

ORIGINAL ARTICLE

Combining circulating tumor cells and tumor-derived extracellular vesicles for prognosis stratification in patients with metastatic breast cancer

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Background: Treatment strategies for patients with metastatic breast cancer (MBC) are becoming increasingly complex, underscoring the need for reliable biomarkers to inform clinical decision making. Circulating tumor cells (CTCs) are well-established prognostic biomarkers in MBC, while tumor-derived extracellular vesicles (tdEVs) have recently demonstrated complementary significance in patients undergoing first-line chemotherapy. This study seeks to validate the complementary role of tdEVs in a larger patient population and establish a clinically relevant cut-off.

Materials and methods: Archived baseline CellSearch® images from 355 patients with MBC (NU16B06 study) were reanalyzed using the Automated CTC Classification, Enumeration and PhenoTyping (ACCEPT) software to quantify CTCs and tdEVs. Multiple tdEV cut-offs were evaluated for association with overall survival (OS), alone and with CTCs (≥ 5 , CTC^{high}). The selected cut-off was validated in a subcohort of 385 patients from the phase III STIC CTC trial.

Results: In the NU16B06 cohort, higher tdEV levels were consistently associated with shorter OS, regardless of cut-off. As models performed similarly (Brier Score difference < 0.005), a cut-off of > 20 tdEVs/7.5 ml (tdEV^{high}) was selected based on prior literature and clinical relevance. Patients with tdEV^{high} had nearly doubled risk of death compared with tdEV^{low}. Combining tdEVs with CTCs identified distinct prognostic subgroups, with CTC^{high}/tdEV^{high} patients showing the shortest OS. Among CTC^{low} patients, tdEV^{high} was associated with increased mortality risk. In the STIC CTC cohort, the cut-off was validated for OS and progression-free survival, with tdEV^{high} consistently associated with poorer survival. When combined, patients with CTC^{high}/tdEV^{high} had the poorest outcomes, while tdEV^{high} remained associated with worse prognosis among CTC^{low} patients.

Conclusion: These findings support the use of tdEVs as complementary prognostic biomarkers to CTCs, potentially enhancing clinical trial design and informing treatment decisions in MBC.

Key words: biomarkers, breast cancer, circulating tumor cells, extracellular vesicles, liquid biopsy, prognosis

INTRODUCTION

Breast cancer (BC) is the second leading cause of cancer-related mortality in women and the incidence of metastatic disease continues to rise.¹ Although therapeutic

advances have extended survival in patients with metastatic breast cancer (MBC), the increasing complexity of treatment options underscores the need for biomarker-driven strategies to optimize clinical decision making. The intrinsic heterogeneity of MBC² further emphasizes the urgency for reliable tools to inform therapy selection and support drug development.

Over the past decade, liquid biopsy has emerged as a minimally invasive approach capable of providing dynamic, *real-time* insights into tumor biology. The study of blood-based biomarkers has shown promise for improving prognostication, treatment selection, and monitoring in MBC.

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Among these, circulating tumor cells (CTCs) were the first to demonstrate prognostic significance in MBC.³ Enumeration of CTCs using the Food and Drug Administration (FDA)-cleared CellSearch® system (Menarini Silicon Biosystems Inc., Huntingdon Valley, PA) has been extensively validated across multiple studies and has consistently been shown to stratify MBC patients' prognosis independently of clinical and pathological features, with recent evidence supporting its clinical utility.⁴

In recent years, attention has focused on extracellular vesicles (EVs), a heterogeneous population of lipid-bound particles released by cells into the extracellular space, which act as mediators of intercellular communication and play a crucial role in cancer progression and metastasis.⁵ Among these, large tumor-derived EVs (tdEVs), ranging from 1 to 12 µm in size, can be co-isolated with CTCs following immunomagnetic epithelial cell adhesion molecule (EpCAM)-based enrichment. These particles, once considered background debris, have been demonstrated to be clinically relevant biomarkers in different solid tumors reflecting tumor burden and biological aggressiveness.⁶⁻⁹ In patients with MBC, elevated tdEV levels were observed in approximately three-quarters of cases and associated with worse survival outcomes.^{7,10} Moreover, data from the SWOG S0500 trial showed that tdEVs provided complementary prognostic information when evaluated alongside CTCs in patients with hormone-insensitive MBC receiving first-line chemotherapy.¹⁰ These findings suggest that in addition to CTCs, tdEVs may represent a valuable biomarker to refine prognostic stratification and inform treatment strategies in MBC using a single blood-based assay. However, their broader clinical applicability and optimal threshold definition remain insufficiently validated.

The present study was designed to address these gaps by evaluating the independent and complementary prognostic value of tdEVs in a broader cohort of patients with MBC. In addition, we aimed to identify a clinically meaningful tdEV cut-off and to validate its prognostic relevance in an independent phase III clinical trial population, to support their integration into future clinical research and, ultimately, their evaluation for routine clinical use.

