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Transcript-based circulating tumor and cancer stem cell detection in inflammatory breast cancer: a six-year prospective evaluation of assay yield and prognostic performance

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Abstract

Inflammatory breast cancer (IBC) is a rare and aggressive presentation for which no validated blood-based monitoring tool exists, and the tumor emboli that define its biology have motivated interest in circulating tumor cell (CTC) assays. We prospectively evaluated a transcript-based platform combining epithelial CTC markers (MUC1, HER2, GA733-2) and cancer stem cell (CSC)-associated markers (TWIST, AKT, PI3K, ALDH1) in patients with stage III breast cancer enrolled from 2013 to 2020, comparing 54 patients with IBC against 23 with non-IBC disease across 222 quality-controlled serial samples. Using the manufacturer-defined positivity thresholds, 14% of samples were CTC-positive and 29% CSC-positive. No marker distinguished IBC from non-IBC at baseline: GA733-2 was positive in 7 of 54 IBC versus 1 of 23 non-IBC patients ($p = 0.42$), and the composite positive categories did not differ ($p = 0.71$ for CTC, $p = 0.11$ for CSC). HER2 and TWIST transcripts were undetectable at threshold in all 77 patients. Among 44 IBC patients evaluable for event-free survival over a median 65 months of follow-up, neither baseline CTC positivity (log-rank $p = 0.10$) nor CSC positivity ($p = 0.55$) stratified outcome. Threshold sensitivity analysis showed that detectable signal clustered an order of magnitude below the positivity cutoffs, so that no threshold produced both an adequate positivity rate and outcome discrimination. Positivity at any draw during follow-up, and an additive classification combining both marker classes, likewise showed no association with outcome. Benchmarked against a tumor-informed ctDNA assay evaluated by our group in the same disease setting, the two platforms showed near-identical sample-level positivity (29% versus 27%) and technical QC performance, yet only ctDNA positivity tracked disease trajectory and preceded radiographic relapse. These findings indicate that transcript-based CTC and CSC detection, as implemented here, does not provide clinically actionable risk stratification in IBC, and they support prioritizing sequencing-based circulating tumor DNA and next-generation CTC platforms for minimal residual disease monitoring in this population.

Introduction

Inflammatory breast cancer (IBC) accounts for 1% to 5% of breast cancer diagnoses but a disproportionate share of breast cancer mortality, with dermal lymphatic tumor emboli as its pathognomonic feature [1,2]. Because the disease disseminates through lymphovascular channels from the outset, and because response assessment is complicated by the diffuse nature of the primary presentation, IBC has long been considered a setting in which blood-based disease monitoring could be particularly valuable.

Circulating tumor cells (CTC) are established prognostic biomarkers in breast cancer [3–6], and their presence predicts inferior outcome across disease stages, including in the randomized SWOG S0500 trial[7]. Most clinically implemented platforms, including the FDA-cleared CellSearch system, capture cells using epithelial markers such as EpCAM [8]. This design may systematically miss tumor cells that have undergone epithelial-to-mesenchymal transition (EMT), a process closely linked to acquisition of cancer stem cell properties and therapeutic resistance [9–11]. Transcript-based platforms that add stemness-

associated targets, including ALDH1 and the EMT regulator TWIST, were developed in part to address this gap [12,13].

Whether such combined platforms perform adequately in IBC has not been established. IBC cohorts large enough to evaluate a circulating biomarker prospectively are difficult to assemble, and the assumption that a rare, embolus-forming disease should yield abundant detectable circulating tumor cells has rarely been tested directly. We therefore conducted a prospective evaluation of a combined CTC and CSC transcript assay in patients with stage III breast cancer, enriched for IBC, with serial sampling over six years. Our objectives were to determine assay yield in IBC, to test whether marker prevalence differs between IBC and non-IBC disease, and to assess whether baseline marker status stratifies event-free survival.

Results

Cohort and sampling

Of 81 patients with records in the study database, 77 had CTC and CSC profiling performed, contributing 226 peripheral blood samples. Four samples were excluded for absent beta-actin internal control in one or both assay panels, leaving 222 evaluable samples; no patient lost all draws to quality control exclusion. The analysis population comprised 54 patients with IBC and 23 with non-IBC stage III disease (Fig. 1).

