Assessment of molecular relapse detection in early-stage breast cancer.

Isaac Garcia-Murillas[1*] PhD, Neha Chopra[1*] MD, Iñaki Comino-Mendez[1] PhD, Matthew Beaney[1] BSc, Holly Tovey[2] BSc, Rosalind J Cutts[1] PhD, Claire Swift[1] BSc, Divya Kriplani[1] MD, Maria Afentakis[3] BSc, Sarah Hrebien[1] BSc, Giselle Walsh-Crestani[1] BSc, Peter Barry[4] MS, Stephen RD Johnston[4] PhD, Alistair Ring[4] MD, Judith Bliss[2] MSc, Simon Russell[5] MD, Abigail Evans[6] MD, Anthony Skene[7] MS, Duncan Wheatley[8] MD, Mitch Dowsett[3] PhD, Ian E Smith[4] MD and Nicholas C Turner[1,4] PhD

1. Breast cancer Now Research Centre, The Institute of Cancer Research, London SW3 6JB, UK
2. ICR-CTSU, The Institute of Cancer Research, London SM2 5NG, UK
3. Ralph Lauren Centre for Breast Cancer Research, London SW3 6JJ, UK
4. Breast Unit, Royal Marsden Hospital, London SW3 6JJ, UK
5. Hinchingbrooke Hospital, Hinchingbrooke Park, Huntingdon PE29 6NT, UK
6. Poole General Hospital, Dorset BH15 2JB, UK
7. Royal Bournemouth Hospital, Bournemouth BH7 7DW, UK
8. Department of Oncology, Royal Cornwall Hospitals NHS Trust, Truro TR1 3LQ, UK

*** These authors contributed equally to this work**

Corresponding author:

Nicholas Turner, The Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB, UK, Fax: +44 (0) 207 51535340, Email: nicholas.turner@icr.ac.uk

This study was presented in part at the 2018 San Antonio Breast Cancer Symposium, December 4-December 8, 2018, San Antonio, Texas (abstract number 549).

Word Count: 1194

Revision Date: 23 February 2019

Key Points

Question: Clinical validity of molecular relapse detection with circulating tumor DNA (ctDNA) analysis in early stage breast cancer.

Findings: We present the results of an independent prospective, multi-center, validation study of ctDNA mutation tracking in early breast cancer. Detection of ctDNA during follow-up had a median lead-time of 10.7 months over clinical relapse, anticipating relapse in all major breast cancer subtypes. Brain only metastasis was detected less frequently by ctDNA analysis, potentially requiring alternative surveillance.

Meaning: Molecular relapse detection has high levels of clinical validity. Clinical trials of treatment initiated on molecular relapse, without waiting for incurable metastatic disease to develop, are required.

Abstract

Importance: The majority of patients presenting with early stage, primary breast cancer are cured by current treatment. Better techniques are required to identify which patients are at risk of relapse.

Objective: Small proof-of-principle studies have demonstrated that detection of circulating tumor DNA (ctDNA) in follow-up associates with future relapse. We assessed the clinical validity of molecular relapse detection with an independent validation study.

Design: A prospective, multicenter sample collection study conducted in 5 UK centers.

Setting: Patients with early stage breast cancer, irrespective of hormone receptor and HER2 status, receiving either neoadjuvant chemotherapy followed by surgery, or surgery prior to adjuvant chemotherapy.

Participants: The study recruited 170 women, with mutations identified in 101 patients forming the main cohort. Secondary analyses were conducted on a combined cohort of 144 patients, including 43 patients previously analyzed in a prior proof-of-principle study.
Interventions: Primary tumor was sequenced to identify somatic mutations, and personalized tumor specific digital PCR assays were used to monitor these mutations in serial plasma samples taken every three months for the first year of follow-up, and subsequently every six months.
Main Outcome and Measure: The primary endpoint was relapse free survival analyzed with Cox proportional hazards models.
Results: In the main cohort with median follow-up of 35.5 months, detection of ctDNA in follow-up was strongly prognostic (relapse free survival time-dependent Hazard Ratio (HR) 25.2, 95% Confidence Interval (CI) 6.7-95.6, P<0.001). Detection of ctDNA at diagnosis, prior to any treatment, was also associated with relapse free survival (HR 5.8, 95% CI 1.22-27.1, P=0.013).
In the combined cohort, ctDNA detection had a median lead-time of 10.7 months (95% CI 8.1-19.1) over clinical relapse, and was highly prognostic in all breast cancer subtypes. Distant extra-cranial metastatic relapse was detected in 96% (22/23) of patients. Brain only metastasis was less commonly detected (P=0.0003), suggesting relapse sites less readily detectable by ctDNA analysis.
Conclusions and Relevance: Detection of ctDNA in follow-up is associated with a very high risk of future relapse in early-stage breast cancer. Prospective studies are required to assess the potential of molecular relapse detection to guide adjuvant therapy.

