BIOMARKERS

original reports abstract

Tumor-Derived Extracellular Vesicles as Complementary Prognostic Factors to Circulating Tumor Cells in Metastatic Breast Cancer

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PURPOSE Circulating tumor cells (CTCs) are strongly prognostic for overall survival (OS) in metastatic breast cancer although additional prognostic biomarkers are needed. We evaluated the complementary prognostic value of tumor-derived extracellular vesicles (tdEVs) next to CTCs.

METHODS We applied the open-source ACCEPT software to archived CellSearch images from the prospective clinical trial SWOG0500 to enumerate CTCs and tumor-derived extracellular vesicles (tdEVs) before and after one cycle of chemotherapy.

RESULTS CTCs enumerated by ACCEPT were strongly correlated with classical ocular enumeration (correlation $r = 0.98$). OS was worse with elevated tdEVs (median OS for high/medium/low groups: 17.1 *v* 29.0 *v* 43.3 months; $P < .0001$). In patients with longer OS by CTC counts (< 5 CTC/7.5 mL blood), elevated tdEV levels were independently associated with poorer OS (multivariable analysis $P < .001$). OS was also longer for patients with low tdEVs after one cycle of chemotherapy (median OS for high/medium/low group: 10.8 *v* 17.8 *v* 26.7; $P < .0001$).

CONCLUSION This study highlights the complementary prognostic significance of tdEVs in metastatic breast cancer before and after one cycle of chemotherapy.

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INTRODUCTION

Circulating tumor biomarkers, designated liquid biopsies,[1] are gaining an important role in the prognosis, prediction, and treatment monitoring of patients with malignancies.[2,3] Circulating tumor cells (CTCs) detected with the CellSearch system are elevated in 25%-50% and 10%-15% of patients with metastatic and early-stage breast cancer, respectively.[4,5] In each of these settings, elevated CTCs are associated with higher risk of progression and poorer overall survival (OS).[6,7]

Failure to eliminate CTCs in patients with metastatic breast cancer after the first weeks of therapy indicates a futile therapy.[4] This observation generated the hypothesis that patients might benefit from an earlier switch to a more effective therapy rather than waiting for classic evidence of progression.[8] To test this theory, SWOG conducted the S0500 clinical trial, in which patients with hormone-insensitive metastatic breast cancer who failed to experience a CTC response, defined as failure to reduce CTCs to < 5/7.5 mL whole blood (WB), after a single cycle of first-line chemotherapy were randomly assigned to either remain on their initial first line of chemotherapy or switch to an alternative chemotherapy.[9] The results of S0500 confirmed that CTC levels at baseline are prognostic and that failure to experience a CTC response reflects very high relative resistance to the administered systemic therapy, which was chemotherapy. Unfortunately, prognosis in the group that did not exhibit a CTC response was dismal, regardless of whether they continued the originally prescribed or switched to an alternative chemotherapy regimen, with a median OS of roughly 13 months.[9] These results highlight the major and still unmet need for more informed, biomarker-driven decisions to better guide cancer therapy. CTC enumeration, coupled with other prognostic and predictive biomarkers, may help address this issue.[10]

Extracellular vesicles (EVs) defined by the International Society for Extracellular Vesicles as "particles naturally released from cells that are delimited by a lipid bilayer and cannot replicate"[11] are released from normal and malignant cells into the extracellular space facilitating the intercellular communication between contiguous and cells of distant sites.[12-16] Furthermore, tumor-derived EVs (tdEVs) have various biologic functions, such as induction of apoptosis; stimulation of

ASSOCIATED CONTENT

Data Sharing Statement
Data Supplement
Protocol

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Can tumor-derived extracellular vesicle (tdEV) levels complement circulating tumor cell (CTC) levels in the risk assessment of patients with metastatic breast cancer? To our knowledge, this is the first study addressing the complementary prognostic value of tdEVs to CTCs in metastatic patients before and after chemotherapy.

Knowledge Generated

Automatically enumerated tdEVs before and after one cycle of chemotherapy are significantly prognostic. The different arms in the SWOG S0500 study were based on CTC counts. Within each of these arms, tdEVs were robust in further stratifying patients and complemented the prognostic effect of CTCs. These tdEVs revealed patient subpopulations with more aggressive/indolent disease at baseline and patients who had possibly benefited from the treatment.

Relevance

Both CTC and tdEV levels of patients can be determined using a single assay, and the two factors can provide clinicians with a more accurate measure of the tumor load, the aggressiveness of the disease, and predict the response of patients to treatment.

proliferation, angiogenesis, and metastasis; and immune activation and regulation.[12-14]

We have previously reported the application of open-source, image analysis software, designated ACCEPT,[17] to evaluate archived image data sets from prior CTC studies using the CellSearch platform, where we observed that elevated levels of circulating large tdEVs, defined as particles of a size between 1 and 12 μm that coexpress epithelial cell adhesion molecules and cytokeratin but not leukocyte-specific CD45 and no DNA stain, were detected in nearly 75% of patients with metastatic breast cancer.[18,19] Importantly, elevated tdEVs (≥ 20 tdEVs/7.5 mL WB) were associated with shorter OS in metastatic cancers of epithelial origin.[18]

These results suggest that tdEVs could serve as valuable biomarkers to aid in the disease management of patients with cancer. We therefore evaluated whether tdEVs enumerated in the CellSearch images collected from patients enrolled in S0500 might enhance the information obtained from CTCs on prognosis and treatment response.[9]

METHODS

The methodology of this prospectively designed retrospective study of S0500 is described in the Data Supplement that accompanies this article.

