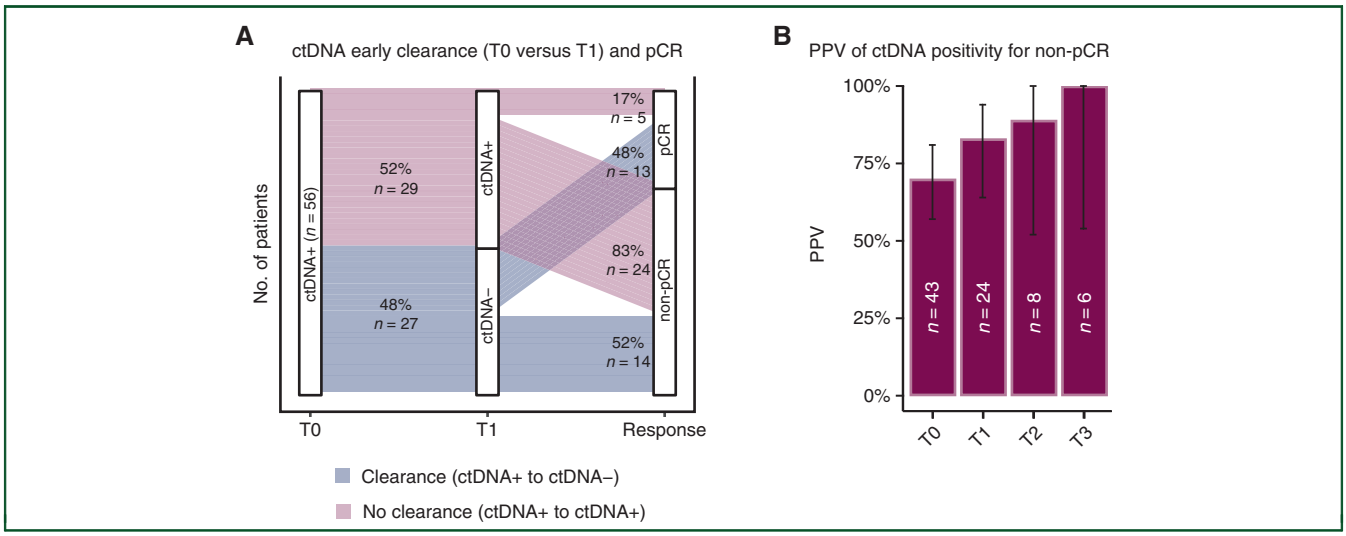

Figure 4. Association of circulating tumor DNA (ctDNA) with response to neoadjuvant chemotherapy and its positive predictive value.
(A) Sankey plot showing ctDNA dynamics (clearance or non-clearance) early during treatment versus response [pathologic complete response (pCR) or no pCR]. Analysis was focused on patients who were ctDNA positive at baseline (T0) and had corresponding ctDNA testing results at T1, 3 weeks after initiation of therapy. (B) Positive predictive value (PPV) of ctDNA positivity in predicting failure to achieve pCR. PPV is the proportion of patients with a positive ctDNA test (at a specific time point) with residual cancer after neoadjuvant chemotherapy (NAC).

Clearance of ctDNA after NAC (T3) is associated with improved survival

Patients were stratified according to pCR and ctDNA status after NAC ($n = 60$). The proportion of subtypes varied across groups based on pCR and ctDNA positivity (Fisher's exact $P = 0.0257$, Figure 5B). As much as 17 patients who achieved pCR (100%), all of whom were ctDNA negative, showed favorable DRFS (Figure 5C). In patients who did not achieve pCR ($n = 43$), ctDNA positivity ($n = 6$; supplementary Figure S7, available at https://doi.org/10.1016/j.annonc.2020.11.007) was significantly associated with worse DRFS ($n = 37$; HR 10.4, 95% CI 2.3-46.6). Interestingly, risk of metastatic recurrence in patients who failed to achieve pCR but were ctDNA negative was similar to those who achieved pCR (HR 1.4; 95% CI 0.15-13.5). Positive predictive value and negative predictive value were 67% (4/6) and 93% (50/54), respectively. A landmark analysis (using T3 as the starting point) was performed and revealed similar results (supplementary Figure S8, available at https://doi.org/10.1016/j.annonc.2020.11.007). In an exploratory multivariable Cox regression analysis, ctDNA-positivity after NAC was a significant predictor of poor DRFS (Table 1).