Baseline characteristics are shown in Table 1. Among the 54 IBC patients, median age at diagnosis was 54 years (range, 29 to 85), and receptor subtypes were triple-negative in 19, HR-positive/HER2-negative in 14, HR-positive/HER2-positive in 11, and HR-negative/HER2-positive in 10. Pathologic complete response was achieved in 15 of 48 patients with evaluable response data. IBC patients contributed 164 quality-controlled samples with a median of 3 draws per patient (range, 1 to 7) and median follow-up from the baseline draw of 65.1 months. The non-IBC comparator group was similar in age ($p = 0.59$) and subtype distribution ($p = 0.48$) but accrued far fewer events, with 3 progressions and 1 death versus 26 and 24 in the IBC group, so it supports marker prevalence comparison but not survival comparison. The serial sampling architecture and patient-level marker results are shown in Fig. 2.

Table 1
Baseline characteristics

Characteristic	IBC (n = 54)	Non-IBC (n = 23)	p value
Median age at diagnosis, years (range)	54 (29–85)	56.5 (32–72)	0.59
Stage IIIA	0	5	
Stage IIIB	27	11	
Stage IIIC	19	7	
Stage III with M1 disease	6	0	
Stage III not otherwise specified	2	0	
TNBC	19	9	0.48
HR-positive/HER2-negative	14	9	
HR-positive/HER2-positive	11	3	
HR-negative/HER2-positive	10	2	
Pathologic complete response	15 of 48 evaluable	4 of 22 evaluable	
Samples contributed	164	56	
Median draws per patient (range)	3 (1–7)	2 (1–7)	
Median follow-up, months (range)	65.1 (0.6-121.2)	73.1 (17.4-127.6)	
Progression events	26	3	
Deaths	24	1	

Assay yield

Across all 222 quality-controlled samples, 14% met the threshold for CTC positivity and 29% for CSC positivity. At the patient level, using each patient's earliest evaluable draw, 8 of 54 IBC patients (14.8%) were CTC-positive and 13 (24.1%) were CSC-positive. Two markers were never positive: HER2 and TWIST transcripts did not reach threshold in any of the 77 patients, and TWIST was detectable at any level in only a single patient across the entire cohort. Both markers were therefore uninformative as implemented and were not analyzed further.

Marker prevalence in IBC versus non-IBC

No marker distinguished IBC from non-IBC disease at baseline (Fig. 3c). GA733-2, the epithelial marker most plausibly linked to embolus biology, was positive in 7 of 54 IBC patients (13.0%) versus 1 of 23 non-IBC patients (4.3%), a difference that was directionally consistent with expectation but not statistically

significant (p = 0.42). MUC1 was positive in 3 of 54 versus 1 of 23 (p = 1.00), and PI3K in 9 of 54 versus 2 of 23 (p = 0.49). ALDH1 was positive in 8 of 54 IBC versus 8 of 23 non-IBC patients, a numerically higher prevalence in non-IBC disease (p = 0.067). The composite categories showed the same pattern, with CTC positivity in 14.8% versus 8.7% (p = 0.71) and CSC positivity in 24.1% versus 43.5% (p = 0.11). The four-group classification combining CTC and CSC status did not differ by disease type (p = 0.33).

Detectable signal falls below the positivity thresholds

To understand the low positivity rates, we examined the distribution of actin-normalized expression relative to the assay cutoffs (Fig. 3a). Detectable signal was common but small in magnitude: among samples with any measurable transcript, median normalized values ranged from 0.009 for HER2 to 0.046 for ALDH1, one to two orders of magnitude below the 0.15 and 0.25 positivity thresholds. Only ALDH1 and GA733-2 produced appreciable numbers of values approaching the cutoffs.

We then examined how the positivity rate varies as a function of the chosen threshold (Fig. 3b). The curves fall steeply near zero and flatten well before the manufacturer cutoffs, meaning that the assay output in this cohort is dominated by low-level signal that a threshold either accepts wholesale or rejects wholesale. Defining positivity as any detectable transcript classified 34% of samples as CTC-positive and 66% as CSC-positive, and at the patient level rendered 39 of 41 serially sampled IBC patients ever-positive, a prevalence incompatible with risk discrimination. Applying the manufacturer thresholds to normalized rather than raw values reduced positivity to under 5% for most markers. No intermediate threshold produced both an adequate positivity rate and outcome separation.

Marker status and event-free survival

Forty-four of 54 IBC patients were evaluable for event-free survival, with 16 events and 28 censored observations; 10 patients recorded as having progressed lacked a documented progression date and were excluded rather than censored, since censoring them would bias survival estimates upward (Fig. 1). Median follow-up among censored patients was 85.6 months.