MATERIALS AND METHODS

Study population and design

This was a retrospective, observational study conducted in two independent cohorts. The training set was based on the NU16B06 cohort of patients with BC prospectively enrolled between 2016 and 2021 at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University (Chicago, IL) under an institutional review board-approved Investigator Initiated Trial (NU16B06). All patients provided written consent to the study protocol. For this analysis, we included all patients with histologically confirmed MBC, regardless of subtype, who had a blood sample collected before initiating a new line of therapy and digitally stored CellSearch® images available. Baseline staging, treatment assignment, and follow-up were carried out according to the investigators' choice. Clinical information was gathered

through electronic medical record review. This population was used to validate the independent and complementary prognostic value of tdEVs in combination with CTCs, and to identify a clinically relevant tdEV cut-off. These findings were subsequently confirmed in an independent validation cohort of patients treated at Institut Curie (France) within the STIC CTC phase III randomized clinical trial (NCT01710605), between 2012 and 2016. A detailed description and results of the trial have been previously published.^{11,12} Briefly, patients with hormone receptor (HR)-positive/human epidermal growth factor receptor 2 (HER2)-negative MBC were randomly assigned to a clinician-driven versus CTC-driven choice of first-line treatment. A national ethics committee approved the STIC CTC clinical trial, and all patients provided written informed consent before enrollment. Reporting of the present study followed the reporting recommendations for tumor marker prognostic studies (REMARK).¹³

Sample preparation and image acquisition

For both cohorts, at baseline 7.5 ml of peripheral blood was collected into CellSave™ Preservative Tubes (Menarini Silicon Biosystems Inc.), maintained at room temperature, and processed within 96 hours using the FDA-cleared CellSearch® system, as previously described.¹⁴ Briefly, the system enriches the sample for objects expressing EpCAM with antibody-coated ferrofluids. Enriched objects are then identified through immunofluorescent staining of cytokeratins (CKs) and leukocyte antigen CD45, and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI). Image acquisition of the cartridge content was carried out using the CellTracks Analyzer II® and candidate CTCs (CK+CD45-DAPI+) were enumerated through a semiautomated imaging algorithm, verified by an operator. Fluorescence images of processed samples were stored on digital versatile disks.

Automated CTC and tdEV enumeration

For the automated CTC and tdEV enumeration, the digitally stored fluorescence image files were reanalyzed with the open-source Automated CTC Classification, Enumeration and Phenotyping (ACCEPT) software (<http://github.com/LeonieZ/ACCEPT>) using the 'Full Detection' function.¹⁵ CTCs were defined as EpCAM+, CK+, DAPI+, CD45– particles of a diameter > 4 µm, while tdEVs were identified as EpCAM+, CK+, DAPI–, CD45– particles of a diameter < 14 µm (Supplementary Figure S1, available at <https://doi.org/10.1016/j.esmoop.2026.108331>). The analyzed tdEVs therefore represent CellSearch/ACCEPT-defined extracellular tumor-associated particles identified through phenotypic and morphometric criteria, rather than EVs fully characterized according to current International Society for Extracellular Vesicles (ISEV) recommendations.¹⁶ Previously optimized and published gates for CTC and tdEV identification were applied to archived images and the counts of objects falling within them were automatically extracted (Supplementary Table S1, available at <https://doi.org/10.1016/j.esmoop.2026.108331>).⁷ All evaluations were carried out blinded to clinical data.

Statistical analysis

Clinical and pathological variables were reported using descriptive statistics. Categorical variables were reported as absolute and relative frequencies, and continuous variables as medians with interquartile ranges (IQRs). Group comparisons were carried out using the Mann–Whitney *U* test or Kruskal–Wallis test for continuous variables, and chi-square or Fisher exact test for categorical variables, as appropriate. Linear regression was used to assess correlations between biomarkers. The association between manual and ACCEPT CTC counts, and between ACCEPT CTCs and tdEVs, was assessed using Spearman rank correlation coefficient. Overall survival (OS) was defined as the time from baseline blood draw (NU16B06) or from randomization (STIC CTC) to death from any cause, with censoring at last follow-up if the patient was still alive. Progression-free survival (PFS) was assessed solely in the STIC CTC cohort and defined as the time from randomization to disease progression per RECIST v1.1 criteria, death, or last follow-up. Median OS and PFS were estimated using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Cox proportional hazards regression analysis was used to determine the univariable hazard ratios for OS with 95% confidence interval (CI) for each variable. A final multivariable Cox model for OS was fit including known prognostic variables and potential confounding variables identified from univariable analyses. For CTCs, patients with ≥ 5 CTCs/7.5 ml were classified as CTC^{high}, and those with < 5 CTCs/7.5 ml as CTC^{low}.¹⁷ For tdEVs, a range of cut-off values were evaluated in the NU16B06 cohort (the training set) and selected based on prior literature [< 20 (low), 20–79 (intermediate), and ≥ 80 (high)]^{7,10} and the observed tdEV distributions in the population (i.e. median value, optimal value [obtained by using the Youden index based on a time-dependent receiver operating characteristic curve], and using percentile-based divisions). Predictive performance of the models based on the various cut-offs was compared using the Brier Score. The tdEV cut-off selected to stratify patients into prognostic groups was then validated in the STIC CTC subcohort (the validation set). Training and validation cohorts were analyzed separately, with the STIC CTC population used exclusively for external validation of the predefined tdEV cut-off. The sample size was determined based on the availability of patients with complete data on CTC and tdEV enumeration in both the training and validation cohorts. Flow of patients through the study is reported in [Supplementary Figure S2](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>. Statistical analyses were carried out using R statistical software (version 4.2.2 and 4.4.1; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