Introduction

Breast cancer is the most frequently diagnosed cancer worldwide, with approximately 95% of women presenting with early stage breast cancer without macroscopic metastatic disease. There is substantial need to develop better tools to establish who is at risk of relapse. Detecting which patients have molecular residual disease (MRD) that has not been

eradicated by treatment would allow clinical trials of adjuvant therapies focused on those who are at highest risk. Several small proof-of-principle studies have also shown that detection of circulating tumor DNA (ctDNA) may present a strategy to identify MRD in patients with breast,[1,2] colon[3,4] and lung cancer.[5,6]

Here we assess the potential of MRD detection in a prospective, multi-center series of patients with primary breast cancer, demonstrating that ctDNA analysis can accurately detect MRD and identify patients at high risk of relapse.

Methods

Patients and Sample Collection

One hundred and seventy patients were recruited from five hospitals in the UK into two prospective ctDNA sample collection studies, the ChemoNEAR study and Plasma DNA study approved by Research Ethics committees (East of England – Essex and London – and Bromley, respectively). Written informed consent was obtained from all participants. All patients had primary breast cancer without evidence of distant metastatic disease, with staging scans conducted as per local guidelines. Patients scheduled to receive standard treatment with neoadjuvant chemotherapy followed by surgery (N=140) consented for sample collection prior to chemotherapy, and patient scheduled to received adjuvant chemotherapy (N=30) consented after surgery and before chemotherapy. Plasma samples were collected every three months for the first year of follow-up, and subsequently every six months until five years (eMethods, eFigure1).

Sample analysis

Tumor DNA was extracted from the diagnostic biopsy, and sequenced to identify somatic mutations to track in plasma with a breast cancer driver gene panel (eMethods). Personalized digital PCR (dPCR) assays were designed to track individual somatic mutations in plasma samples. Plasma DNA was extracted and analyzed on a Bio-Rad QX-200 system (eFigure 2). dPCR analysis criteria were pre-specified.[1]

Statistical analysis

The primary study objective was to assess whether patients with ctDNA detected in follow-up blood samples had worse Relapse Free Survival (RFS) than patients without ctDNA detected, using Cox proportional hazards models both standard and time-dependent (eMethods). Secondary endpoints included lead-time between ctDNA detection and relapse using Kaplan-Meier methods, and association between detection of ctDNA in the diagnosis sample prior to neoadjuvant chemotherapy using a Cox proportional hazard model.

Results

Patient cohort

Primary tumor from the 170 patients was sequenced to identify somatic mutations, identifying a mutation in 101 patients, which formed the primary analysis cohort (eFigure3, eTable1). In total, 165 mutations were identified, 78 patients (77.22%) with one mutation and 23 patients (22.78%) with multiple mutations, with median allele frequency (AF) of 26% (eFigure4A). Validated personalized dPCR assay were developed for 150 (91.46%) mutations from 101 patients (eFigure5).

Plasma DNA was extracted from 695 samples (median/patient=7, inter-quartile range (IQR)= 5-8) and analyzed for presence of ctDNA. Buffy coat DNA was analyzed to control for Clonal Hematopoiesis of Indeterminate Potential (eMethods), with CHIP detected in 2.97% (3/101) patients (eFigure6). In blood samples taken at diagnosis prior to any treatment, ctDNA was detected in 51% (41/80) patients, at median AF 0.36% (eFigure4B). Detection of ctDNA at diagnosis associated with relapse free survival (RFS, hazard ratio (HR) 5.8, 95% confidence interval (CI) 1.2-27.1, Figure1A).