RESULTS

Enumeration of CTCs and tdEVs by ACCEPT

In S0500, at baseline, patients were assigned to arm A or arms B/C according to ocular enumeration of CTC/7.5 mL WB[9] (arm A: 0-4 CTC/7.5 mL WB; arms B/C: ≥ 5 CTC/7.5 mL WB). Arms B and C were further determined on the basis of ocular enumeration of CTC/7.5 mL WB at first follow-up, approximately 22 days after the first dose of chemotherapy (arm B: 0-4 CTC/7.5 mL WB; arm C: ≥ 5 CTC/7.5 mL WB). Of the CellSearch images used for the ocular primary analysis, 98% (831 of 852) were available for reanalysis with ACCEPT (Data Supplement). For 578 of the 596 (97%) eligible patients enrolled in the SWOG S0500 clinical trial, ACCEPT could analyze CellSearch images corresponding to 547 samples collected at baseline and 284 samples at first follow-up (Data Supplement). Patients in arm A did not have CTC enumeration beyond baseline.

Enumeration of CTCs

Examples of objects classified as CTCs after applying the ACCEPT CTC gate are shown in Figure 1A. The distributions of ACCEPT CTCs corresponding to each arm (A, B, and C, assignment per the original study) and time point (baseline and first follow-up) are displayed in box

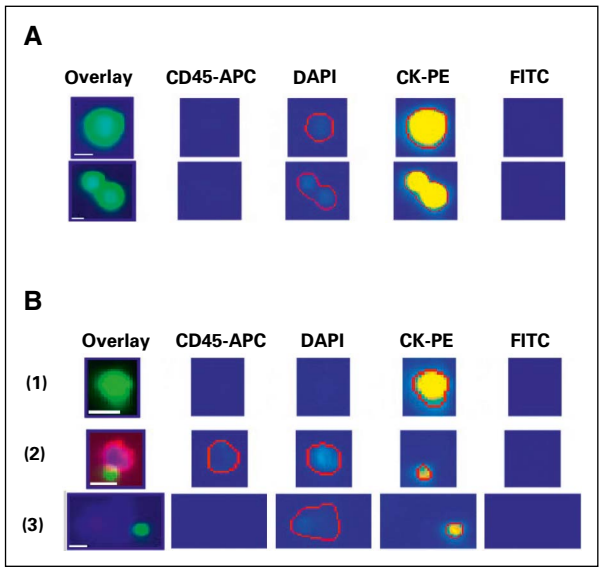

FIG 1. Examples of thumbnails falling in the ACCEPT gate(s) applied for (A) CTC and (B) tdEV automated enumeration. In the case of tdEVs, three different gates were applied to include (1) single tdEVs, (2) tdEVs attached to WBCs, and (3) tdEVs attached to undefined nucleated events. Scale bars indicate 6.4 μm. CTC, circulating tumor cell; tdEV, tumor-derived extracellular vesicle.

