Clinical Cancer Research

Circulating Tumor Cell Clusters in Patients with Metastatic Breast Cancer: a SWOG S0500 Translational Medicine Study

Costanza Paoletti[1], Jieling Miao[2], Emily M. Dolce[1], Elizabeth P. Darga[1], Madeline I. Repollet[3], Gerald V. Doyle[4], Julie R. Gralow[5], Gabriel N. Hortobagyi[6], Jeffrey B. Smerage[1], William E. Barlow[2], and Daniel F. Hayes[1]

Abstract

Purpose: Metastasis requires malignant cell circulation from the primary to a distant tissue. Elevated levels of circulating tumor cells (CTC) portend a poor prognosis in breast and other cancers. Recent studies have suggested that CTC clusters may be a factor in the metastatic process. We conducted a prospective retrospective study of the SWOG0500 clinical trial to test whether CTC clusters are associated with poorer prognosis.

Experimental Design: CTC CellSearch galleries from SWOG0500 trial were reread using prespecified criteria for CTC clusters, doublets, and enumeration. Survival analysis methods include Kaplan–Meier plots and log-rank tests.

Results: Patients were classified into three prognostic subgroups based on baseline CTC/7.5 mL whole blood (WB): Arm A: <5CTC; Arm B/C: ≥5CTC and then B (<5CTC) and C (≥5CTC)/7.5 mL WB at first follow-up. At baseline, 19% of patients had CTC doublets or clusters, which were more likely in Arm B/C versus Arm A (38% vs. 1.4%; $P < 0.0001$). Furthermore, doublets or clusters were significantly more common in patients who were ultimately assigned to Arm C versus B (54% vs. 25%; $P < 0.0001$). In Arm C, doublets and clusters were associated with worse overall survival than only doublets, clusters, or no doublets nor clusters at baseline ($P = 0.008$) and first follow-up ($P = 0.010$). When compared with enumeration alone, doublets, clusters, or both were not prognostic in patients who had 5–19 or ≥20 CTC/7.5 mL WB.

Conclusions: In patients with metastatic breast cancer starting first-line chemotherapy, mortality is independent of the presence of CTC clusters, but rather depends on the number of CTC/7.5 mL WB.

Introduction

The metastatic process is complex, requiring several distinct biological steps, including invasion of surrounding normal tissue, intravasation into the circulation, extravasation into distant organs, and establishment of viable clones (1). In this regard, recently reported studies have suggested that clusters of circulating tumor cells (CTC), rather than elevated levels of single cells, are the driving force for subsequent metastases and death (2). The proposed mechanisms for this hypothesis are based on propensity of clusters for survival in a hostile environment and ability to extravasate and establish metastases (2). In addition, heterotypic clusters consisting of CTC and leukocytes appear to have higher viability and confer advantage to the metastatic process (3, 4). More recently, a comprehensive genome-wide analysis of DNA-methylation events has shown that CTC found in clusters have hypomethylation of critical stemness- and proliferation-related sites (5). Furthermore, CTC clusters may play a key role in the metastatic process and may also reflect resistance to chemotherapy (2, 6–12). Indeed, preliminary clinical investigations have suggested that the presence of CTC clusters are prognostic in metastatic breast, prostate, and small-cell lung cancers (SCLC; refs. 2, 6, 11, 12).

Several assays have been developed over the past two decades to identify, enumerate, and characterize CTCs (13). Of these, the most widely used is the CellSearch system, which is based on anti-EpCAM capture and subsequent validation with immunofluorescent staining for DAPI and cytokeratin (14). Elevated CTC levels using this assay are highly prognostic in breast, prostate, colorectal, and SCLC as well as in early-stage breast cancer (6, 15–19). However, for the most part, prognosis in these studies was based on enumeration of CTC, without regard to the presence of CTC-clusters.

SWOG S0500, a prospective randomized clinical trial, addressed whether patients with metastatic breast cancer (MBC) who had residual CTC [≥5/7.5 mL whole blood (WB)] after 1 cycle of first-line chemotherapy (indicating lack of a CTC-response) benefit from continuing that therapy or changing to an alternate chemotherapy. Although switching chemotherapy

[1]Department of Internal Medicine, University of Michigan Rogel Cancer Center, Ann Arbor, Michigan. [2]SWOG Statistical Center, Seattle, Washington. [3]Menarini Silicon Biosystems, Huntingdon Valley, Pennsylvania. [4]BioMagnetic Solutions LLC, State College, Pennsylvania. [5]Seattle Cancer Care Alliance, Seattle, Washington. [6]Department of Breast Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, Texas.