We evaluated marker status four ways (Fig. 4). Baseline CTC positivity was associated with numerically inferior event-free survival, with 2-year estimates of 50% versus 72%, but the difference was not significant and rested on 6 positive patients (log-rank p = 0.10). Baseline CSC positivity showed no association (p = 0.55). Because a single baseline draw may miss transient positivity, we also classified patients by positivity at any draw during follow-up; 28 of 44 patients were ever positive, and this did not stratify outcome (p = 0.48), nor did ever-positivity restricted to CTC markers (p = 0.63) or CSC markers (p = 0.69). Finally, we tested an additive classification counting the number of positive marker classes at baseline, on the hypothesis that concurrent epithelial and stemness positivity might identify a higher-risk group. Only 1 of 44 patients was positive for both classes, 13 were positive for one, and 30 for neither; the additive classification did not stratify outcome (p = 0.28), and the comparison of any positivity versus

none gave p = 0.19. We note that the ever-positive analyses are susceptible to immortal time bias, since a patient must remain in follow-up to accrue a positive draw, and that this bias would act in the direction of favoring the ever-positive group.

Comparison with tumor-informed ctDNA in the same disease setting

To place these results in context, we compared assay performance against a personalized tumor-informed ctDNA assay evaluated by our group in an overlapping disease setting, namely HR-positive stage III IBC without pathologic complete response (NCT02971748)[17]. Restricting the present cohort to the same clinical population yielded 18 patients contributing 59 quality-controlled samples (Table 2). Technical performance was comparable, with sample QC pass rates of 98% and 99%, and sample-level positivity rates were also similar, at 29% for combined CTC or CSC positivity versus 27% for ctDNA. The platforms differed entirely in what a positive result conveyed. In the ctDNA study, positivity tracked disease trajectory, with persistent and rising levels preceding radiographic relapse by up to 18 months, two patients converting from negative to positive 10 and 15 months before clinical progression, and all patients negative at post-operative baseline and throughout treatment remaining progression-free at 2 years. In the present cohort, 11 of 18 patients in the matched subset were ever positive while 9 of 18 progressed, with no correspondence between the two. Notably, 96% of ctDNA-positive calls in that study fell below 0.01% tumor fraction, a range in which the expected number of intact circulating tumor cells in a standard blood draw approaches zero.

Table 2
Platform comparison in HR-positive stage III IBC without pathologic complete response

Parameter	AdnaTest CTC and CSC (present study)	Tumor-informed ctDNA (NCT02971748)
Analyte	Marker transcripts in enriched cells	Cell-free tumor DNA
Patients	18	19 of 23 enrolled with successful panel design
Samples analyzed	59	154
Sample QC pass rate	98% (cohort-wide)	99%
Sample-level positivity	29% (CTC 8%, CSC 24%)	27%
Sampling context	Baseline and serial follow-up	Post-operative MRD surveillance
Association with relapse	None detected	Persistent and rising levels preceded relapse
Lead time over imaging	Not demonstrated	Up to 18 months
Negative predictive value	Not assessable	All sustained-negative patients progression-free at 2 years
Analytical floor	Intact cell capture	96% of positive calls below 0.01% tumor fraction

Because these are separate protocols with non-overlapping analysis populations and different sampling schedules, this is a cross-study benchmark rather than a paired comparison, and the ctDNA data are reported in abstract form.

Discussion

In a prospectively collected IBC cohort with six years of follow-up, a transcript-based assay combining epithelial CTC and stemness-associated CSC markers did not identify patients at higher risk of progression, did not distinguish IBC from non-IBC stage III disease, and yielded positivity in a minority of samples. Two of the seven markers were never positive. We report these findings because negative evaluations of established platforms are underrepresented in the IBC literature, and because the cost of pursuing an unproductive monitoring strategy in a rare disease is high.

The central observation is that assay output was dominated by low-level signal sitting well below the manufacturer-defined thresholds, creating a dichotomization problem with no satisfactory solution. Treating any detectable transcript as positive classifies nearly every patient as positive and discriminates nothing, while applying the defined cutoffs, particularly to normalized values, leaves too

few positive cases to analyze. The comparison with tumor-informed ctDNA in the same disease setting suggests why. In that study, 96% of ctDNA-positive calls fell below 0.01% tumor fraction[17]. At such tumor fractions the expected number of intact circulating tumor cells in a 5 to 10 mL draw approaches zero, meaning that any platform relying on capture of whole cells is operating below its physical detection floor. On this interpretation, the transcript signal we observed is not a diluted version of the disease signal that ctDNA detects; it is a different quantity altogether, plausibly reflecting background transcript carryover from leukocytes or non-malignant epithelial cells rather than residual tumor. That would explain why positivity is common, low in magnitude, and uncorrelated with outcome. This framing also offers a caution for reanalysis of legacy CTC datasets: the choice between raw and normalized values, and between manufacturer and data-derived thresholds, reclassified roughly half of the present cohort and can generate apparent associations that do not reflect biology.