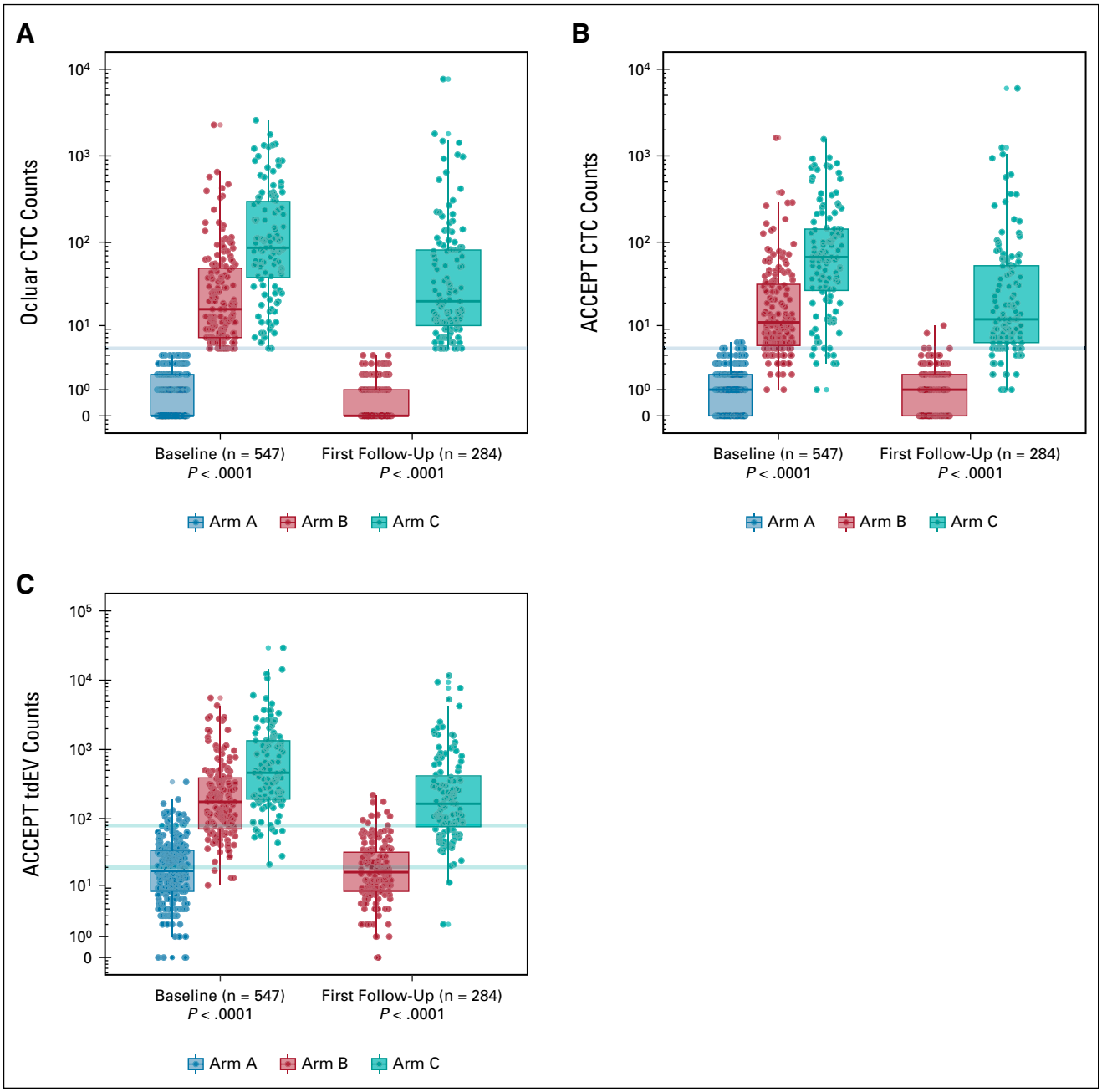

FIG 2. Distributions of (A) ocular CTC counts, (B) ACCEPT CTC counts, and (C) ACCEPT tdEV counts of patients with metastatic breast cancer enrolled in SWOG S0500 at baseline (images evaluable for 547 of 564 patients enrolled in SWOG S0500) and first follow-up (images evaluable for 284 of 288 patients enrolled in SWOG S0500). The levels of patients in arms A, B, and C are depicted in blue, red, and teal, respectively. The blue bold line (in A and B) represents the cutoff point of 5 CTC/7.5 mL WB and indicates the population for which ACCEPT CTC would have led to a different assignment than originally determined by ocular enumeration. The teal bold lines (in C) represent the cutoff points for low (0-19), intermediate (20-79) and high (≥ 80) tdEV levels. All the values in the box plots were log 10 transferred, whereas y-axes were labeled with actual value. The *P* value comparing the biomarker distributions among arms A, B, and C is indicated at the bottom, using the Kruskal-Wallis ANOVA test. In the box and whisker plots, the box surrounds the 25-75 percentiles (Q1, Q3), the bold line indicates the median, the upper whisker extends from Q3 to the largest value not further than 1.5 × IQR from the Q3, and the lower whisker extends from the Q1 to the smallest value at most 1.5 × IQR from the Q1. CTC, circulating tumor cell; tdEV, tumor-derived extracellular vesicle; WB, whole blood.

plots in Figure 2B and are compared with the respective distributions of ocular CTC levels (Fig 2A and Data Supplement).[9] ACCEPT was similar to ocular CTC enumeration, with a correlation of 0.98. Correlations were similar for baseline ($r = 0.98$) and first follow-up ($r = 0.995$; Data Supplement). Much of the discordance between the two methods is at very low CTC levels, mostly less than the clinically validated cutoff point of 5 CTC/7.5 mL WB.

If ACCEPT had been applied in the original trial (Data Supplement, Figs 2A and 2B), 10 of 265 (4%) patients originally assigned at baseline to arm A would have been assigned to arm B/C, and 33 of 282 (12%) assigned at baseline to arm B/C would have been assigned to arm A. At follow-up, 5 of 161 (3%) assigned to arm B would have been assigned to arm C and 15 of 123 (12%) assigned to arm C would have been assigned to arm B. In all 15 cases in which CTC counts went from < 5 CTC by the ocular method to ≥ 5 CTC/7.5 mL WB because of the enumeration by ACCEPT, the ACCEPT CTC counts were < 10 CTC/7.5 mL WB.

There was a concordance in known factors important in metastatic breast cancer evaluation and treatment when the S0500 patient cohort was dichotomized on the basis of either ocular or ACCEPT CTC levels (< or ≥ 5 CTC/7.5 WB; Data Supplement).