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Corresponding Author: Costanza Paoletti, University of Michigan Rogel Cancer Center (UMRCC), 1500 E. Medical Center Drive, 7130 CCC, SPC 5948, Ann Arbor, MI 48109. Phone: 734-647-7250; Fax: 734-647-9480; E-mail: pcostanz@med.umich.edu

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Translational Relevance

Several assays have been developed to enumerate circulating tumor cells (CTC). Elevated CTC levels portend a poor prognosis in several cancers including metastatic and early-stage breast cancer. Recent studies have suggested that, beyond elevated numbers of CTC, CTC clusters may be a driving force in the metastatic process. In this study, we tested the prognostic significance of CTC clusters compared with elevated CTC alone within the SWOG0500 clinical trial. Our findings suggest that the presence of doublets or clusters contributed little, if any, added prognostic information beyond the absolute number of CTC. We conclude that cluster evaluation in patients with metastatic breast cancer starting first-line chemotherapy has little or no clinical significance and it is unlikely to provide additional information to direct patient care in standard or investigational clinical settings. Additional trials in nonmetastatic patients, or using different CTC enrichment assays specifically designed to capture clusters, warrant further investigation.

regimens did not improve overall survival (OS), S0500 demonstrated that lack of CTC response after only a single cycle of chemotherapy portends chemoresistant MBC (18).

On the basis of the previously published preclinical and clinical data, we hypothesized that CTC doublets or clusters might identify relatively poorer prognosis in patients with MBC who participated in S0500 (18).

Materials and Methods

S0500 conduct/study design

This was a prospectively designed retrospective translational medicine study of S0500, for which the conduct and primary results have been previously reported (18). S0500 was conducted in accordance with the Declaration of Helsinki, and all applicable laws. All subjects provided written informed consent approved by local Institutional Review Boards. In S0500, Arm A included patients with <5 classic CTC/7.5 mL WB at baseline, whereas Arms B and C had ≥5 classic CTC/7.5 mL WB at baseline, but either had <5 classic CTC/7.5 mL WB (Arm B) or ≥5 classic CTC/7.5 mL WB (Arm C) after 1 cycle of single-agent chemotherapy. A prospectively written study plan of this study was approved by the SWOG Breast Translational Medicine Working Group (Supplementary Material).

Patient staging and follow-up

Details regarding patient eligibility, accrual, and overall conduct of the trial have been reported (18). As previously described, estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor 2 (HER2) status were determined locally by routine pathology at the treating institutions (18).

Blood draw, isolation, and enumeration of CTC

Blood draw, mailing, and processing for CTC using the CellSearch System (Menarini, Silicon Biosystems, Inc.) has been described previously (18). If a patient had <5 classic CTC/7.5 mL WB (Arm A), no further blood draws were collected. Patients with ≥5 classic CTC/7.5 mL WB at baseline (Arm B/C) had additional blood draws after 1 cycle of chemotherapy (C2D1).

Reanalyses of S0500 galleries

Classic versus revised algorithm. In the CellSearch System, candidate fluorescence–generated images from each of the filters, plus a composite image, are arranged as thumbnail images in a gallery format for classification by reviewers (Supplementary Fig. S6A and S6B as examples). A CTC event is defined as DAPI positive, expresses cytokeratin, but CD45 negative (10, 15, 18, 20). In the "classic" algorithm for the FDA-approved CellSearch System, thumbnail images are only counted as one CTC event, even if they contain several single cells or a doublet or cluster of cells (Supplementary Fig. S1A and S1B).

To determine the presence or absence of CTC doublets or clusters, and to compare the incidence of these to absolute numbers of CTC/7.5 mL WB, we applied a "revised" CellSearch CTC enumeration algorithm, in which CTC enumeration was calculated by counting each individual CTC, or each CTC within a doublet or a cluster, if captured in one thumbnail image. Thus, a patient may have originally had only 1–4 CTC/7.5 mL WB, which is below the cutoff for positivity in the classic algorithm, and yet, in the revised algorithm, might have more CTC (Supplementary Fig. S1A and S1B).