Mutation tracking to identify molecular residual disease and anticipate relapse

At median follow-up of 35.5 months, MRD was detected in 16 patients at median AF 0.16% (eFigure4C). Median RFS of patients with ctDNA detected MRD was 38.0 months (95% CI

20.8-undetermined) with median not reached in patients without ctDNA detected (standard HR:16.7, 95% CI 3.45-80.5, P<0.001, Figure1B). The majority of patients with ctDNA detected were negative at the first time-point in follow-up, and became ctDNA positive in a follow-up sample (Figure1B and 1C). To account for this, a Cox time-dependent model was fitted (time-dependent HR:25.2, 95%CI:6.7-95.6, P<0.001, eFigure7). MRD detection remained highly prognostic in a multi-variable model (time-dependent HR:35.7, 95%CI:6.0–212, P<0.001) (eTable 2), adjusted for clinical-pathological factors (subtype, tumor size, nodal status and tumor grade), pathological complete response, and ctDNA detection at diagnosis.

Mutation tracking in breast cancer subtypes

To investigate individual breast cancer subtypes we conducted a combined analysis of the current study with our prior proof-of-principle study (eFigure8).[1] The combined cohort of 144 patients had 210 trackable mutations (eTable3, eFigure9) and 36.3 months median follow–up. MRD was detected in 29 patients, highly prognostic in a standard (HR:17.4, 95%CI:6.3-47.8, P<0.001, eFigure10) and time dependent model (HR:32.8, 95%CI:13.5–79.2, P<0.001, eFigure10), with median lead-time between ctDNA detection and relapse of 10.7 months (95%CI:8.1–19.1, eFigure10). Detection of ctDNA in follow-up was highly prognostic in all major breast cancer subtypes (Figure 2).

We investigated the characteristics associated with ctDNA detection at diagnosis in samples taken prior to treatment. TNBC patients had the highest level of ctDNA (median:4.96 copies/ml, IQR:0-17.0), HER2+ intermediate (median:0.81 copies/ml, IQR:0-5.4), and ER+HER2- the lowest (median:0 copies/ml, IQR:0-4.4) (p=0.0036, eFigure11, eTable4). Detection at diagnosis also associated with larger tumor size (p=0.012) and higher grade (p=0.045).

Metastatic sites not detected by mutation tracking

Of the 26 relapsed patients, 23 (88.4%) relapsed with prior ctDNA detection, whereas 6 (21.6%) patients relapsed without ctDNA detection prior to, nor at, the time of relapse. All six patients had a single site of relapse; three brain only relapse without extracranial relapse, one ovarian solitary metastasis and two solitary locoregional relapse (P=0.016, Table1). Brain only relapse was unlikely to be detected (P=0.0003, Table1), similar to the low rates of ctDNA detection in primary brain tumors[7-9]

Discussion

We present the results of an independent prospective, multi-center, validation study of mutation tracking. Detection of ctDNA in follow-up was strongly prognostic for future relapse, overall and in all major breast cancer subtypes, with ctDNA detected prior to relapse in 96% (22/23) patients with extra-cranial distant metastatic relapse. TNBC cancers had the highest ctDNA level at diagnosis, likely representing high proliferative rates and cell turnover. Detection of ctDNA at diagnosis, before any treatment, was also associated with risk of relapse, suggesting the potential for incorporation of this feature into future prognostic models if validated in future studies.

Clonal haematopoiesis of indeterminate potential (CHIP) is common with increasing age,[10,11] potentially causing false positives in ctDNA analysis.[12-14] We prospectively assessed controls detecting CHIP, all *TP53* mutations, in three patients that would otherwise had generated false-positive ctDNA results. These patients remained relapse free after 18.4, 42.3 and 50.7 months follow-up.

Our results demonstrate a high level of clinical validity for ctDNA mutation tracking with dPCR but do not demonstrate clinical utility. Without evidence that mutation tracking can improve patient outcome, our results should not be recommended yet for routine clinical practice. For example protein tumor marker assessment, with lead times of just few months, did not improve overall survival when assessed in large studies.[15]

Prospective clinical trials are now required to assess whether detection of ctDNA can improve outcome for patients, and we have initiated a phase II interventional trial in triple negative breast cancer (NCT03145961). This may develop a new treatment paradigm for treating breast cancer, where treatment is initiated on molecular relapse, without waiting for symptomatic incurable metastatic disease to develop.