Enumeration of tdEVs

Examples of objects classified as tdEVs after applying the (1-3) ACCEPT tdEV gates are shown in Figure 1B. ACCEPT tdEV levels are illustrated in Figure 2C. Using previously defined cutoff points of 0-19 (low), 20-79 (intermediate), and ≥ 80 (high) tdEV/7.5 mL WB,[18] at baseline, 150 (27%), 150 (27%), and 247 (45%) patients had low, intermediate, and high tdEV levels, respectively. ACCEPT CTCs were moderately correlated with tdEVs combining both baseline and first follow-up samples ($r = 0.56$; Data Supplement). However, tdEV levels were widely distributed within each arm, as originally assigned (Data Supplement). At baseline, of the 265 patients in arm A, 146 (55%), 100 (38%), and 19 (7%) patients had low, intermediate, and high tdEV levels, respectively. At first follow-up of the 161 patients in arm B, 97 (60%), 55 (34%), and 9 (6%) had low, intermediate, and high tdEV levels, and of the 123 patients in arm C, 2 (2%), 29 (24%), and 92 (75%) fell into these respective three categories. The odds of having low, intermediate, or high tdEV levels were independent of hormone receptor, human epidermal growth factor receptor 2 (HER2) status, or age (Data Supplement).

Association of CTCs and tdEVs With Clinical Outcomes

OS was the primary end point of the S0500 study.[9] In the primary analysis, OS was directly related to baseline and first follow-up ocular CTC levels.[9] The median OS was 35 months for patients with baseline ocular CTC < 5/7.5 mL WB (arm A), 23 months for those who had elevated ocular CTC levels at baseline (≥ 5/7.5 mL WB) that declined to < 5/7.5 mL WB after one cycle of chemotherapy (arm B), and 13 months for those who had elevated ocular CTC at baseline that did not decline to < 5/7.5 mL WB (arm C).[9] ACCEPT CTC and tdEV levels were evaluated for their association with the clinical outcome of 547 patients, for whom ACCEPT analysis was available (Table 1).

OS by CTC Enumeration

As expected, increased ACCEPT CTC levels were adversely associated with OS of patients (Fig 3A and Table 1). The median OS from baseline was 34.2, 20.7, and 11.3 months for patients determined by ACCEPT to have 0-4 CTC/7.5 mL WB at baseline or ≥ 5 CTC/7.5 mL WB at baseline but who were determined later, at first follow-up, to have declined CTC or elevated CTC, respectively (Fig 3A and Table 1). These median OS differences were highly statistically significant ($P < .0001$). Importantly, however, median OS did not differ when it was determined by ACCEPT or ocular CTCs for each group: median OS for arm A (ACCEPT *v* ocular = 34.2 *v* 35), arm B (ACCEPT *v* ocular = 20.7 *v* 23), and arm C (ACCEPT *v* ocular = 11.3 *v* 13). These data confirm that the original selection of 5 CTC/7.5 mL WB as a cutoff separates favorable from unfavorable prognosis in metastatic breast cancer[4,6,8,9] and that CTC enumeration by ACCEPT gives nearly identical results to those obtained by the ocular method. Similarly, median OS from first follow-up for patients in arms B and C according to ACCEPT CTC levels was very similar to those we observed using the ocular method: median OS for arm B (ACCEPT *v* ocular = 20.0 *v* 22.9) and for arm C (ACCEPT *v* ocular = 10.6 *v* 12.5; Data Supplement; Table 1).

OS by tdEV Levels

OS was adversely associated with increasing tdEV levels (Fig 3B and Table 1). In all patients, the median OS from baseline was 43.3, 29.0, and 17.1 months for patients with low, intermediate, and high tdEV, respectively (Fig 3B). Importantly, the association of elevated tdEV counts with worsening OS was complementary to ACCEPT CTC levels, with a more robust separation of the Kaplan-Meier curves within each arm (Fig 4). For example, subdividing arm A on the basis of the CTC counts showed that OS for patients with 1-4 CTC was slightly worse than for those with 0 CTC/7.5 mL WB (median OS 39.8 *v* 31.4 months; $P = .045$; Data Supplement and Table 1). However, there was a stepwise decrement in median OS according to increasing enumeration of tdEVs, with the median OS ranging from 42.5 months with low (< 20/7.5 WB) to only 23.2 months with high (≥ 80/7.5 mL WB) tdEV levels ($P = .002$ for trend; Fig 4A; Table 1). Similar observations were found in arm B, in whom few if any CTC were detected at first follow-up, but in whom elevated tdEV levels were associated with a highly significant worse OS. As illustrated in Figure 4C and Table 1, the median landmark OS from first follow-up in arm B was 26.7, 17.1, and 14.3 months for patients with low, intermediate, or high tdEV levels ($P = .0009$). Even in arm C, for which prognosis is quite poor on the basis of CTC levels (median OS = 13 months regardless of original or switched chemotherapy), tdEV provided additional prognostic information (Fig 4D and Table 1). The median OS was 19.2 versus 9.7 months for patients with intermediate versus high tdEV levels, and only two patients in this group had low tdEVs. The multivariable analysis demonstrated

TABLE 1. Median Overall Survival (in months) According to CTCs and Tumor-Derived Extracellular Vesicles Analyzed by ACCEPT Within Arms Determined by ACCEPT CTCs or Ocular CTCs