Doublet and cluster analysis. CTC clusters were defined as a group of CTC containing three or more distinct nuclei, and with contiguous cytoplasmic membranes, as described previously (6, 7, 10). However, to maximize our sensitivity, we included 2 cells containing 2 distinct nuclei with contiguous cytoplasmic membranes as a "doublet." Examples of single CTC, CTC cluster, and CTC doublet are provided in Supplementary Table S1.

Existing, archived CellSearch images were reread blindly by two operators (EMD or EPD) without knowledge of which arm the patient was assigned or clinical outcomes. To test interlaboratory variability, images from 20 samples were read in parallel by Hayes laboratory (EMD; EPD), and Menarini Silicon Biosystems' laboratory (MR).

Statistical analysis

Results were returned to SWOG Statistical Office for clinical associations. Patients were reassigned to Arm A, B, or C based on CTC values under the revised algorithm and analyses investigated the association between CTC enumeration, doublets, clusters, and clinical outcomes (OS). Survival curves were estimated using Kaplan–Meier method, 95% confidence intervals for median (m) OS were calculated using the method of Brookmeyer and Crowley, and were compared by using log-rank test. χ^2 testing was performed to find the proportional differences among groups. All tests performed were two-sided.

Results

Patient characteristics

A total of 595 evaluable patients were enrolled in S0500 (Fig. 1; ref. 18). Of these, 31 patients (10%) were not retested due to death, disease progression, or patient withdrawal before the second blood draw was due, accounting for a total of 564 patients. For this translational medicine study, using the revised algorithm, 15 patients had unreadable images (11 at baseline and four at C2D1); thus, 549/564 (97%) of the original enrolled

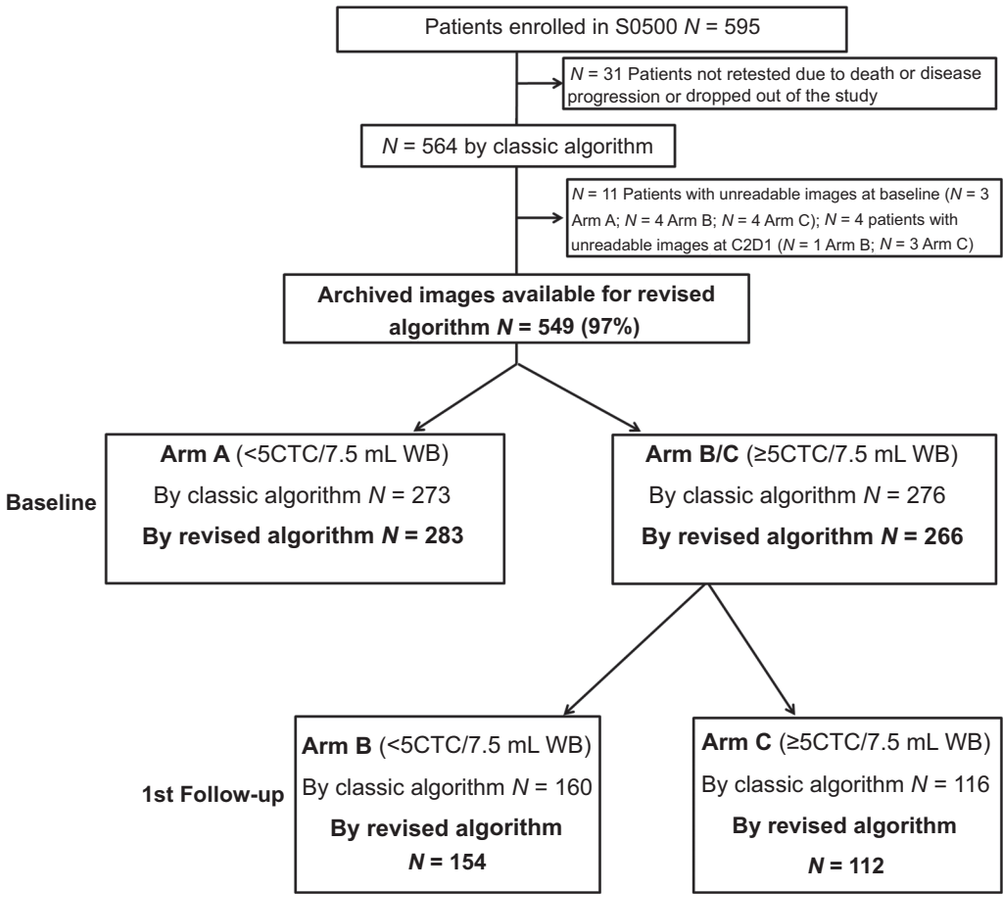

Figure 1.