DISCUSSION

In this study, we examined the role of personalized ctDNA as a predictive biomarker for response and outcome in the neoadjuvant setting. The cohort included early-stage breast cancer patients with high risk of recurrence and who were treated with standard NAC alone or combined with MK-2206 (AKT inhibitor) treatment in the I-SPY 2 TRIAL.

ctDNA studies in the neoadjuvant setting in breast cancer have recently been reported[28,31-33] (supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2020.11.007). Two of the four studies were limited to a particular breast cancer subtype (i.e. only TNBC[28] or only HER2+[31]). Rothé and colleagues[31] observed that ctDNA detection before NAC was associated with decreased likelihood of achieving a pCR. McDonald and colleagues[32] showed that nonresponding patients have higher ctDNA levels after NAC compared with those who achieved a pCR. Two of the studies examined association between ctDNA and survival,[28,31] but none was able to demonstrate the prognostic impact of residual ctDNA after NAC. To our knowledge, our work represents the most comprehensive study on ctDNA detection in all subtypes, before, during, and after NAC and examined for the first time its association with response and survival in early breast cancer.

Here, we report on the use of a personalized ctDNA test informed by each patient's tumor genotype. We found that ctDNA is frequently detected in untreated high-risk early stage population (~70% of patients). The patterns of change in ctDNA during NAC were significantly correlated with risk of metastatic recurrence. We also found that ctDNA testing early during NAC (at 3 weeks) provided potentially actionable information as persistent ctDNA identified patients who were unlikely to achieve a pCR, whereas clearance was associated with improved response.

ctDNA positivity rate at baseline was significantly different among breast cancer subtypes (HR+/HER2−: 52%, HER2+: 82%, TNBC: 89%). We speculate that the lower rate of ctDNA positivity in HR+ breast cancer compared with HER2+ and TNBC is due in part to the lower proliferation rates (lower expression of Ki67[34]) in this subtype, as was observed by Abbosh and colleagues in lung cancer.[25] Elucidating molecular and genomic factors predictive of ctDNA presence in the blood may shed on light on the biology of ctDNA release and clearance during treatment.

We examined whether ctDNA status at different time points was associated with risk of metastatic recurrence. We found that ctDNA-positive patients at T1, T2, and T3