	Arms on the Basis of ACCEPT CTCs				Arms on the Basis of Ocular CTCs			
	Baseline		First Follow-Up		Baseline		First Follow-Up	
Arms	**A**	**B/C**	**B**	**C**	**A**	**B/C**	**B**	**C**
No.	**288**	**246**	**142**	**104**	**265**	**282**	**161**	**123**
ACCEPT enumerations[a]								
CTC								
0	39.8 (34.0 to 52.5)	NA	16.8 (11.8 to 28.5)	NA	39.8 (34.0 to 52.5)	NA	20.0 (15.1 to 28.5)	NA
1-4	31.4 (27.9 to 35.6)	NA	21.0 (17.4 to 26.0)	NA	31.4 (27.0 to 36.5)	31.5 (22.6 to 44.2)	25.4 (20.2 to 30.3)	17.4 (9.5 to 23.5)
≥ 5	NA	16.8 (14.3 to 18.2)	NA	10.6 (8.7 to 13.4)	28.2 (20.9 to NR)	17.1 (14.4 to 18.4)	13.5 (8.3 to 19.9)	11.0 (8.8 to 13.8)
Log-rank *P*	.04	NA	.77	NA	.12	.0035	.04	.30
HR (95% CI)								
1-4 *v* 0	1.37 (1.01 to 1.85)	NA	0.95 (0.65 to 1.37)	NA	1.37 (1.0 to 1.87)	NA	0.93 (0.65 to 1.31)	NA
≥ 5 *v* 0	NA	NA	NA	NA	1.11 (0.51 to 2.44)	NA	2.92 (1.15 to 7.45)	NA
OR								
≥ 5 *v* 1-4	NA	NA	NA	NA	NA	1.8 (1.2 to 2.7)	NA	1.3 (0.77 to 2.3)
tdEV								
0-19	43.0 (35.2 to 51.4)	NR	26.2 (17.8 to 32.3)	NR	42.5 (34.8 to 51.3)	83.7 (51.7 to NR)	26.7 (21.2 to 34.8)	NR
20-79	31.8 (26.8 to 35.5)	21.8 (14.9 to 35.7)	17.1 (11.4 to 21.0)	16.3 (10.3 to 22.2)	30.9 (26.3 to 35.5)	26.5 (20.7 to 36.5)	17.1 (14.7 to 21.3)	19.2 (13.4 to 29.1)
≥ 80	23.2 (15.2 to 28.9)	15.3 (13.1 to 17.5)	14.8 (7.3 to 22.2)	9.3 (7.5 to 11.9)	23.2 (10.0 to 28.3)	16.0 (13.7 to 17.7)	14.3 (2.6 to 27.9)	9.7 (8.1 to 12.5)
Log-rank *P*	< .0001	.03	.005	.006	.002	.0002	.0009	.002
HR (95% CI)								
20-79 *v* 0-19	1.44 (1.10 to 1.91)	NA	1.58 (1.07 to 2.34)	NA	1.45 (1.09 to 1.94)	NA	1.76 (1.22 to 2.55)	NA
≥ 80 *v* 0-19	2.52 (1.63 to 3.91)	NA	2.42 (1.29 to 4.54)	NA	2.22 (1.31 to 3.78)	NA	2.66 (1.32 to 5.37)	NA
OR								
≥ 80 *v* 20-79	NA	1.52 (1.02 to 2.26)	NA	2.05 (1.24 to 3.40)	NA	1.67 (1.20 to 2.33)	NA	2.07 (1.33 to 3.21)

Abbreviations: CTC, circulating tumor cell; HR, hazard ratio; NR: not reached; tdEV, tumor-derived extracellular vesicle; WB, whole blood.

[a]CTCs and tdEVs expressed/7.5 mL WB.