Acknowledgments

This research was funded by Breast Cancer Now, Le Cure, and NIHR funding to the Biomedical Research Centre at The Royal Marsden and The Institute of Cancer Research. ICR-CTSU receives programmatic funding from Cancer Research UK (C1491/A15955 and C1491/A25351) which supported the statistical analysis. The funding organizations had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Isaac Garcia-Murillas, Neha Chopra, Holly Tovey, Rosalind J Cutts and Nicholas C Turner conducted and are responsible for the data analysis. Nicholas C Turner had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Garcia-Murillas I, Schiavon G, Weigelt B, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Science Translational Medicine.* 2015;7(302).
2. Olsson E, Winter C, George A, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. *EMBO Mol Med.* 2015;7(8):1034-1047.
3. Tie J, Wang Y, Tomasetti C, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci Transl Med.* 2016;8(346):346ra392.

4. Reinert T, Scholer LV, Thomsen R, et al. Analysis of circulating tumour DNA to monitor disease burden following colorectal cancer surgery. *Gut.* 2016;65(4):625-634.
5. Abbosh C, Birkbak NJ, Wilson GA, et al. Phylogenetic ctDNA analysis depicts early-stage lung cancer evolution. *Nature.* 2017;545(7655):446-451.
6. Chaudhuri AA, Chabon JJ, Lovejoy AF, et al. Early Detection of Molecular Residual Disease in Localized Lung Cancer by Circulating Tumor DNA Profiling. *Cancer Discov.* 2017;7(12):1394-1403.
7. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med.* 2014;6(224):224ra224.
8. De Mattos-Arruda L, Mayor R, Ng CK, et al. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun.* 2015;6:8839.
9. Merker JD, Oxnard GR, Compton C, et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. *Journal of clinical oncology.* 2018.
10. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nature Medicine.* 2014;20(12):1472.
11. Genovese G, Kähler AK, Robert E. Handsaker, et al. Clonal Hematopoiesis and Blood-Cancer Risk Inferred from Blood DNA Sequence. *New England Journal of Medicine.* 2014;371:26.
12. Hu Y, Ulrich BC, Supplee J, et al. False-Positive Plasma Genotyping Due to Clonal Hematopoiesis. *Clinical Cancer Research.* 2018.
13. Ivey A, Hills RK, Simpson MA, et al. Assessment of Minimal Residual Disease in Standard-Risk AML. *N Engl J Med.* 2016;374(5):422-433.
14. Jongen-Lavrencic M, Grob T, Hanekamp D, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. *N Engl J Med.* 2018;378(13):1189-1199.
15. Henry NL, Hayes DF, Ramsey SD, Hortobagyi GN, Barlow WE, Gralow JR. Promoting quality and evidence-based care in early-stage breast cancer follow-up. *J Natl Cancer Inst.* 2014;106(4):dju034.