NU16B06 cohort characteristics

Three hundred and fifty-five patients with MBC were eligible and included in the analysis. The baseline clinical

and pathological characteristics of the study population are reported in [Supplementary Table S2](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>. The median age at the time of blood draw was 56 years (IQR 46–64 years). The most represented histological subtype was ductal (72%) followed by lobular (13%) carcinoma. Eighty-three patients (23%) had a diagnosis of inflammatory BC (IBC). One hundred eighty-eight patients (53%) had HR-positive/HER2-negative BC, 69 (19%) had HER2-positive BC, and 97 (27%) had triple-negative BC (TNBC). Among the study population, 43% of patients had ≥ 3 metastatic sites, with visceral metastases documented in 67% of cases, while nonvisceral metastases were present in 89%.

The majority of the patients (65%) were initiating first-line ($n = 185$) or second-line treatment ($n = 47$) for their metastatic disease at the time of blood draw. The type of treatment administered was mainly represented by chemotherapy (31%), endocrine therapy with or without targeted therapy (namely CDK4/6 inhibitors, mTOR inhibitors, or PI3K inhibitors) (24%), anti-HER2 targeted therapy (22%), and immunotherapy-containing regimens (11%). The estimated median follow-up time, as calculated by the reverse Kaplan–Meier method, was 30 months (95% CI 26.5–33.6).

Enumeration of tdEVs and CTCs in the NU16B06 cohort

The median tdEV count in the NU16B06 population was 20 (IQR 3–107). The association between tdEVs and clinical characteristics is shown in [Supplementary Table S2](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>. Higher tdEVs were observed in patients with invasive lobular carcinoma (ILC) as compared with ductal BC ($P = 0.006$) and in patients with non-IBC than IBC ($P = 0.046$). Patients with a diagnosis of HER2-positive BC had lower tdEV count compared with HR-positive/HER2-negative and TNBC ($P = 0.004$). Higher tdEVs were also associated with the CTC manual count and the number of metastatic sites ($P < 0.001$ for both). Similar associations were observed between CTCs and clinical characteristics ([Supplementary Table S2](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). The median ACCEPT CTC count was 3 (IQR 1–13), strongly correlated with the manual count (Spearman $\rho = 0.848$, $P < 0.001$) ([Supplementary Figure S3](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>) and showed similar association with OS [hazard ratio and 95% CI 2.28 (1.69–3.07) for manual count and 2.37 (1.76–3.21) for ACCEPT count] ([Supplementary Figure S4](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). ACCEPT CTC counts were therefore used for all subsequent analyses.

tdEV and CTC counts were significantly correlated (Spearman $\rho = 0.796$; $P < 0.001$) ([Supplementary Figure S5](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). tdEVs were detected even in patients with no CTCs ($n = 87$, 25%; median tdEV = 2, IQR 0–6) and only 31 patients (9%) had undetectable tdEVs.

