Personalized Detection of Circulating Tumor DNA by Ultra-Deep Sequencing Reflects Response to Neoadjuvant Therapy and Predicts Metastatic Recurrence in High-Risk Early Stage Breast Cancer

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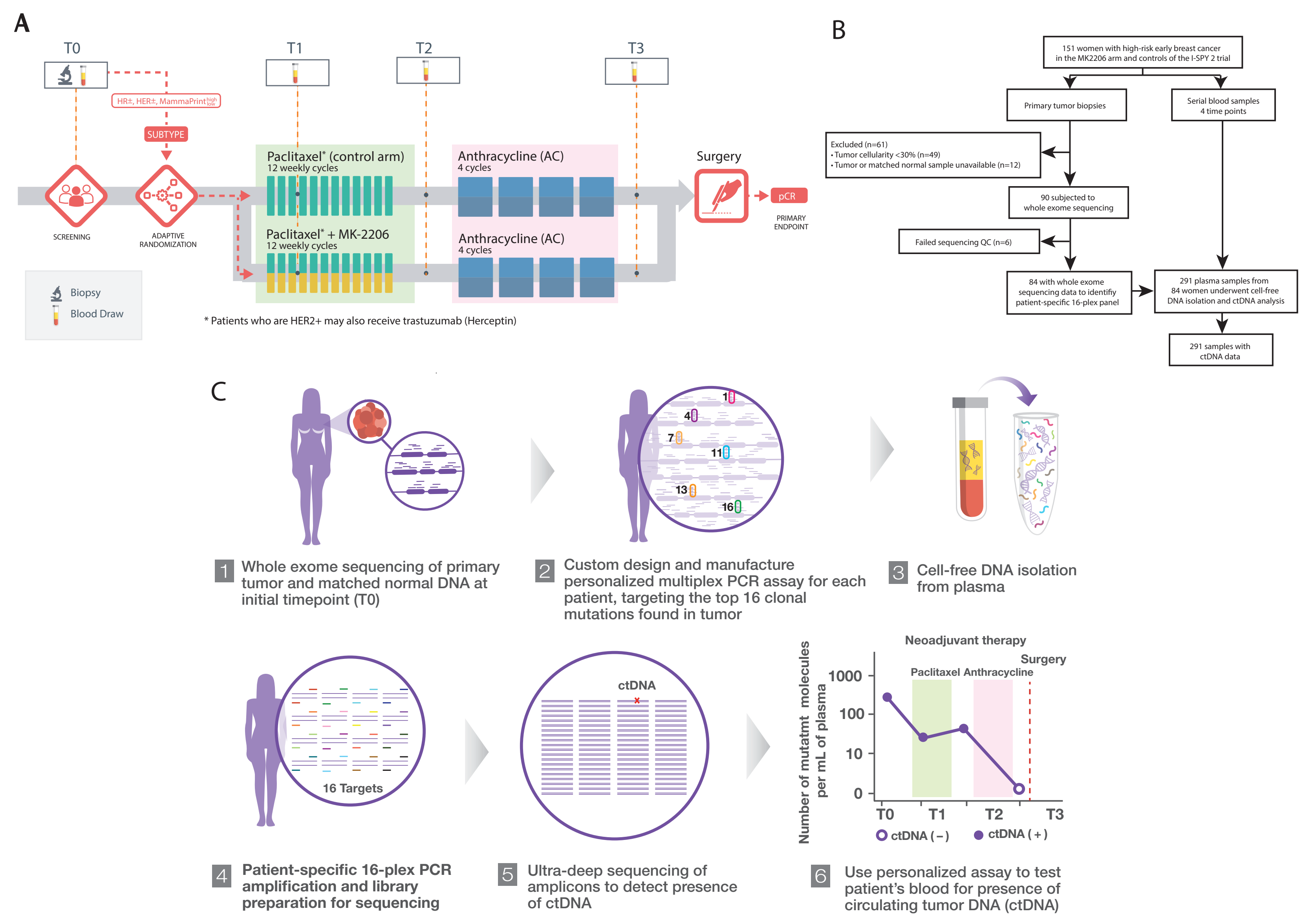
Introduction

- The detection of circulating tumor DNA (ctDNA) during neoadjuvant therapy (NAT) may serve as an early indicator of emerging resistance and disease progression. In this study, we analyzed ctDNA from high-risk early breast cancer patients who received NAT and definitive surgery in the I-SPY 2 TRIAL (NCT01042379). We hypothesized that ctDNA can serve as a biomarker of response and survival in this setting.