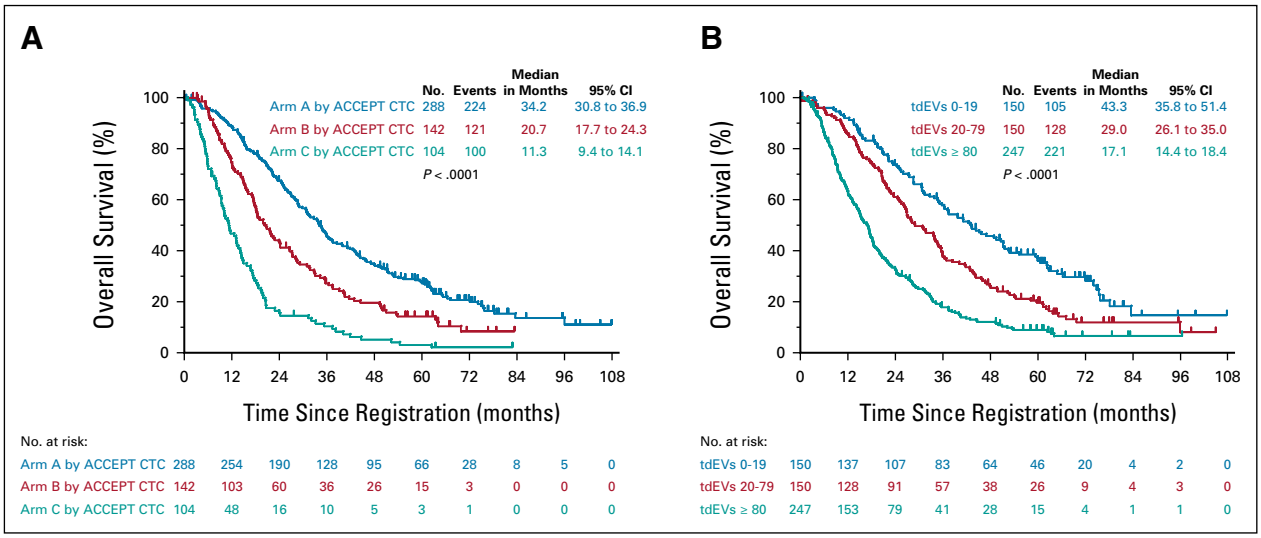

FIG 3. Kaplan-Meier plots of OS of 547 of 564 patients enrolled in SWOG S0500, stratified on the basis of ACCEPT analysis of (A) CTCs and (B) tdEVs (from baseline). The table below the horizontal axis shows the number of patients at risk. Note: the numbers in (A) differ from total in arms B and C because some ACCEPT CTC could not be performed at both time points. For details, see the Data Supplement. (A) Although OS is calculated from baseline, the patients are divided by CTC levels at baseline (arm A of S0500; 0-4 CTC/7.5 mL WB, blue line) or, if ≥ 5 CTC/7.5 mL WB at baseline, by whether they were ultimately assigned to arm B (0-4 CTC/7.5 mL WB, red line) or C (if ≥ 5 CTC/7.5 mL WB, teal line) at first follow-up. The total number of patients included in this figure differs from others, because of several patients with missing ACCEPT CTC evaluation at first follow-up. For detailed information, see the Data Supplement. (B) Curves are separated by tdEVs 0-19 (blue line), 20-79 (red line), and ≥ 80 (teal line)/7.5 mL WB at baseline. CTC, circulating tumor cell; OS, overall survival; tdEV, tumor-derived extracellular vesicle; WB, whole blood.

that tdEVs were the factor most associated with OS at both baseline (Table 2A; hazard ratio for 20-79 and ≥ 80 = 1.56 and 2.76, respectively, $P < .0001$) and first follow-up (Table 2B; hazard ratio for 20-79 and ≥ 80 = 1.58 and 2.85, respectively, $P < .0001$). Uno's C-index was the highest for the multivariable model of OS when it included both ACCEPT CTCs and tdEVs next to the disease site and the biologic subtype, further demonstrating the added value of tdEVs. More specifically, Uno's C index for the model including disease site, biologic subtype and (1) tdEVs and ACCEPT CTCs at baseline was 0.6860, (2) tdEVs at baseline was 0.6790, (3) ACCEPT CTCs at baseline was 0.6610, and (4) ocular CTCs was 0.6543.

DISCUSSION

In the present study, we performed a prospective-retrospective automated image analysis using the open-source ACCEPT platform to determine the levels of CTCs and tdEVs for patients with metastatic breast cancer who were starting first-line chemotherapy and participated in the SWOG S0500 clinical trial. Nearly 100% of the patient data from the primary analysis were available and were included in this reported ACCEPT analysis.

We observed that ACCEPT and ocular CTC counts were strongly correlated and that clinical outcomes according to CTC were nearly identical when determined by either analysis. These findings confirm prior reports that ACCEPT CTC enumeration performs equally well to trained operators using ocular analysis for metastatic breast and prostate cancer patient cohorts.[18,20,21] Although technical in nature, these findings provide a remarkable advantage over ocular evaluation in terms of both time and elimination of inter- and intraoperator bias. Furthermore, ACCEPT enables the enumeration of CTCs and tdEVs in the same computational effort.

Our most intriguing observation was the presence, broad distribution, and complementary prognostic nature of tdEVs with CTCs. At baseline, 73% of patients in S0500 had elevated (intermediate or high) tdEVs (≥ 20/7.5 mL WB) and nearly one half had very high levels (≥ 80/7.5 mL WB). tdEVs were significantly prognostic, independent of CTC counts. Importantly, although CTCs and tdEVs were correlated, tdEVs proved to be a robust biomarker to stratify patients into different risk groups within each CTC-based arm of the clinical trial. For example, within arm A, which included patients with 0-4 CTC/7.5 mL WB and for whom prognosis is considered relatively favorable (median OS = 35 months), patients with intermediate and especially high tdEV levels had significantly, and clinically relevant, worse OS compared with those with low levels. Furthermore, the distribution of tdEV levels in these groups was relatively evenly divided; 55% of patients had low tdEVs although of the 45% with elevated levels, 100 (84%) and only 19 (16%) had intermediate and high levels, respectively.