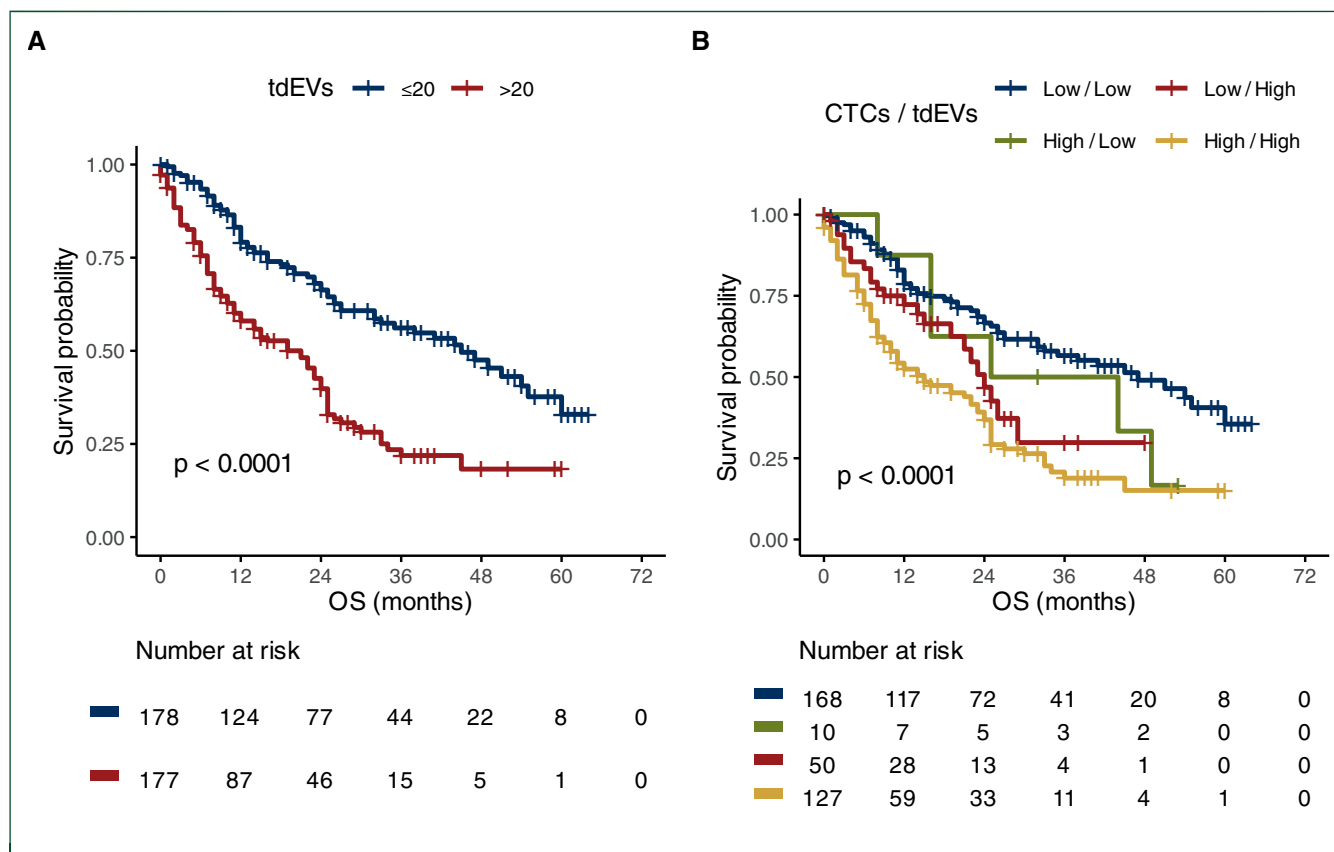


Figure 1. Kaplan–Meier plots for OS of the NU16B06 population by baseline tdEVs (A) and combined CTCs/tdEVs (B). CTC, circulating tumor cell; OS, overall survival; tdEV, tumor-derived extracellular vesicle.

Association of tdEVs with outcomes in the NU16B06 population (training set)

The association of tdEV with OS was assessed using various predefined cut-off values including previously defined 20–80, median value, optimal value (= 67 tdEVs, [Supplementary Figure S6](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>) and percentile-based divisions. Through a systematic evaluation, including univariable and multivariable Cox regression for each cut-off, we observed that multiple thresholds for tdEVs were associated with OS ([Supplementary Tables S3–S7](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). As the performance of models was similar (Brier Score difference <0.005, [Supplementary Figure S7](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>), the cut-off of 20 tdEVs/7.5 ml (>20, tdEV^{high}) was selected based on its clinical relevance informed by prior literature (normal range of 0–20 tdEVs per 7.5 ml of blood in healthy donors and association with outcomes)^{7,10} and easier clinical applicability of a single cut-off. Median OS was significantly shorter in patients with tdEV^{high} versus tdEV^{low} (21 versus 45 months, hazard ratio 2.38, $P < 0.001$; [Figure 1A](https://doi.org/10.1016/j.esmoop.2026.108331)), confirmed in multivariable analysis ([Table 1](https://doi.org/10.1016/j.esmoop.2026.108331)). The prognostic relevance of tdEVs was further supported by sensitivity analyses modeling tdEV as a continuous variable ([Supplementary Table S8](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). The combination of tdEVs with CTCs identified a subgroup of patients with very favorable prognosis (CTC_{low}/tdEV_{low}

median OS 47 months), while those with high levels of both biomarkers had the shortest survival (median OS 15 months; [Figure 1B](https://doi.org/10.1016/j.esmoop.2026.108331)). In univariable analysis, among CTC_{low} patients ($n = 218$), those with tdEV^{high} (23%) had increased risk of death (hazard ratio 1.78, 95% CI 1.09–2.88, $P = 0.020$) compared with tdEV_{low}. No significant difference in OS was observed between CTC^{high}/tdEV_{low} and CTC_{low}/tdEV_{low} (hazard ratio 1.57, 95% CI 0.68–3.65, $P = 0.29$), while CTC^{high}/tdEV^{high} had the highest risk (hazard ratio 2.68, 95% CI 1.93–3.74, $P < 0.001$). Multivariable analysis confirmed these trends ([Table 2](https://doi.org/10.1016/j.esmoop.2026.108331)), although the difference between CTC_{low}/tdEV_{low} and CTC_{low}/tdEV^{high} was no longer statistically significant (hazard ratio 1.53, 95% CI 0.93–2.53).