Methods

- A personalized ctDNA test was designed to detect 16 patient-specific variants (from whole exome sequencing of pretreatment tumor) in plasma from 84 high-risk early breast cancer patients who received NAC +/- investigational agent MK-2206 in the I-SPY 2 TRIAL **(Figure 1)**.

Figure 1. Schematic of Clinical Protocol, Study Design and Molecular Protocol



1 Whole exome sequencing of primary tumor and matched normal DNA at initial timepoint (T0)

2 Custom design and manufacture personalized multiplex PCR assay for each patient, targeting the top 16 clonal mutations found in tumor

3 Cell-free DNA isolation from plasma

4 Patient-specific 16-plex PCR amplification and library preparation for sequencing

5 Ultra-deep sequencing of amplicons to detect presence of ctDNA

6 Use personalized assay to test patient's blood for presence of circulating tumor DNA (ctDNA)

Figure 1: A) Diagram showing the study schema of the I-SPY 2 TRIAL. Prior to study entry, tumor biopsy from each patient was analyzed to assess hormone-receptor (HR) and HER2 status and MammaPrint scores. The results were then fed into the adaptive randomization engine to determine eligibility and assignment to treatment arms. Blood samples were collected at the following time points: T0- baseline/pretreatment, T1- 3 weeks after initiation of therapy, T2- between two treatment regimens (paclitaxel and AC), T3- after neoadjuvant chemotherapy prior to surgery. **B)** Flow chart showing patients and samples evaluated in the study and sample performance at different quality control (QC) points. WES-whole exome sequencing. **C)** Schema of the methods for ctDNA analysis. PCR-polymerase chain reaction.

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Results

- **73% of patients were ctDNA positive at baseline. ctDNA positivity and levels were significantly associated with larger tumors and more aggressive tumor biology and subtype (Figure 2).**