The absence of enrichment for epithelial markers in IBC deserves comment. IBC is defined by dermal lymphatic tumor emboli, and it would be reasonable to predict abundant epithelial transcript detection in blood. GA733-2 was numerically more frequent in IBC, but the difference was not significant and the absolute prevalence was low. One interpretation is that emboli in IBC represent cohesive clusters retained within lymphatic channels rather than a source of abundant single circulating cells amenable to immunomagnetic capture, which would be consistent with reports that CTC clusters possess distinct capture and survival characteristics[14,15]. Testing that interpretation requires platforms that preserve and enumerate clusters, which this assay does not.

Several limitations apply. The non-IBC comparator group was small (n = 23) and accrued only 3 progression events, so it supports prevalence comparison but not survival comparison (expected based on distinct worse survival of IBC). Progression dates were unavailable for 10 IBC patients recorded as having progressed, reducing the survival analysis population and limiting statistical power. Samples were collected between 2013 and 2020 and analyzed on a platform that predates current tumor-informed sequencing approaches, so our findings speak to this assay class rather than to liquid biopsy in IBC generally. Finally, the analyses were exploratory and were not adjusted for multiple comparisons; given that no comparison approached significance, this does not alter the interpretation.

The implication for the field is directional. Blood-based monitoring in IBC remains an important unmet need, and the serial sampling infrastructure described here demonstrates that longitudinal collection is feasible in this population over many years. Our results argue that the effort should be directed toward tumor-informed circulating tumor DNA assays, which detect molecular residual disease (MRD) at allele fractions far below the sensitivity of transcript-based cell capture and have shown lead time over radiographic recurrence in stage III IBC [16], and toward CTC platforms capable of capturing clusters and EMT-phenotype cells independent of epithelial marker expression. The near-identical sample-level positivity rates but starkly divergent clinical utility of the two platforms in Table 2 argue that the relevant question for a circulating biomarker is not how often it is positive but whether positivity corresponds to residual disease, at least based on the platforms we have tested.

As the field converges on ctDNA for MRD detection, this study provides reassurance that little is being lost by that choice in IBC: a technically demanding multi-marker CTC platform added no prognostic information in the same disease setting, and ctDNA can reasonably remain the primary modality.

Methods

Patients and study design

Patients with stage II to IV breast cancer initiating a new line of therapy were prospectively enrolled at The University of Texas MD Anderson Cancer Center from December 2013 through January 2020 under an Institutional Review Board-approved biomarker protocol (PA12-0097). The study was conducted in accordance with the Declaration of Helsinki, and all participants provided written informed consent. Eligibility required histologically confirmed breast cancer, age 18 years or older, and ECOG performance status 0 to 2. Patients with local or regional recurrence, or a non-breast malignancy within the preceding two years, were excluded. Peripheral blood was collected at baseline and every six months (plus or minus three months) for up to five years or until recurrence, progression, second primary malignancy, death, or loss to follow-up. IBC status was assigned clinically according to international consensus criteria[1] and recorded in the study database.

Sample processing and marker detection

Blood was collected in ACD-A tubes (BD, Cat# 364606) and processed using AdnaTubes (Qiagen, Cat# T-1-610-R). Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, followed by immunomagnetic enrichment using EpCAM- and MUC1-coated beads on an AutoMACS Pro Cell Separator (Miltenyi Biotec). RNA was extracted and reverse-transcribed for PCR-based detection of epithelial CTC markers (MUC1, HER2, GA733-2; AdnaTest BreastCancer Detect) and CSC-associated markers (TWIST, AKT, PI3K, ALDH1; AdnaTest EMT-2/StemCell). Beta-actin served as the internal control for each panel.

Positivity definition and quality control

Samples were required to have detectable beta-actin in each panel for that panel to be evaluable; samples failing this criterion were excluded from analyses involving the affected panel. Positivity was defined using the manufacturer-specified thresholds applied to raw marker values: 0.15 or greater for any epithelial CTC marker and 0.25 or greater for any CSC-associated marker. Actin-normalized values (marker value divided by the corresponding panel beta-actin value) were used for descriptive distributional analyses and for the prespecified threshold sensitivity analysis. Because the manufacturer thresholds were established for raw values and have not been validated for normalized ratios, we report both, and the sensitivity analysis in Fig. 3b displays positivity rates across the full range of candidate thresholds for raw and normalized values in both panels.