Association of tdEVs with outcomes in the STIC CTC population (validation set)

A subcohort of the STIC CTC phase III trial ($n = 385$) was used as validation set. Characteristics of this population and association with tdEV and CTC counts are shown in [Supplementary Table S9](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>. Median tdEV count was 17 (IQR 5–66). Patients with >20 tdEV/7.5 ml had shorter OS ([Figure 2A](https://doi.org/10.1016/j.esmoop.2026.108331)); tdEV^{high} were associated with worse OS in both univariable (hazard ratio 2.35, 95% CI 1.73–3.19, $P < 0.001$) and multivariable analysis (hazard ratio 2.28, 95% CI 1.52–3.34, $P < 0.001$; [Supplementary Table S10](https://doi.org/10.1016/j.esmoop.2026.108331), available at <https://doi.org/10.1016/j.esmoop.2026.108331>). When combined with CTCs, patients with

Table 1. Multivariable Cox regression analysis for overall survival using the median cut-off for tdEVs (20 tdEV/7.5 ml) in the NU16B06 population

Characteristic	N	HR [95% CI]	P value
CTCs	355		0.040
<5		Reference	
≥5		1.51 [1.02-2.25]	
tdEVs	355		0.019
≤20		Reference	
>20		1.65 [1.09-2.52]	
IHC subtype	355		<0.001
HR+/HER2–		Reference	
HER2+		0.71 [0.44-1.15]	
HR–/HER2–		2.42 [1.74-3.37]	
Unknown		0.00 [0.00-Inf]	
Therapy line	355		<0.001
<3		Reference	
≥3		1.69 [1.24-2.29]	
Isolated bone metastases	355		0.021
No		Reference	
Yes		0.43 [0.21-0.88]	
Visceral metastasis	355		0.3
No		Reference	
NA		0.77 [0.48-1.23]	
Yes		1.20 [0.65-2.23]	
Number of metastatic sites	355		0.4
<3		Reference	
≥3		1.21 [0.81-1.79]	

CI, confidence interval; CTC, circulating tumor cell; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; HR+, hormone receptor positive; IHC, immunohistochemistry; NA, not available; tdEV, tumor-derived extracellular vesicle.

CTC_{low}/tdEV^{high} and CTC^{high}/tdEV^{high} had worse OS compared with patients with CTC_{low}/tdEV_{low} (hazard ratio 1.96, 95% CI 1.15-3.33 and hazard ratio 2.74, 95% CI 1.82-4.11, respectively; Table 3 and Figure 2B). In contrast, high CTC levels in the absence of elevated tdEVs (CTC^{high}/tdEV_{low}) did not significantly affect OS (hazard ratio 0.96, 95% CI 0.50-1.84). The tdEV cut-off derived from the training cohort was subsequently applied to assess PFS in the independent validation cohort, where prospective and standardized RECIST-based assessments ensured reliable PFS measurement. Similar patterns were observed for PFS (Figure 2C and D), with patients in the CTC^{high}/tdEV^{high} group experiencing the poorest outcomes and elevated tdEVs alone still associated with an increased risk in the CTC_{low} group, further supporting the independent and complementary prognostic role of tdEVs.

DISCUSSION

In this retrospective study involving two independent cohorts of patients with MBC, we demonstrated that the number of tdEVs before treatment, quantified through automated analysis of CellSearch® images, was independently associated with survival outcomes. Importantly, tdEVs provided complementary prognostic information to CTCs, identifying subgroups of patients with distinct outcomes. Among patients with low CTC counts, typically considered to have favorable prognosis,¹⁷ tdEVs may reveal a subgroup (tdEV^{high}) with poorer outcomes, corresponding to nearly a twofold higher risk of death, that would otherwise remain undetected. This finding is clinically meaningful, as this represents 23% and 11% of the population in the training and validation cohorts, respectively.

Even among patients with high CTC counts, for whom prognosis is generally poor,¹⁷ tdEVs provided additional prognostic information. Within this subgroup, CTC^{high}/tdEV_{low} patients had better outcomes, remarkably not significantly different from those with CTC_{low}/tdEV_{low}, suggesting that tdEVs may have a more pronounced prognostic impact than CTCs. This interpretation is further supported by multivariable analyses, where only tdEVs remained significantly associated with patient outcomes in most models when considered separately from CTCs.

These findings build upon prior evidence supporting the clinical relevance of tdEVs in MBC.^{7,10} In the SWOG S0500 clinical trial, tdEVs were shown to provide prognostic information complementary to CTCs¹⁰; however, that analysis was restricted to patients receiving first-line chemotherapy, limiting the ability to exclude potential therapy-related influences and the assessment of their broader clinical applicability.¹⁰ The present study extends these observations by validating the prognostic value of tdEVs across a real-world population exposed to different treatment strategies, and by independently confirming these findings in a prospective phase III clinical trial cohort. Together, these results support the robustness and generalizability of tdEVs as complementary prognostic biomarkers beyond the specific context of first-line chemotherapy. tdEV analyses have not been previously reported for either the NU16B06 or the STIC CTC cohorts. While these patient populations have been included in prior publications focusing on CTC-based analyses, the present study represents the first investigation of tdEVs in these cohorts. Across cohorts, we observed a median tdEV count of 20 and 17 for the NU16B06 and STIC CTC populations, respectively, with a wider distribution in the former. This likely reflects differences in cohort composition, as the STIC CTC study includes a homogeneous clinical trial population of untreated patients with HR-positive/HER2-negative MBC, which may explain the narrower tdEV distribution. In contrast, the NU16B06 cohort includes a more heterogeneous population, with patients across all subtypes and treatment settings. In this context, we observed a trend toward higher tdEV levels with increasing lines of therapy, suggesting that cumulative disease burden and treatment exposure may contribute to greater interpatient variability. This interpretation aligns with previous studies including pretreated and clinically heterogeneous populations of patients with MBC reporting higher and more variable tdEV levels.^{7,10} Notably, a finding consistently observed across cohorts was the significantly higher tdEV levels in patients with ILC compared with ductal histology, an association not previously investigated in tdEV studies. While a higher count of CTCs in ILC has been previously described^{18,19} and is also observed in our cohort—potentially reflecting the impaired cell–cell adhesion characteristic of this subtype—the biological mechanisms underlying increased tdEV levels in this subgroup remain to be elucidated.