Figure 2. Association Between ctDNA and Clinicopathological Characteristics

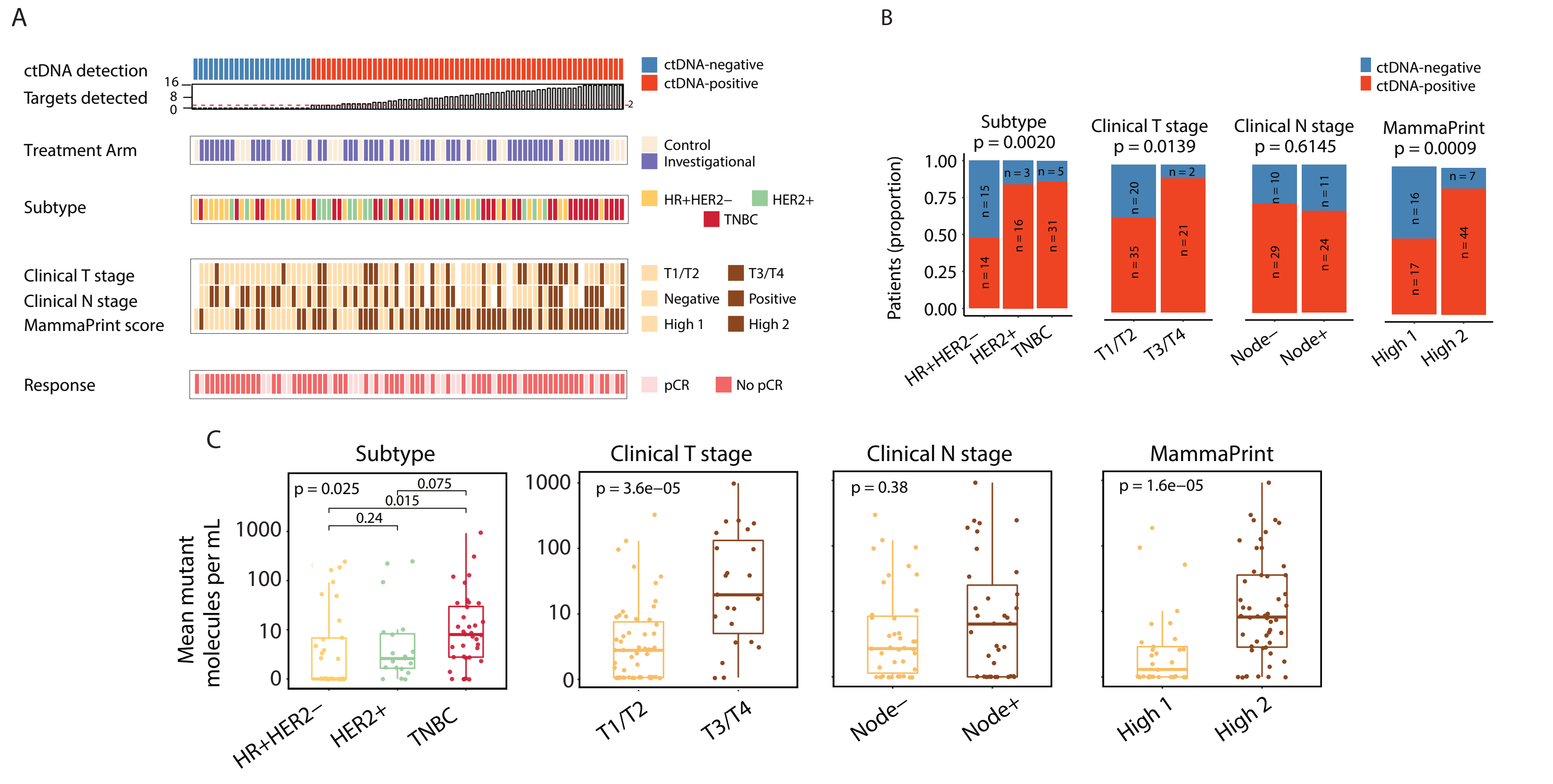


Figure 2: A) Overview of patient and tumor characteristics according to ctDNA status at baseline (T0). HR-hormone receptor, TNBC-triple negative breast cancer, pCR- pathological complete response. **B)** Proportion of ctDNA-positive and negative patients at baseline (T0) according to clinical characteristics. P values were calculated using Fisher's exact test. **C)** Mean mutant molecules per mL of plasma according to clinical characteristics. Distributions were compared using Wilcoxon rank sum (binary variable) or Kruskal Wallis (ternary variable) tests.

- **ctDNA levels during NAT decreased over time. Five ctDNA clearance patterns were observed (Figure 3).**