Statistical analysis

The baseline sample was defined as each patient's earliest quality-control-passing draw. Marker prevalence was compared between IBC and non-IBC patients using Fisher exact tests for binary positivity and Mann-Whitney tests for continuous normalized values; the four-group distribution was compared using a chi-square test. Event-free survival was measured from the baseline draw to documented progression, with patients not experiencing progression censored at last follow-up. Patients recorded as having progressed but lacking a progression date were excluded from survival analyses rather than censored, since censoring them would systematically overestimate survival. Survival was estimated using the Kaplan-Meier method with log-rank comparison, and the four-group comparison used a multivariate log-rank test. All tests were two-sided, no adjustment for multiple comparisons was applied, and analyses were exploratory. Analyses were performed in Python 3.12 using pandas, SciPy, and lifelines.

Data availability

De-identified data supporting the findings of this study are available from the corresponding author on reasonable request.

Code availability

Analysis and figure generation code is available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare no competing interests.

Author Contribution

B.L. conceived and designed the study and supervised the work. J.M.R. and H.G. developed and performed the CTC and CSC assays. D.C.O., M.K., E.R.L., J.H., L.X., A.S. curated clinical data and performed analyses, created figures and tables. V.V. contributed to patient enrollment and clinical oversight. E.R.L, L.X., and B.L. drafted the manuscript, and all authors reviewed and approved the final version.

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Data Availability

De-identified data supporting the findings of this study are available from the corresponding author on reasonable request.