Beyond introducing tdEV analyses, this work supports the implementation of a fully automated approach for CTC enumeration, allowing a comprehensive and reproducible

Table 2. Multivariable Cox regression analysis of overall survival for the combined tdEV and CTC assessment in the NU16B06 population

Characteristic	N	HR [95% CI]	P value
Combined automated CTCs (<5 versus ≥5) and tdEVs (median cut-off: ≤20 versus >20)	355		<0.001
CTCs low/tdEVs low		Reference	
CTCs low/tdEVs high		1.53 [0.93-2.53]	
CTCs high/tdEVs low		1.22 [0.52-2.86]	
CTCs high/tdEVs high		2.49 [1.74-3.56]	
IHC subtype	355		<0.001
HR+/HER2–		Reference	
HER2+		0.71 [0.44-1.15]	
HR–/HER2–		2.44 [1.75-3.41]	
Unknown		0.00 [0.00-Inf]	
Therapy line	355		<0.001
<3		Reference	
≥3		1.70 [1.25-2.32]	
Isolated bone metastases	355		0.020
No		Reference	
Yes		0.43 [0.21-0.88]	
Visceral metastasis	355		0.3
No		Reference	
NA		0.76 [0.47-1.22]	
Yes		1.19 [0.64-2.20]	
Number of metastatic sites	355		0.4
<3		Reference	
≥3		1.18 [0.79-1.77]	

CI, confidence interval; CTC, circulating tumor cell; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; HR+, hormone receptor positive; IHC, immunohistochemistry; NA, not available; tdEV, tumor-derived extracellular vesicles.

assessment of both biomarkers within the same analytical framework, maximizing data yield from a single blood sample without added cost. In line with previous studies,^{10,20,21} we found a strong correlation between manual CTC enumeration (which is the current gold standard for CTC count) and automated analysis, with nearly identical clinical outcomes. The use of automation offers significant advantages over operator-based assessment, including time efficiency and elimination of interoperator and intraoperator variability, which is crucial for integrating CTCs and tdEVs into clinical trials and practice.

Findings from the STIC CTC and PACE trials demonstrated the clinical utility of CTCs to tailor treatment in HR-positive/HER2-negative MBC.^{11,12,22} The present study suggests that integrating tdEVs can further refine risk stratification in patients with MBC and potentially improve clinical decision making beyond use of CTCs alone, with prospective studies needed. In the era of precision medicine, marked by rapidly expanding treatment options for patients with MBC, the demand for sensitive biomarkers to assess patients' risk and guide therapeutic decisions is growing. Recent trials are shifting highly effective treatment to earlier lines, but this approach requires careful patient selection.²³⁻²⁵ In this context, combining CTCs and tdEVs may offer an objective, noninvasive tool to identify patients with more indolent disease (i.e. CTC_{low}/tdEV_{low}), for whom treatment de-escalation could be evaluated, while patients with high levels of both biomarkers may benefit from treatment escalation, particularly if associated with use of targeted therapies or antibody–drug conjugates if needed.

For stratification purposes, while the threshold of five CTCs per 7.5 ml of blood is well established, a standardized cut-off for tdEVs has yet to be determined. In this study, we

evaluated multiple tdEV thresholds, all of which were associated with outcomes and showed a similar performance. We selected a cut-off of 20 tdEVs per 7.5 ml based on prior literature identifying 0-20 as the normal range in 93 healthy donors⁷ and supporting its clinical validity.^{7,10} Additionally, using a single cut-off facilitates integration with CTC-based stratification and application in clinical practice. The prognostic relevance of this tdEV cut-off was confirmed in an independent validation cohort, where it showed consistent performance for both OS and PFS, further supporting its clinical validity. Future research should focus on optimizing the integration of tdEVs and CTCs, potentially combining them into a single biomarker, as previously proposed.²⁰ Also, integration with other liquid biopsy components, mainly circulating tumor DNA (ctDNA), may further refine personalized treatment approaches.