Figure 3. ctDNA Dynamics Over the Course of Neoadjuvant Chemotherapy

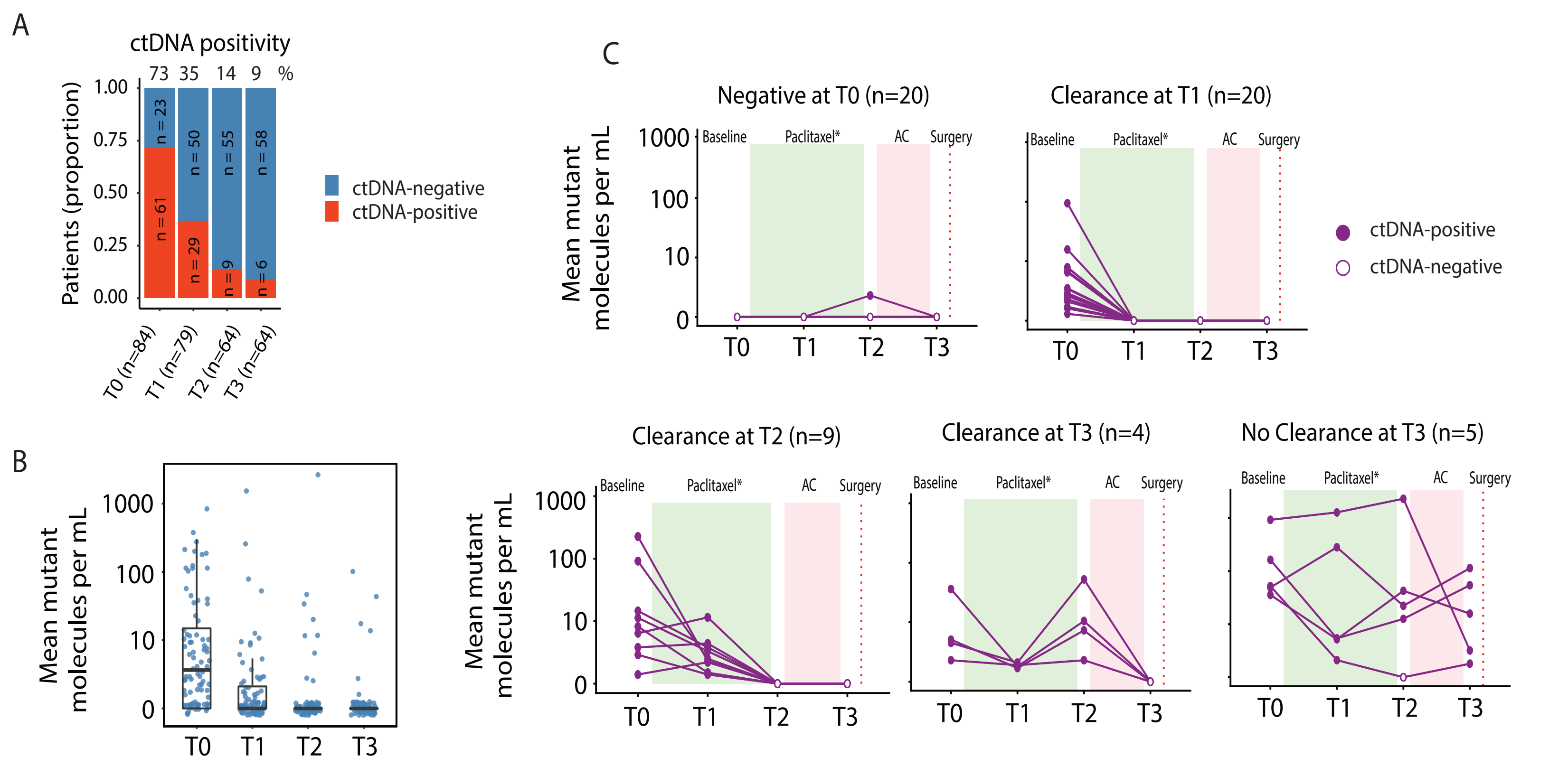


Figure 3: A) Proportion of patients according to ctDNA positivity across time points. **B)** Mean mutant molecules per mL of plasma across time points. **C)** Patients with complete ctDNA data for four time points (n=58) grouped according to observed patterns of ctDNA clearance or non-clearance.

- **Patients who remained ctDNA-positive at T1 were significantly more likely to have a non-pCR (83%) compared to those who cleared ctDNA (52%; OR 4.33, P=0.012) (Figure 4).**