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Figures

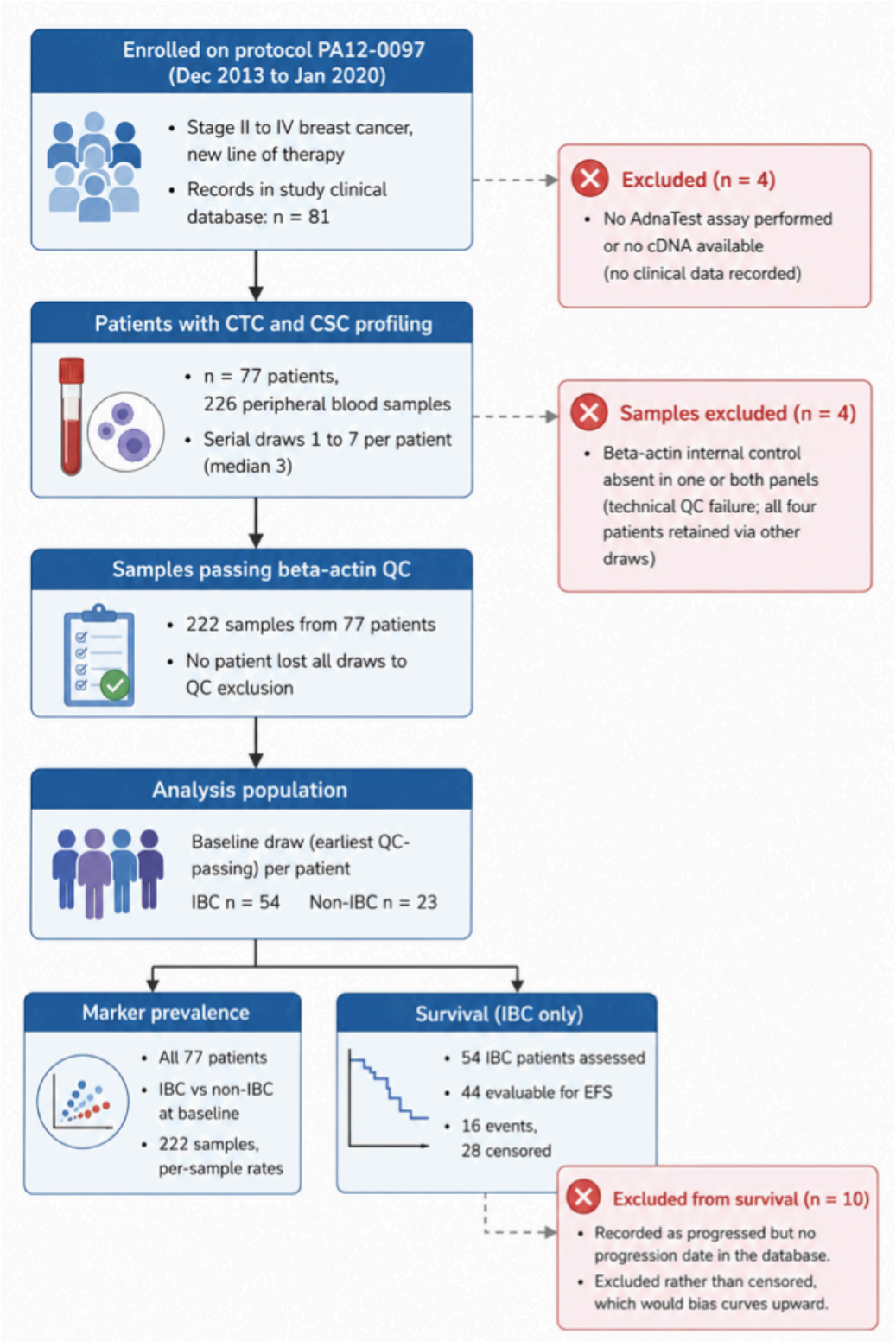


Figure 1

Study flow diagram. Patient and sample accounting from enrollment through the marker prevalence and survival analysis populations, with reasons for exclusion at each stage.

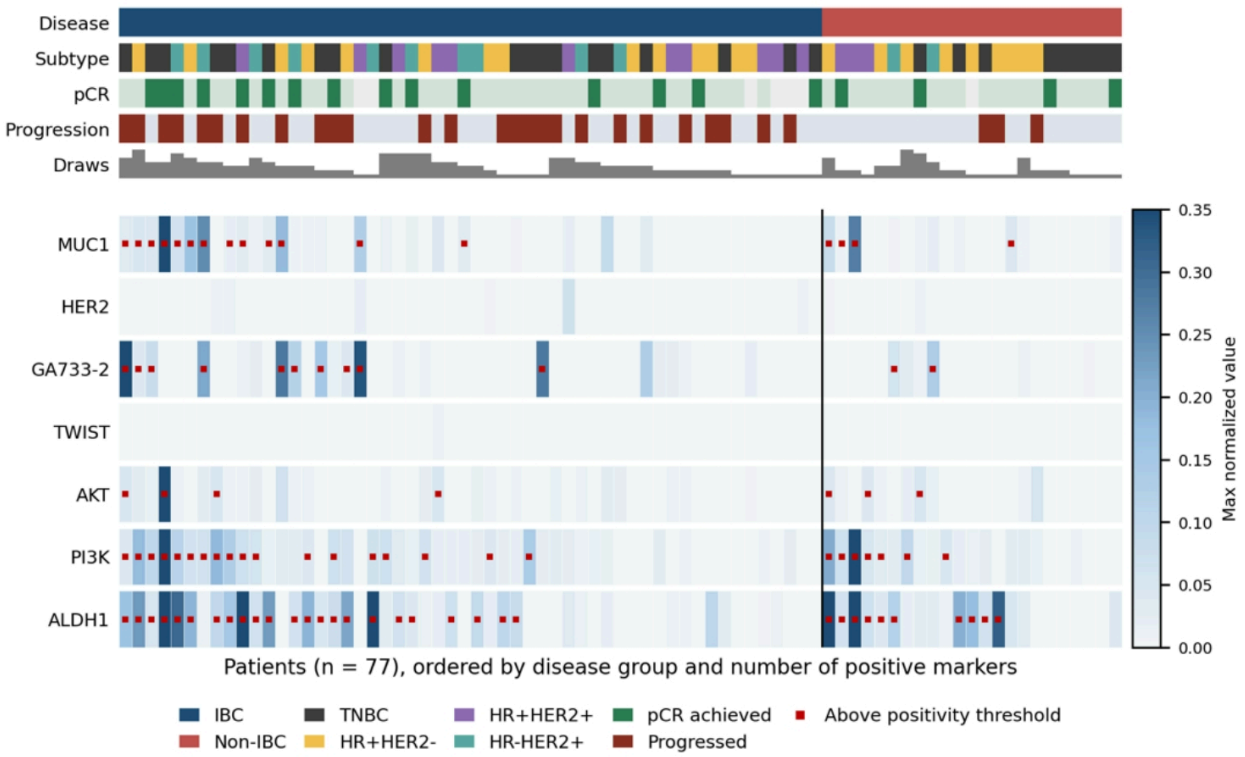


Figure 2

Patient-level marker results. Columns represent the 77 analyzed patients, ordered by disease group and number of positive markers, with IBC to the left of the vertical divider. Cells are shaded by each patient's maximum actin-normalized value across all draws, and red squares mark values reaching the positivity threshold at any draw. Annotation tracks above show disease group, receptor subtype, pathologic complete response, progression status, and number of draws contributed.