REMARK diagram for doublet/clusters analysis of S0500. Of the 564 patients originally eligible for S0500, 549 had images stored and readable for analysis for doublets and clusters. When the CellSearch galleries were reread, 10 patients originally assigned to Arms B/C were reassigned to Arm A due to revised total CTC <5 due to interlaboratory variability, and likewise, 4 patients originally assigned to Arm C were assigned to Arm B due to revised total CTC <5 at 1st follow-up. Two patients originally assigned to Arm A were revised to ≥5 CTC/7.5 mL WB, but they could not be reassigned to Arm B or C due to the lack of assessments at first follow-up and therefore, they were still assigned to Arm A ($N = 1$ due to interlaboratory variability; $N = 1$ due revised algorithm)(see text for details).

patients were eligible (Fig. 1). Using the classic CellSearch algorithm, 273 patients (50%) did not have increased CTC levels at baseline (Arm A) and 276 patients had ≥5 classic CTC/7.5 mL WB at baseline (Arm B/C). Patients in Arm B/C were then divided in Arms B ($N = 160$) and Arm C ($N = 116$) according to reduction of CTC to <5 or ≥5 classic CTC/7.5 mL WB at first follow-up, respectively.

We used the revised CellSearch algorithm to permit counting of all CTC, regardless of whether they were in one or several CellSearch thumbnail images, and regardless of whether they are present singly, in doublets, or in clusters. A total of 283/549 eligible patients (52%) were placed in Arm A. Ten patients originally in Arm B/C were deemed to have <5 CTC/7.5 mL WB upon rereading, and were moved to Arm A. Two patients (0.4%) originally assigned to Arm A were determined to have ≥5 revised CTC/7.5 mL WB, either due to interlaboratory reading variability ($N = 1$) or to the revised algorithm ($N = 1$). Because they only had baseline and not subsequent follow-up blood draw, they could not be reassigned to Arm B or C and were included in the revised Arm A. Of the 266 patients who had ≥5 revised CTC/7.5 mL WB at baseline (Arm B/C), 154 were assigned to Arm B and 112 were assigned to Arm C. Four patients from the original Arm C were moved to Arm B due to interlaboratory reading variability. Overall, assignment of only 2.9% (16/549) of patients differed between the original and revised arm due to interlaboratory variability ($N = 15$) or due the revision of the algorithm ($N = 1$).

Incidence of single CTC, doublet(s), and cluster(s)

For analysis of outcomes based on CTC-clusters, we divided the patients into five groups: (i) no clusters or doublets, (ii) doublets only, (iii) clusters only, (iv) doublets and clusters, and (v) any doublets or clusters (sum of groups 2–4).

Baseline. The incidence of single CTC, CTC doublet(s) only, CTC cluster(s) only, CTC doublet(s) and cluster(s), and any CTC doublet(s) or cluster(s) across Arms A, B/C, B, and C are shown in Table 1A. Of those in Arm A, using the revised algorithm, 158/283 patients (56%) had zero CTC (Table 1A). Among 125 (44%) patients in Arm A with at least 1 CTC, four (1.4%) had CTC doublet(s) or cluster(s).

Table 1. Incidence of single CTC, doublets(s), and cluster(s) at baseline

A. Incidence of single CTC, doublets(s), and cluster(s) at baseline using the revised CellSearch algorithm

		Single CTC (no doublets or clusters)			CTC with doublets or clusters present			
	Baseline	0 CTC	1–4 CTC	≥5 CTC	Doublet(s) only	Cluster(s) only	Doublet(s) and cluster(s)	Any doublet(s) or cluster(s)
All	$N = 549$	158 (29%)	120 (22%)	167 (30%)	58 (11%)	11 (2%)	35 (6%)	104 (19%)
Arm A	$N = 283$	158 (56%)	120 (42%)	1 (<1%)	3 (1%)	0	1 (<1%)	4 (1.4%)
Arm B/C	$N = 266$	NA	NA	166 (62%)	55 (21%)	11 (4%)	34 (13%)	100 (38%)
Arm B	$N = 154$	NA	NA	115 (75%)	20 (13%)	4 (2%)	15 (10%)	39 (25%)
Arm C	$N = 112$	NA	NA	51 (46%)	35 (31%)	7 (6%)	19 (17%)	61 (54%)