Figure 4. Association of ctDNA with response to neoadjuvant chemotherapy

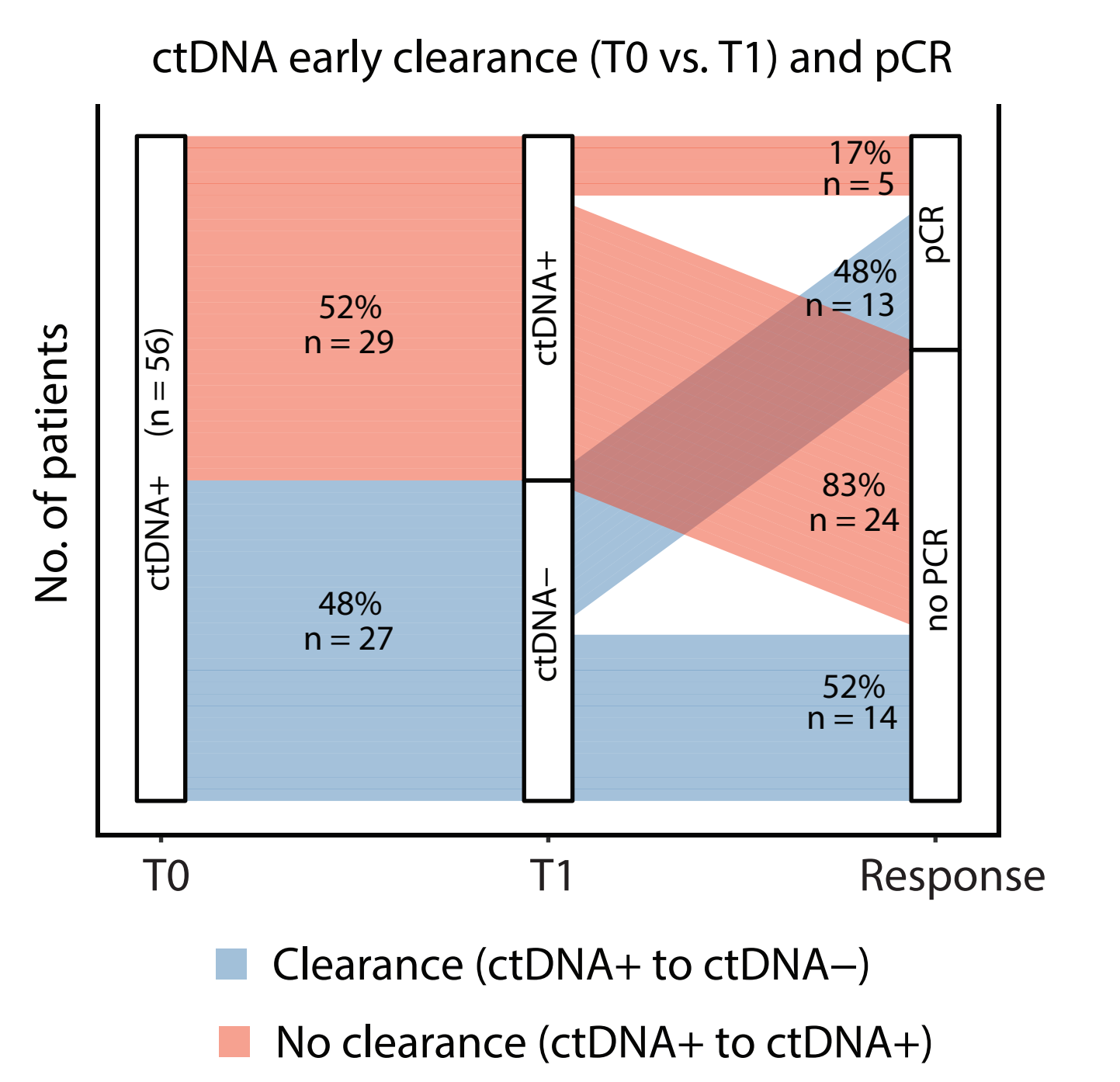


Figure 4. Association of ctDNA with response to neoadjuvant chemotherapy and its positive predictive value. A) Sankey plot showing ctDNA dynamics (clearance or non-clearance) early during treatment vs. response (pathologic complete response, pCR, or no pCR). Analysis was focused on patients who were ctDNA-positive at baseline (T0) and had corresponding ctDNA testing results at T1, 3 weeks after initiation of therapy.

- **All eight patients who died and nine out of 10 patients who had distant recurrence had detectable ctDNA in at least one timepoint (Figure 5).**