These results validate our prior observation that high tdEVs (≥ 80/7.5 mL WB) at baseline distinguish patients with higher-risk metastatic breast cancer with favorable CTC

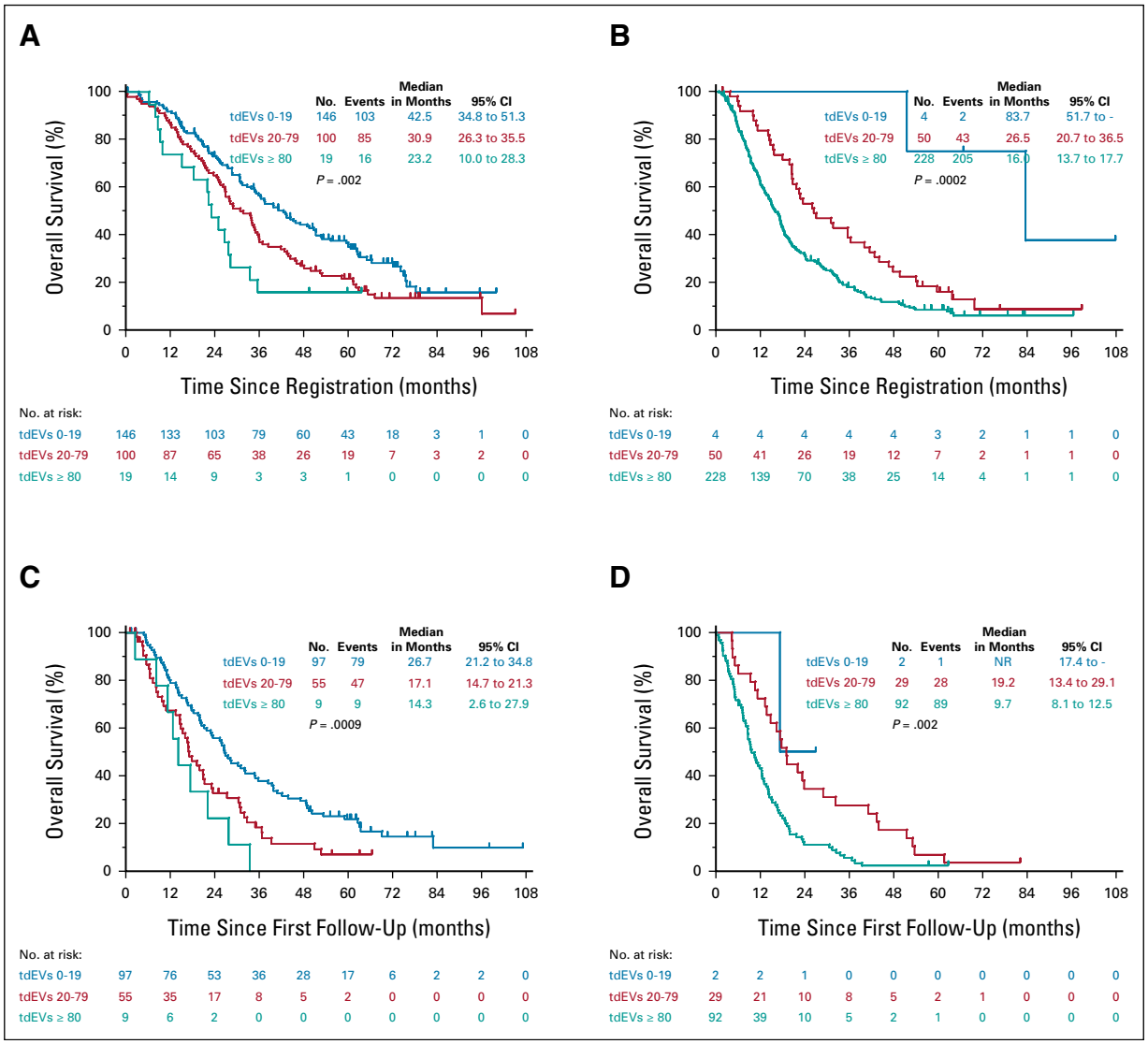

FIG 4. Kaplan-Meier plots of OS according to tdEVs in arms (A) A from baseline and (B) B and C combined from baseline and landmark analysis of OS according to tdEVs in arms (C) B from first follow-up and (D) C from first follow-up. Assignment to arms according to ocular CTC enumeration. CTC, circulating tumor cell; OS, overall survival; tdEV, tumor-derived extracellular vesicle.

levels.[18] In that study, patients were enrolled at various times in their metastatic process and they were treated with a variety of therapies, depending on their circumstances. Nonetheless, taken together, these results suggest that tdEVs, as quantified by automated analysis in the CellSearch images, might identify patient subpopulations originally left to a relatively favorable prognosis, by virtue of low CTCs either at baseline or after one cycle of single-agent chemotherapy, that could benefit from an alternative treatment strategy other than single-agent chemotherapy.

Our data also suggest that low tdEV levels might identify patients originally assigned to arm C by ocular CTC analysis and who were thought to have a dismal prognosis, but who instead appear to have more indolent disease. We speculate that perhaps these patients with low tdEVs might represent a small subgroup of such patients who did not have a CTC response, but who might have, in fact, benefited from the chemotherapy. Overall power was insufficient to evaluate tdEV prognosis within each of the randomized arms of arm C.