Figure Legends

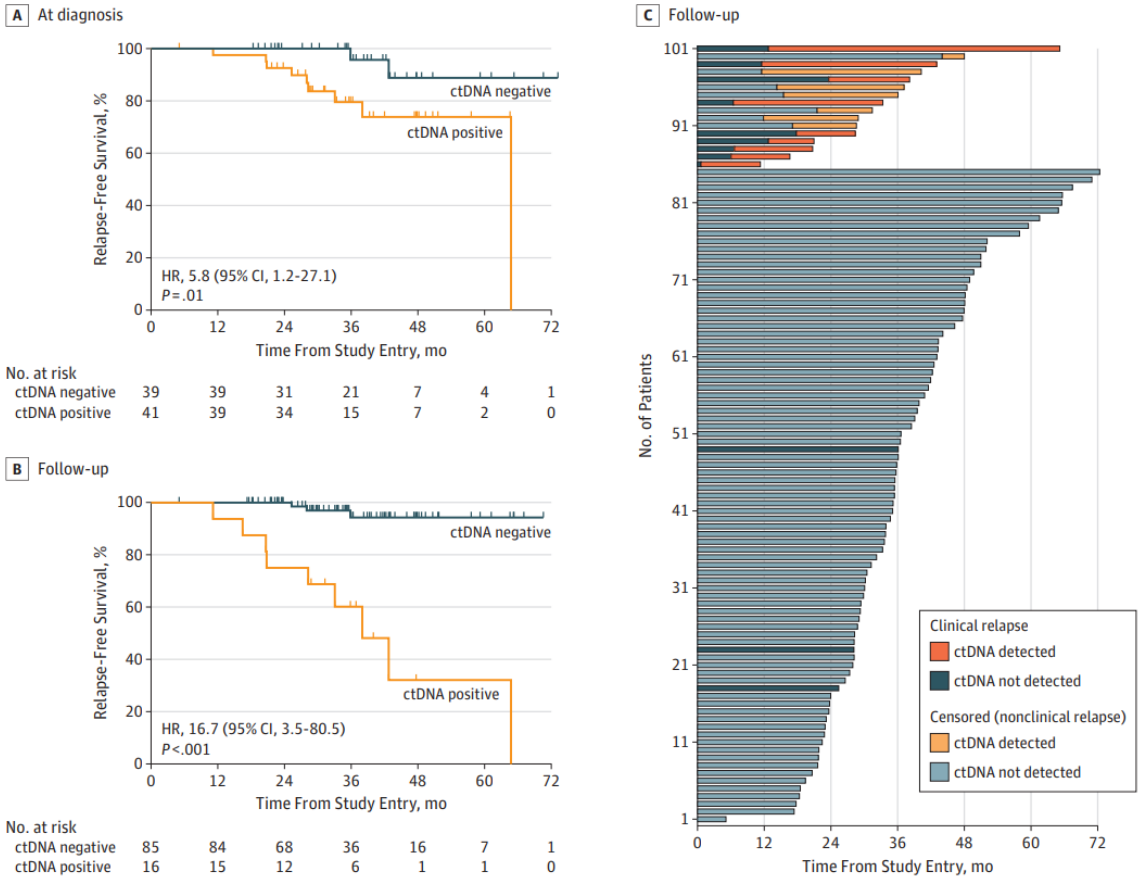

Figure 1: Relapse free survival in patients with ctDNA detected molecular residual disease

(A) Relapse free survival by ctDNA detection at diagnosis prior to any treatment, in patients who subsequently received neoadjuvant chemotherapy.

(B) Relapse free survival in 101 patients with ctDNA detected molecular residual disease in follow-up and patients without ctDNA detected (*left*). The population consisted of 35 estrogen receptor positive and HER2 negative (ER+HER2-), 41 HER2 positive, and 25 triple negative breast cancers (TNBC, eTable1)

(C) Relapse free survival for individual patients with or without ctDNA detection along the study (*right*). Censored patients did not have a clinical relapse at the time of the data collection.