Figure 5. Correlation of ctDNA with Risk of Metastatic Recurrence

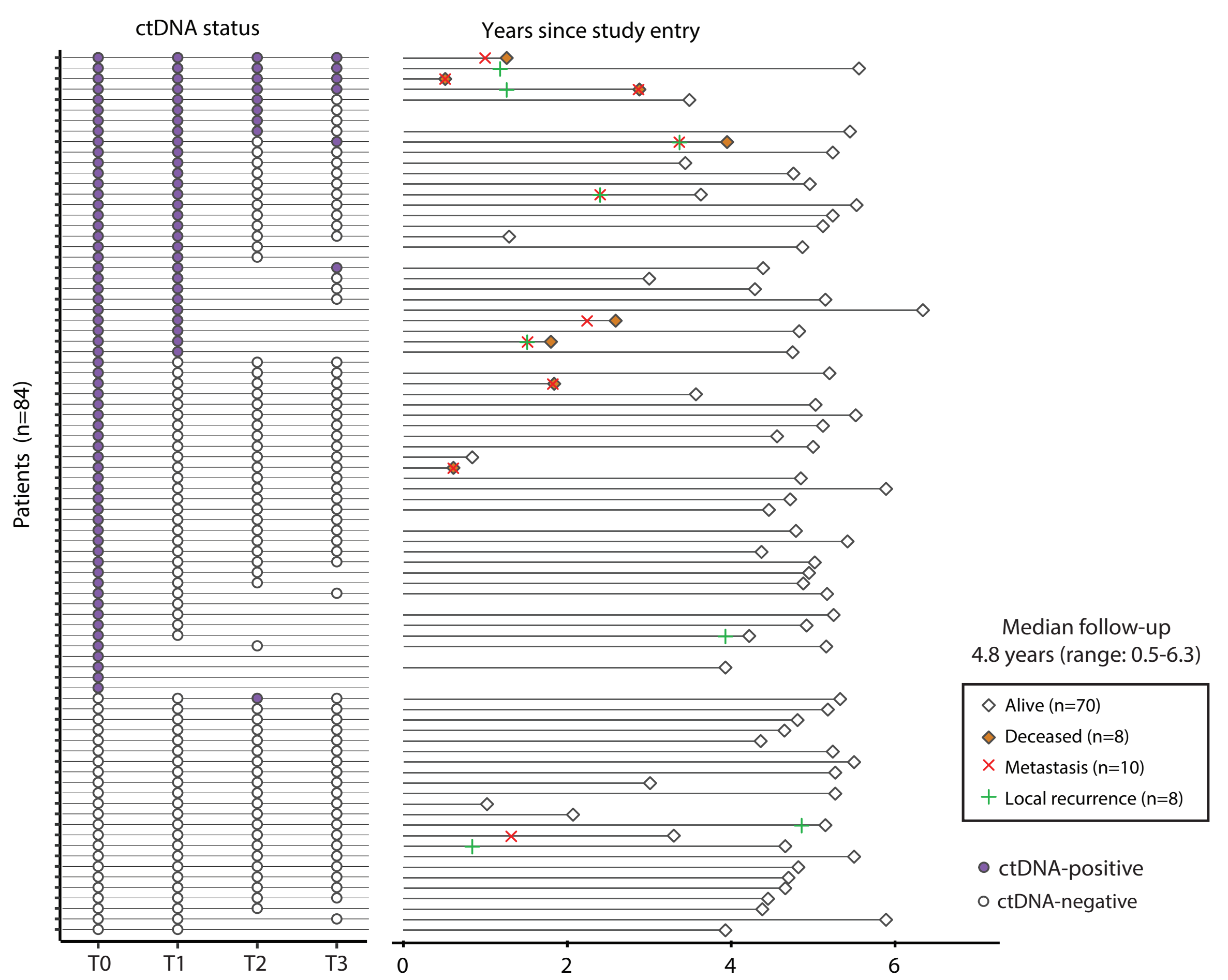
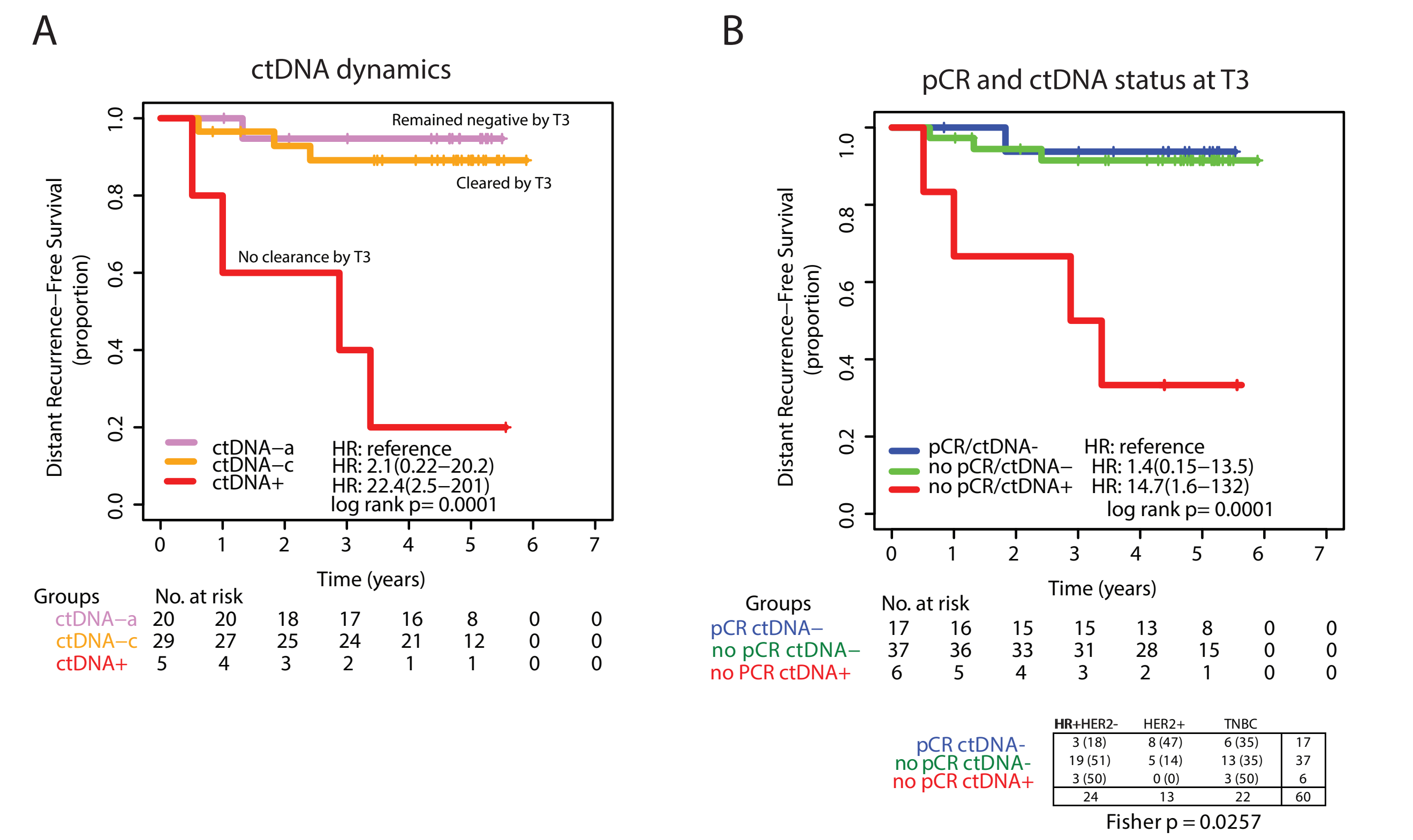