Although we were not able to fully characterize these tdEVs on the basis of the isolation methodology, their sizes are consistent with large oncosomes.[22]

A larger fraction of smaller tdEVs will reside in the plasma fraction, and their role has not yet been explored.[23,24] A typically applied EV definition includes a size smaller than 1 μm, which means that the number of antigens detectable on a single EV is low because of their small surface area.[25] As a result, the smaller and more numerous tdEVs may be more difficult to enumerate and phenotype. Technological developments, such as, for example, microfluidic devices,[23,26]

TABLE 2. Multivariable Cox Regression Model for OS

A. From Baseline

Parameter	Reference Group	HR	95% CI	*P*
tdEVs				< .0001
20-79	0-19	1.56	1.19 to 2.03	
≥ 80	0-19	2.76	1.97 to 3.86	
ACCEPT CTC				
≥ 5	0-4	1.30	0.98 to 1.72	.065
Disease site[a]				
Nonmeasurable disease only, including bone metastasis	Measurable disease	0.80	0.62 to 1.04	.099
Biologic subtype				< .0001
Hormone receptor–positive; HER2-negative	Triple-negative	0.46	0.36 to 0.58	
HER2-positive	Triple-negative	0.25	0.18 to 0.34	

B. From First Follow-Up

Parameter	Reference Group	HR	95% CI	*P*
tdEVs				
20-79	0-19	1.58	1.11 to 2.24	< .0001
≥ 80	0-19	2.85	1.87 to 4.35	
ACCEPT CTC				
≥ 5	0-4	1.16	0.83 to 1.63	.39
Disease site[a]				
Nonmeasurable disease only, including bone metastasis	Measurable disease	0.95	0.67 to 1.33	.76
Biologic subtype				< .0001
Hormone receptor–positive; HER2-negative	Triple-negative	0.43	0.31 to 0.59	
HER2-positive	Triple-negative	0.43	0.27 to 0.68	

NOTE. Model for OS including tdEVs at time point + ACCEPT CTCs at time point + disease site + biologic subtype.

Abbreviations: CTC, circulating tumor cell; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival; tdEV, tumor-derived extracellular vesicle.

[a]Patients who only have nonmeasurable disease without bone involvement were not eligible.

may enable enumeration of small tdEVs in clinical routine. Until that time, the CellSearch and ACCEPT-based solution presented here is reproducible and clinically applicable.

Previous studies by members of our team[19] and others[24,27,28] have reported specific tumor-associated antigens (TAA) present on tdEVs, such as HER2, KIT, and programmed death-ligand 1 (PD-L1).[29,30] Identification and quantification of these and several other TAA on CTCs using the CellSearch platform, such as estrogen receptor, HER2, BCL2, Ki67, markers of apoptosis, and PD-L1, have been reported.[31-35] We are now initiating studies to examine clinical associations with many of these tdEV-related TAA from our past and ongoing breast cancer clinical trials.

The biologic explanation for the prognostic effect of tdEVs remains unclear. tdEVs facilitate the intercellular communication and cancer metastatic process, either in the local environment where they have been secreted or in distant tissues by virtue of circulation in lymph or plasma.[36-38] Importantly, cellular production of tdEVs is increased with exposure to toxic stimulants, such as therapeutic radiation and cytotoxic agents, perhaps leading to drug resistance.[39] In a preclinical study, the transient receptor potential cation channel subfamily C member 5 (TRPC5), transported in tdEVs, mediated resistance to the chemotherapeutic agent doxorubicin when taken up by endothelial cells.[40] In another study of 55 patients with locally advanced breast cancer, increased expression of the breast cancer–resistant protein (in both mRNA and protein level) in the circulating EVs in the blood of patients was associated with chemoresistance and progression of the disease.[41] We hypothesize that, in patients with hormone refractory metastatic breast cancer, the poor prognosis related to increased tdEV in S0500 might stem from either baseline/de novo or induced resistance to chemotherapy.

This study has several strengths. First, it was performed within a prospectively conducted clinical trial led by a major cancer clinical trial cooperative group (SWOG S0500). Second, the analyses were performed by an automated system, reducing interoperator biases and strengthening

the analytical validity of our findings, which are problems in the field of CTCs and tdEVs.[36,42] Furthermore, ACCEPT analyses were performed in a blinded fashion to clinical outcomes, and correlations with clinical outcomes were performed by SWOG statisticians. Finally, the cutoff levels used to evaluate tdEV were prospectively chosen, on the basis of data-derived cut points from previous studies.[18] Taken together, our observations are unlikely to be due to chance, analytical issues, or overfitting.[43]

In conclusion, enumeration of CTC using the ACCEPT platform within the CellSearch system permits standardized and rapid evaluation, minimizing inter- and intraoperator variabilities. Importantly, these results strongly confirm the prognostic role of quantification of circulating tdEVs in patients with metastatic breast cancer, particularly those initiating first-line chemotherapy. These data suggest that tdEV levels, in association with CTC, could be used to design future clinical trials and, in the long run, help select more effective therapies for patients than are currently used.

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DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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