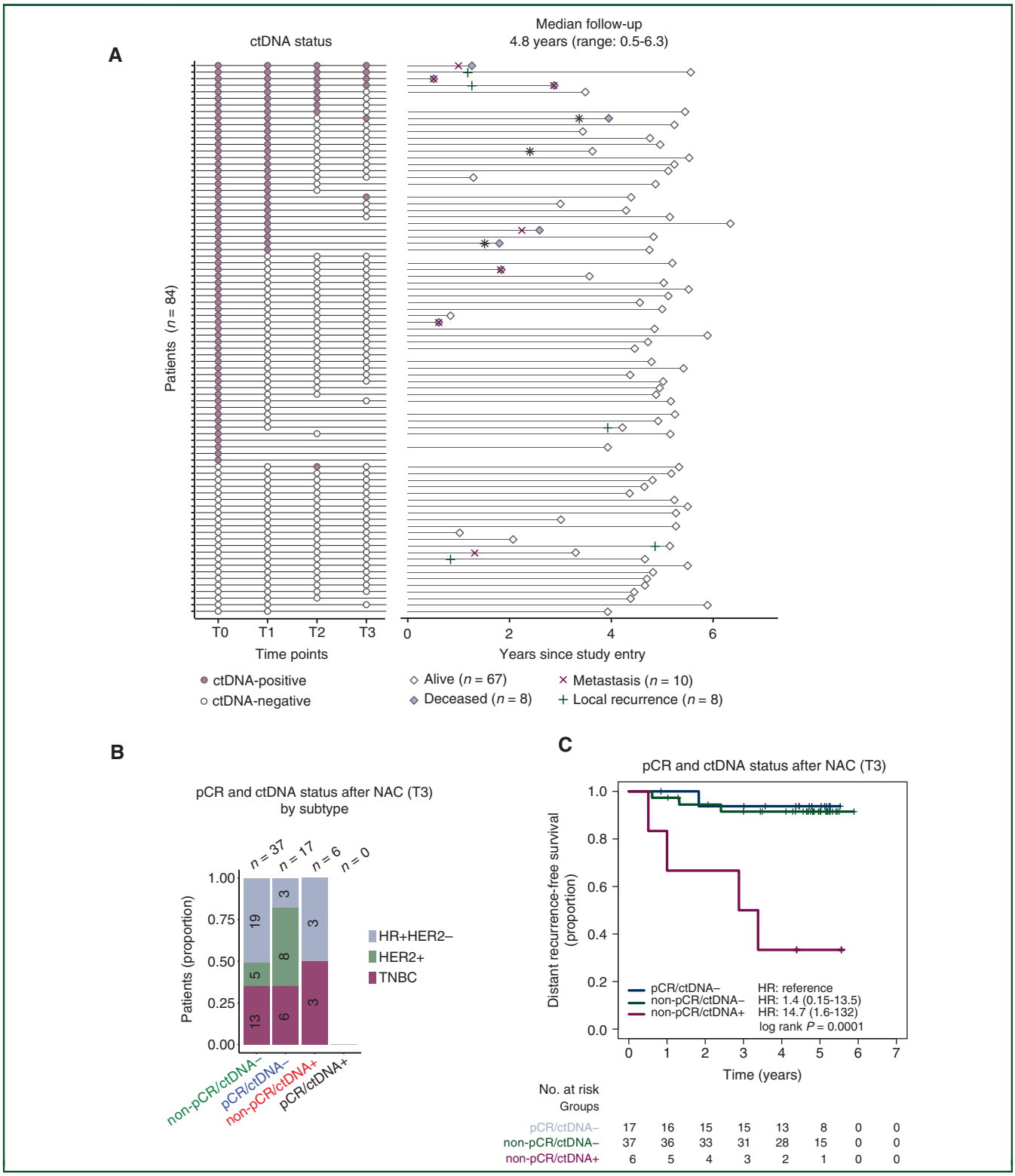

Figure 5. Circulating tumor DNA (ctDNA) and clinical outcomes.
(A) Overview of the ctDNA detection across different time points [T0, baseline/pretreatment; T1, 3 weeks after initiation of therapy; T2, between two treatment regimens [paclitaxel and anthracycline (AC)]; T3, after neoadjuvant chemotherapy (NAC) prior to surgery]. The right panel shows a swimmer plot depicting the length of follow-up and events in 75 patients with survival data. The primary endpoint of the study was distant recurrence-free survival. (B) Proportion of subtypes according to groups based on pathologic complete response (pCR) and ctDNA status at T3. (C) Patient survival stratified based on ctDNA status after NAC (T3) and response to treatment [pathological complete response (pCR)]. HER2, human epidermal growth factor receptor 2; HR, hazard ratio.

Table 1. Multivariate Cox regression analysis to determine association between ctDNA positivity after NAC (T3) and distant disease-free survival (DRFS) while controlling for pCR and subtype

Variable	DRFS			
	Hazard ratio	Lower 0.95 CI	Upper 0.95 CI	*P*-value
ctDNA+ versus ctDNA− at T3	14.9	2.66	83.11	0.0021
pCR versus no pCR	0.4	0.04	4.45	0.4579
HR+ HER2− versus TN	0.8	0.16	3.98	0.7833
HER2+ versus TN	3.6	0.39	33.80	0.2559

CI, confidence interval; HR, hormone receptor; pCR, pathologic complete response; TN, triple negative.

(but not at T0) had significantly inferior DRFS compared with those who were ctDNA negative. We observed that PPV and HR increased with time, indicating that the last time point (i.e. after NAC before surgery) may be most informative for risk stratification of patients, and thus potentially help guide treatment in the adjuvant setting.

Our study showed that ctDNA status after NAC can potentially stratify patients who did not achieve pCR into low- and high-risk groups (Figure 5C). We found that clearance of ctDNA after NAC was associated with improved survival even in patients who did not achieve pCR. If validated, these findings could have a profound impact on treatment management in the neoadjuvant and adjuvant settings.

Recent clinical studies in breast cancer have shown that additional adjuvant therapy for nonresponders to NAC can lead to improvements in patient outcomes.[35,36] Future studies should take into account the potential confounding effects of adjuvant treatment on the prognostic performance of ctDNA and other biomarkers analyzed in the neoadjuvant setting.

Differences in prognostic value of pCR by subtype have been reported, including its poor association with prognosis in HR+ breast cancer.[21,37] Survival analysis in I-SPY 2 involving 950 patients has shown that pCR and subtype (including HR+) were strongly associated with DRFS.[23] In this subset, the individual prognostic impact of pCR and subtype was not observed, perhaps due to the modest sample size. Our exploratory survival analysis did show that ctDNA after NAC was a strong prognostic factor for DRFS. Further studies in larger cohorts are warranted to examine the contributions of ctDNA, pCR, and subtypes in predicting outcomes of patients who received NAC.

The I-SPY 2 schema includes the collection of serial magnetic resonance imaging (MRI) data during NAC to assess tumor response.[38,39] We have previously analyzed paired ctDNA and MRI data collected at the same time points in the same cohort as this present study.[40] We found that MRI-based functional tumor volume—a clinically established measure of residual disease in the breast[39,41]—was significantly correlated with ctDNA levels at all time points.[40] Furthermore, we found that ctDNA status after NAC improved the performance of functional tumor volume as predictor of metastatic recurrence and death. ctDNA testing could therefore serve as complementary tool to MRI for risk stratification of patients post-NAC.