Figure 4: The overview plot indicates ctDNA detection across different timepoints [T0: baseline/pretreatment, T1: 3 weeks after initiation of therapy, T2: between two treatment regimens (paclitaxel and AC), T3: after neoadjuvant chemotherapy prior to surgery]. The right panel shows a swimmer plot depicting the length of follow-up and events in 75 patients with survival data. The primary endpoint of the study was distant recurrence-free survival.

- **Patients who did not clear ctDNA had the worst survival compared to those who cleared ctDNA or remained ctDNA-negative. Interestingly, the non-pCR/ctDNA-negative patients had similar risk of metastatic recurrence to those who achieved a pCR, while the 6 non-pCR/ctDNA-positive patients had significantly increased risk of metastatic recurrence (Figure 6).**

Figure 6. ctDNA Status and Recurrence Free Survival



	HR+HER2-	HER2+	TNBC	
pCR ctDNA-	3 (18)	8 (47)	6 (35)	17
no pCR ctDNA-	19 (51)	5 (14)	13 (35)	37
no pCR ctDNA+	3 (50)	0 (0)	3 (50)	6
	24	13	22	60

Fisher p = 0.0257

Figure 6: A) Survival in patients grouped according the ctDNA clearance patterns. **B)** Patient survival stratified based on ctDNA status after neoadjuvant chemotherapy (T3) and response to treatment (pathological complete response, pCR). Inset table shows the numbers and percentages of patients according to subtype and ctDNA/response groups. Of the six patients who were positive at T3, three were hormone receptor-positive (HR+HER2-) and three had triple negative breast cancer (TNBC).

Conclusions

- Early clearance of ctDNA during NAT was significantly associated with increased likelihood of achieving pCR.
- Clearance of ctDNA at any point during NAT was associated with improved outcomes.
- The no pCR/ctDNA-negative group represents a novel cohort of patients that have improved clinical outcomes.
- Residual ctDNA after NAT was a significant predictor of metastatic recurrence.
- Taken together, personalized monitoring of ctDNA during NAT may aid in real-time assessment of treatment response and help fine-tune pCR as a surrogate endpoint of survival.
- Validation studies in a larger cohort are warranted.

Patient Advocate's Perspective

"Undergoing multiple core biopsies to determine if cancer has progressed in the neoadjuvant setting is concerning to patients due to pain during and post procedure, and time to heal the biopsy site. In addition, the biopsy site may not represent the diversity of the tumor burden so may not suffice as a predictor of the most appropriate therapeutic intervention. Breast cancer may also be spreading beyond local tumor tissue and invading other organs. Liquid biopsies (via blood draws) could be a less invasive, real time, alternate to multiple neoadjuvant biopsies while also identifying metastatic potential. ctDNA liquid biopsies will provide supplemental information to imaging and limit the number of pathological studies required to customize therapies." -Amy L. Delson, UCSF Breast Science Advocacy Core

Acknowledgments

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