Beyond risk stratification, integrating tdEVs into the management of patients with MBC may offer additional advantages. Consistent with previous studies,^{7,10,26} we observed a broader distribution of tdEVs compared with CTCs, with tdEV counts typically ranging from 5-fold to 10-fold higher and rarely undetectable. This suggests that tdEVs may better capture tumor heterogeneity and allow for a more reliable evaluation of therapeutic targets than CTCs, such as the HER2 status.^{20,26} Moreover, tdEVs could serve as a more sensitive indicator of tumor burden, making them a promising biomarker for treatment monitoring beyond CTC enumeration alone.^{9,20} The lack of longitudinal monitoring in our cohorts precluded the evaluation of this aspect.

This study has several strengths. Firstly, the use of automated image analysis enhances the analytical robustness, by increasing reproducibility and reducing operator-related biases, an essential improvement in this area of

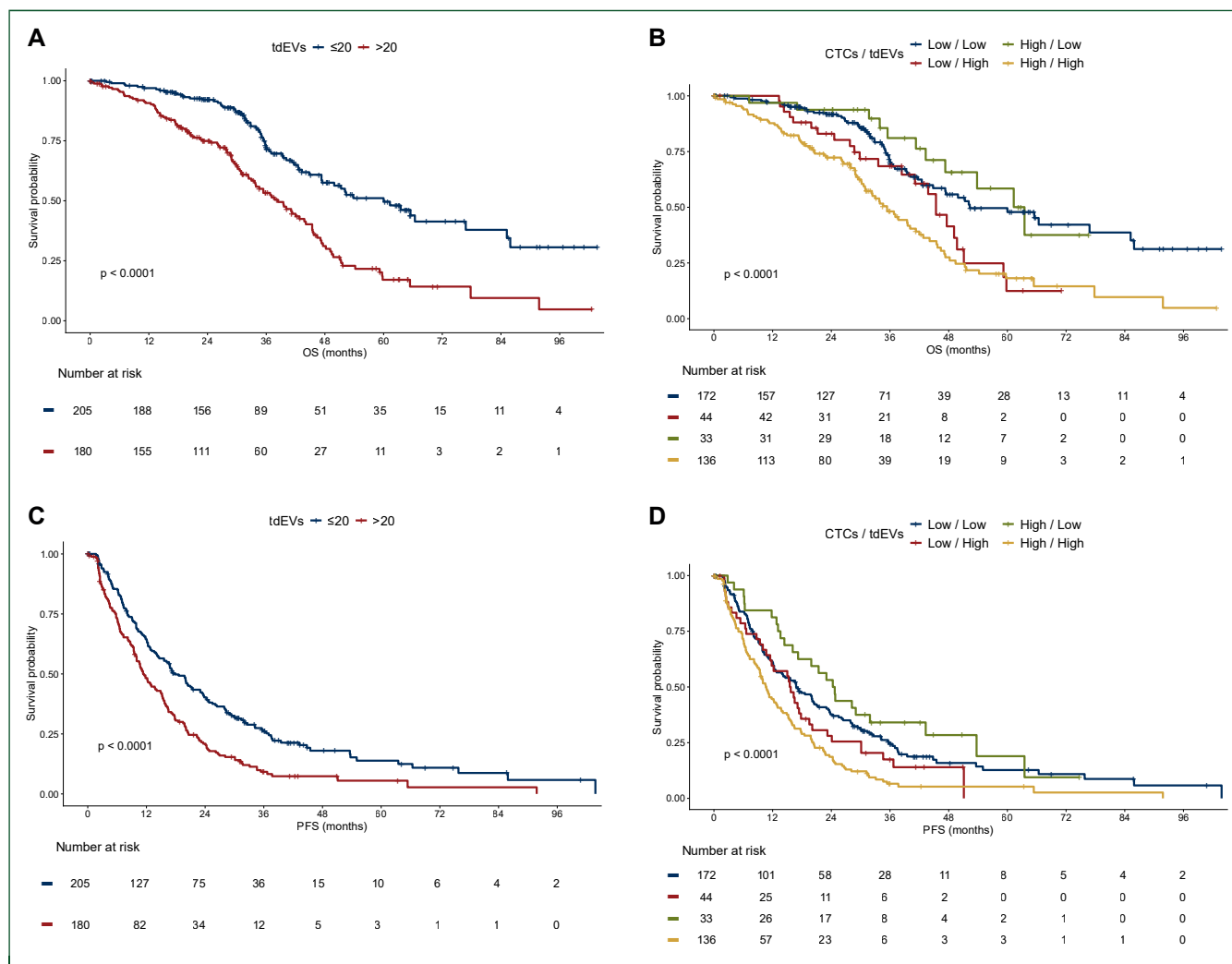


Figure 2. Kaplan–Meier plots for OS (A and B) and PFS (C and D) of the STIC CTC population by baseline tdEVs (A and C) and combined CTCs/tdEVs (B and D). CTC, circulating tumor cell; OS, overall survival; PFS, progression-free survival; tdEV, tumor-derived extracellular vesicle.