A number of technologies for detection of ctDNA have been developed and are described in detail in a recently published review.[42] Our approach provides several advantages over other methods of ctDNA analysis. The upfront whole-exome sequencing of primary tumors enables personalized selection of ctDNA targets that is independent of driver status. Our assay simultaneously tracks up to 16 patient-specific somatic variants and thus offers a more robust representation of the heterogeneity of a patient's tumor.[26,27,30] By contrast, other methods such as droplet digital polymerase chain reaction[8] or BEAMing[43] can track only one to a few somatic variants. Our ctDNA test does have certain limitations including the inability to detect new second primary cancers which are often genetically unrelated to the original cancer[44]; also, it will miss novel somatic variants that arise during tumor evolution in response to therapy-mediated selection pressures.[45]

Clonal hematopoiesis of indeterminate potential mutations are potential sources of false positives in sequencing analyses of cfDNA.[46,47] The ctDNA detection approach used in this study filters out clonal hematopoiesis of indeterminate potential mutations by focusing only on tumor-specific mutations that were initially detected by whole-exome sequencing of paired pretreatment tumor and germline DNA.

In the light of our findings, novel paradigms for ctDNA-directed treatment can be envisioned in future clinical trials. The current I-SPY 2 schema provides patients a single therapeutic opportunity to achieve a pCR[48] (Figure 1A). In the next iteration of the trial, patients will be given options to receive additional treatment to improve their chances of achieving a pCR, that is, if the initial agent does not result in a predicted complete response. For example, the decision to switch therapy for a patient without an early clinical or imaging response to a novel therapeutic agent would be supported if the patient fails to clear ctDNA. By contrast, patients who clear their ctDNA could continue treatment. Furthermore, information from ctDNA testing after NAC may help guide clinical decisions on whether to escalate or de-escalate treatment in the adjuvant setting. For example, if clearance of ctDNA is confirmed as a predictor of low risk of metastatic recurrence, such information can support treatment de-escalation.

Analysis of pooled serial circulating tumor cell (CTC) data obtained during neoadjuvant treatment of early breast cancer revealed that the prognostic impact of CTCs was the strongest at pretreatment (prior to NAC) compared with other time points.[49] By contrast, our study showed that ctDNA status after NAC appeared to be the most important time point for prognostication. While both blood-based biomarkers display prognostic impact, their clinical value may not be redundant.[50] Further studies that contemporaneously assess ctDNA and CTCs in the neoadjuvant setting are needed to elucidate the relative contributions of each biomarker in predicting response and outcome.

The focus of this study was to examine the clinical significance of ctDNA monitoring in the neoadjuvant setting. The detection of minimal residual disease after surgery is of great clinical importance and can provide a unique opportunity for treatment redirection to delay metastatic recurrence and improve patient outcomes. We have now expanded our studies to include postsurgical monitoring of ctDNA in the adjuvant setting with the focus on residual disease detection after surgery and recurrence prediction.

In summary, our study shows promise that early response prediction by highly sensitive ctDNA analysis in high-risk early breast cancer patients may facilitate a timely and judicious change in treatment to improve patients' chances of achieving favorable long-term outcomes. The I-SPY 2 TRIAL provides an excellent platform to investigate how personalized ctDNA testing can complement imaging[51] and pathologic evaluation[52] of tumor response to fine-tune pCR as a surrogate endpoint for improved survival. Dynamic monitoring of ctDNA during NAC could facilitate evaluation of new agents by providing an early endpoint of treatment efficacy. Response over time as measured by imaging and ctDNA in the setting of early (pCR) and late (DRFS) outcomes will provide a robust framework for elucidating the potential clinical utility of ctDNA in the neoadjuvant setting.

ACKNOWLEDGEMENTS

The authors thank the patients and their families and the I-SPY 2 TRIAL Investigators for their participation in this study.

FUNDING

This study was funded in part by the Breast Cancer Research Foundation (BCRF-17-162) (LJVV), and the Breast Cancer Research Foundation at UCSF (LJVV, no grant number) and NIH/NCI P01CA210961 (LJE). We are grateful to Dr Matthew Rabinowitz for his support and insightful discussions of the project. ctDNA analysis was provided by Natera without cost.

DISCLOSURE

The following authors are employees of Natera, Inc. (HS, H-TW, RS, AT, SS, HP, PB, AA, ML, BZ). LJVV is co-founder, stockholder, and part-time employee of Agendia NV. The remaining authors have declared no conflicts of interest.

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