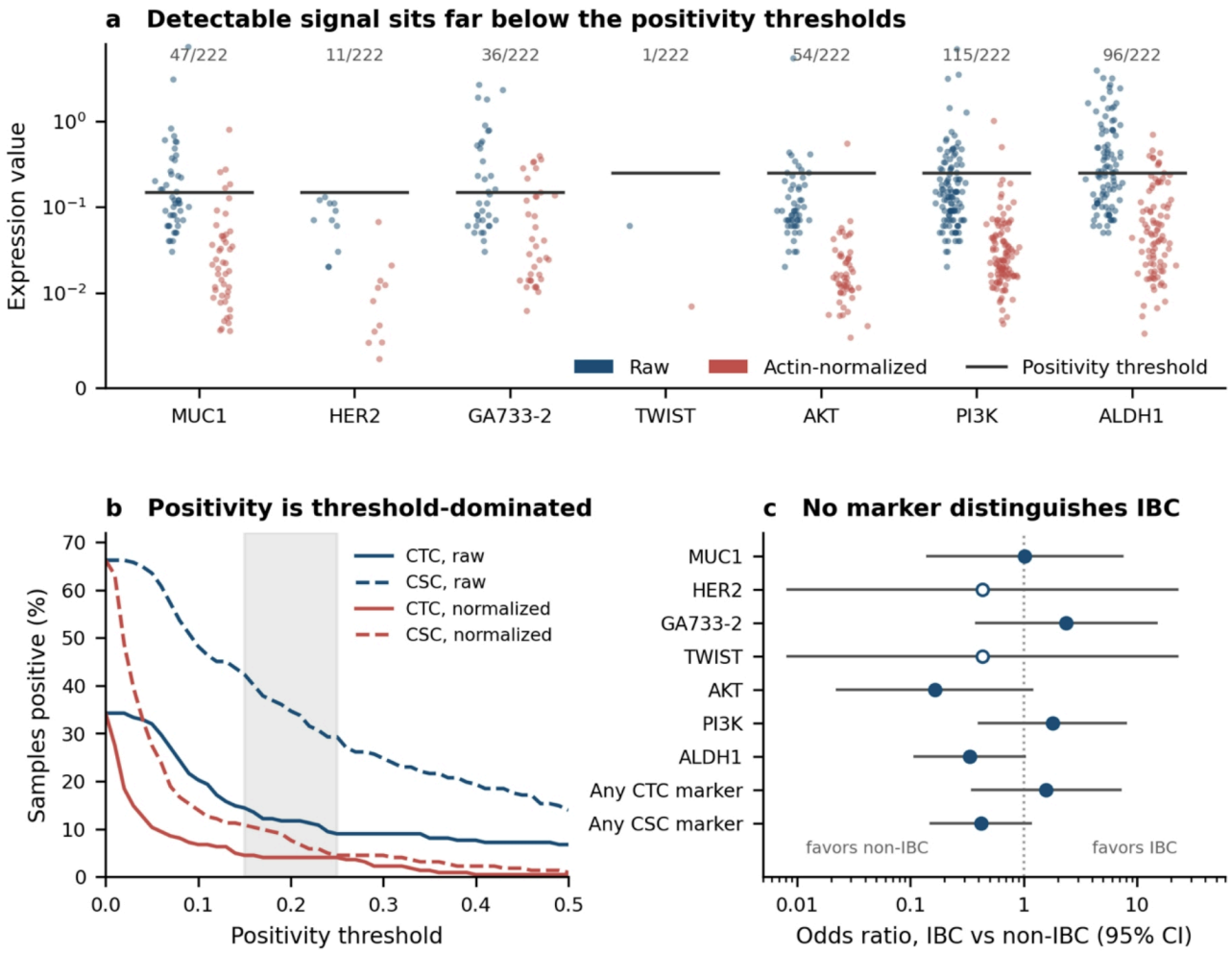


Figure 3

Assay performance. a Raw and actin-normalized expression values for each marker across all 222 quality-controlled samples, with the positivity threshold shown as a horizontal line and the fraction of samples with detectable raw signal above each marker. The vertical axis is symmetric-log scaled. **b** Percentage of samples classified positive as a function of the chosen threshold, for the CTC and CSC panels using raw and normalized values; the shaded band spans the manufacturer thresholds. **c** Odds ratios with 95% confidence intervals for baseline marker positivity in IBC versus non-IBC patients. Open circles denote markers with zero positive patients in both groups, for which the estimate derives from continuity correction and is uninformative.