research. ACCEPT analyses were carried out in a blinded fashion with respect to clinical outcomes. Another strength is the prospective design of sample collection, with one cohort derived from a phase III prospective clinical trial, ensuring standardized procedures and high-quality data. Moreover, we used two large independent cohorts to allow for the external validation of the selected tdEV cut-off, chosen among a set of thresholds predefined before analysis, and confirmed its clinical relevance across both OS and PFS outcome measures. Nevertheless, some limitations should be acknowledged. Firstly, the retrospective nature of the analysis and the inclusion of only a subset of STIC CTC trial participants may introduce selection bias. Secondly, while the training cohort was heterogeneous in terms of patient characteristics, types, and lines of treatment, to enhance the generalizability of the findings across a broader MBC patient population, the validation cohort included only patients with HR-positive/HER2-negative MBC treated in the first-line setting (before the introduction of CDK4/6i). Although this represents a clinically relevant setting, it may limit the applicability of the results to other subgroups. Additionally, while the selected cut-off

was validated, prospective studies are needed to confirm its utility in guiding treatment decisions. Future studies should also investigate multimodal strategies integrating tdEVs with other circulating biomarkers, particularly ctDNA, which represents a cornerstone of current clinical liquid biopsy applications but was not available in the present cohorts, precluding direct comparative analyses. By leveraging the complementary biological information captured by distinct circulating analytes,²⁷ such strategies have the potential to improve risk stratification and treatment personalization in patients with MBC, and possibly in early-stage disease, where tdEV data are currently lacking.

Finally, the biological basis underlying the prognostic value of tdEVs remains to be fully elucidated. Due to the enrichment and image-based detection method employed, comprehensive characterization of the objects identified as tdEVs was not feasible; therefore, they should be regarded as operationally defined, EV-like particles enriched for epithelial tumor-associated features, rather than fully characterized EVs according to current ISEV guidelines.¹⁶ The tdEVs quantified in this study represent a specific subset of large EV-like particles

Table 3. Multivariable Cox regression analysis of overall survival for the combined tdEV and CTC assessment in the STIC CTC population

	N = 347	HR [95% CI]	P value
Combined automated CTCs (<5 versus ≥5) and tdEVs (median cut-off: ≤20 versus >20)			<0.001
CTCs low/tdEVs low	153	Reference	
CTCs low/tdEVs high	39	1.96 [1.15-3.33]	
CTCs high/tdEVs low	29	0.96 [0.50-1.84]	
CTCs high/tdEVs high	126	2.74 [1.82-4.11]	
Hormone receptor status			0.6
Negative	7	Reference	
Positive	340	0.73 [0.26-2.02]	
Isolated bone metastases			0.9
No	254	Reference	
Yes	93	1.04 [0.68-1.58]	
Lung and pleura metastases			0.003
No	241	Reference	
Yes	106	1.86 [1.24-2.80]	
Liver metastasis			0.002
No	282	Reference	
Yes	65	1.91 [1.28-2.85]	
Number of metastatic sites			0.3
<3	234	Reference	
≥3	113	0.81 [0.55-1.19]	
Treatment received			0.5
Chemotherapy	107	Reference	
Endocrine therapy	240	1.13 [0.78-1.63]	

CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; tdEV, tumor-derived extracellular vesicle.

recovered from the cellular blood fraction, with a size distribution consistent with that reported for large oncosomes,²⁸ while smaller vesicles present in the plasma fraction were not assessed. The wide range of tdEV levels observed, including low or undetectable counts, likely reflects interpatient heterogeneity in tumor burden and vesicle shedding dynamics rather than excluding a tumor-associated origin of these particles. Consistently, prior studies reported low but detectable tdEV levels (median <10) in some healthy donors, defining a normal reference range below 20 tdEVs per 7.5 ml of blood, while demonstrating markedly higher levels in patients with metastatic cancer.^{6,7} In addition, the strong correlation observed between tdEVs and CTCs suggests a shared tumor-related source, further supported by prior reports showing concordant decrease of both biomarkers in response to systemic therapy,^{9,20} as well as the expression of tumor-associated antigens such as HER2 on tdEVs.²⁶ Importantly, baseline tdEV levels have shown robust and reproducible associations with clinical outcomes across independent cohorts and across different tumor types,⁶⁻¹⁰ supporting a biologically meaningful and clinically relevant tumor-associated signal. In this context, the designation ‘tumor-derived’ is based on the phenotypic characteristics of these particles and on prior studies showing their clinical relevance, rather than on direct molecular confirmation of tumor origin. From a biological perspective, tumor-derived EVs are increasingly recognized as mediators of cancer progression, contributing to microenvironment modulation, invasion, metastasis, and therapeutic resistance.^{5,29} Further investigations combining molecular and

genotypic characterization of these tdEVs will be essential to clarify their precise origin and functional role.

Conclusions

This study demonstrates that the combined use of tdEVs and CTCs provides valuable and complementary prognostic information in patients with MBC. We identified and independently validated a single, clinically relevant cut-off for tdEVs that effectively stratifies patients based on survival outcomes. This integrated approach could enable more precise risk assessment and enhance personalized decision making for patients with MBC, supporting its potential integration into both clinical trials and routine practice.

DATA AVAILABILITY

De-identified participant data will be available upon reasonable request from the author. Data will be shared for research purposes, following approval of a proposal, with a signed data access agreement.

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