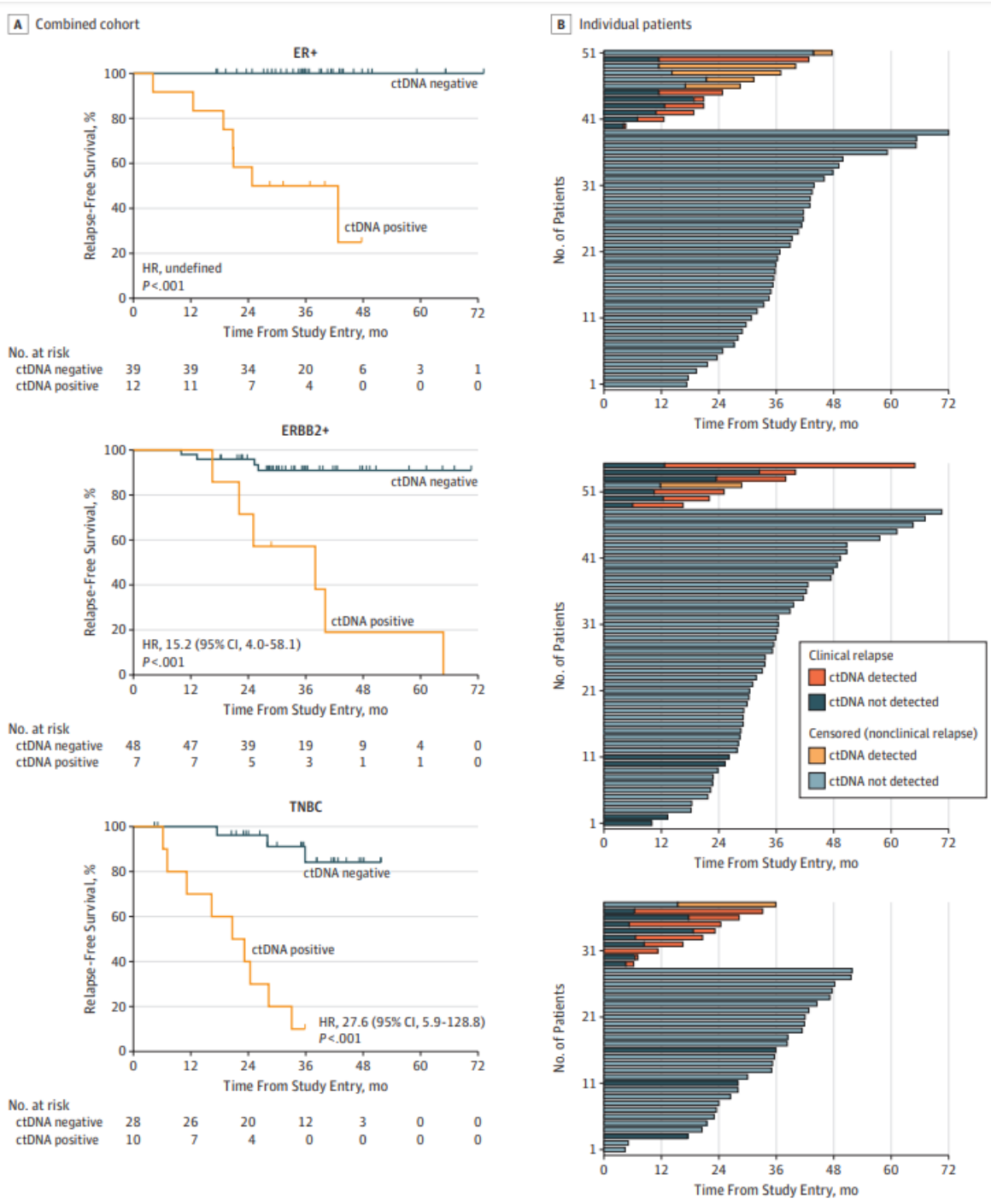

Figure 2: Relapse free survival by tumor subtype in patients with ctDNA detected molecular residual disease.

(A) In the combined cohort, relapse free survival in the major subtypes of breast cancer. ER+HER2- breast cancer - HR was not definable as no patients relapsed in the ctDNA negative group (N=51, P<0.001) with median lead-time 13.3 months (95%CI:2.1–undefined). HER2+ breast cancer - HR 15.2 (N=55, 95%CI:4.0–58.1, P<0.001) with median lead-time

14.5 months (95%CI:7.5-undefined). Triple negative breast cancer (TNBC) - HR 27.6 (N=38, 95%CI:5.9–128.8, P<0.001) with median lead-time 10.6 months (95%CI:0.6–19.1). **(B)** Relapse free survival for individual patients, in the major subtypes of breast cancer, from study entry and during follow-up. Censored patients did not have a clinical relapse at the time of the data collection.

	Recurrence without ctDNA detection	ctDNA detected recurrence	P value
N	6	23	
Median Age (range)	55 (43-65)	51 (45-59)	0.72*
Sites of recurrence			
Single	6	8	0.016
Multiple	0	14	
Brain only	3	0	0.006
Extra-cranial	3	23	
Brain only or loco-regional	5	1	0.0003
Distant extra-cranial	1	22	
Pathology			
IDC	5	19	1
Non-IDC	1	4	
Histological Grade			
Grade 2	0	7	0.28
Grade 3	5	15	
Subtype			
ER+ HER2-	0	7	0.25^{+}
HER2+	3	6	
Triple Negative	3	10	
Clinical size at presentation (cT)			
T2	4	12	0.66
T3/4	2	11	
Nodal status at presentation			
Positive	3	6	0.34
Negative	3	17	

Table 1: Clinical and pathological factors associated with lack of ctDNA detection prior to disease relapse.

P values Fisher's exact text, with the exception of * Mann-Whitney U test and $^{+}$ ChiSquare test.