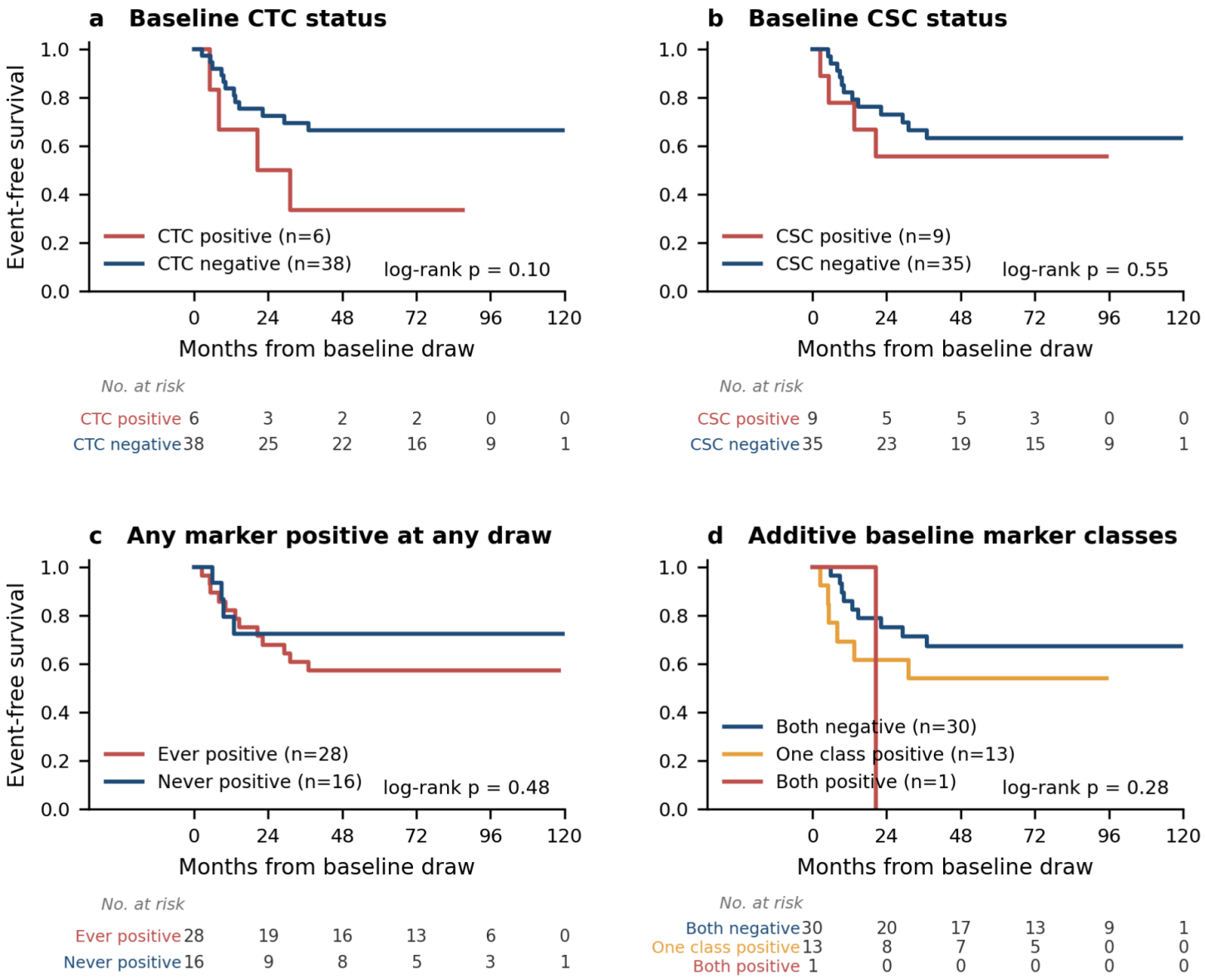


Figure 4

Event-free survival by marker status in IBC. a Baseline CTC status. **b** Baseline CSC status. **c**Positivity for any marker at any draw during follow-up. **d** Additive classification by number of positive marker classes at baseline. Numbers at risk are shown below each panel, and p values are from log-rank tests, with panel d using a multivariate log-rank test across three groups.