B. Incidence of doublets, clusters, doublets and/or clusters at baseline by hormone receptor (HR) and human epidermal growth factor 2 (HER2) status in primary cancer

		Single CTC (no doublets nor clusters)			CTC with doublets or clusters present			
	Baseline	0 CTC	1–4 CTC	>5 CTC	Doublet(s) only	Cluster(s) only	Doublet(s) and cluster(s)	Any doublet(s) or cluster(s)
All	$N = 545$[a]	157 (29%)	120 (22%)	164 (30%)	58 (11%)	11 (2%)	35 (6%)	104 (19%)
HR^+; $HER2^-$	$N = 320$	81 (25%)	72 (22%)	101 (32%)	37 (12%)	10 (3%)	19 (6%)	66 (21%)
$HER2^+$	$N = 95$	30 (32%)	23 (24%)	26 (27%)	11 (12%)	1 (1%)	4 (4%)	16 (17%)
Triple negative	$N = 130$	46 (35%)	25 (19%)	37 (29%)	10 (8%)	0	12 (9%)	22 (17%)

C. Incidence of doublets, clusters, doublets and/or clusters at baseline by sites of metastases

		Single CTC (no doublets nor clusters)			CTC with doublets or clusters present			
	Baseline	0 CTC	1–4 CTC	>5 CTC	Doublet(s) only	Cluster(s) only	Doublet(s) and cluster(s)	Any doublet(s) or cluster(s)
All	$N = 547$[b]	156 (29%)	120 (22%)	167 (30%)	58 (11%)	11 (2%)	35 (6%)	104 (19%)
Bone only	$N = 66$	20 (30%)	15 (22%)	21 (32%)	4 (6%)	3 (5%)	3 (5%)	10 (15%)
Visceral	$N = 372$	97 (26%)	74 (20%)	123 (33%)	43 (11%)	6 (2%)	29 (8%)	78 (21%)
Other	$N = 109$	39 (36%)	31 (28%)	23 (21%)	11 (10%)	2 (2%)	3 (3%)	16 (15%)

NOTE: **A,** P value of presence of doublets or clusters comparing Arm B/C versus Arm A: < 0.0001; P value of presence of doublets or clusters comparing Arm B versus Arm C: < 0.0001.

B, [a]Four patients with missing data; χ^2 test for the presence of doublets according to breast cancer type: $P = 0.93$; χ^2 test for the presence of doublets and/or clusters between breast cancer subtype and clusters: $P = 0.55$.

C, [b]Two patients with missing data. χ^2 test for presence of doublets according sites of metastases: $P = 0.10$ χ^2 test for presence of any doublets or clusters according to sites of metastases: $P = 0.24$.

By definition, all patients in Arms B/C, had ≥5 single CTC/7.5 mL WB, at baseline. Of note, 100 patients (38%) had CTC doublet(s) or cluster(s) (Table 1A). In those ultimately assigned to Arm B, 39/154 (25%) patients had CTC doublet (s) or cluster(s) (Table 1A). In those ultimately assigned to Arm C, 61/112 (54%) patients had CTC doublet(s) or cluster(s) (Table 1A). Overall, we found that doublets(s) or cluster(s) or both were more likely to be present in those with ≥5 CTC/7.5 mL WB (Arm B/C) versus <5 CTC/7.5 mL WB (Arm A; 38% vs. 1.4%; $P < 0.0001$). Additional data are in Supplementary Table S2A. Because the revised algorithm permits counting every cell within a single composite image regardless of whether it is single, in doublet, or cluster, we examined the level of CTC as determined with the classic CellSearch algorithm according to the presence or absence of doublets and clusters (Supplementary Fig. S2). In this analysis, CTC doublets and cluster were more likely in specimens with higher CTC values ($P < 0.0001$).

At baseline, there was no significant difference in the incidence of CTC doublet(s) and/or cluster(s) among different breast cancer subtypes. In particular, 21%, 17%, and 17% of patients with HR^+/$HER2^-$, $HER2^+$, and triple negative had doublet(s) and/or cluster (s), respectively ($P = 0.55$; Table 1B). Likewise, there was no significant difference in the incidence of CTC doublet(s) and/or cluster(s) between different sites of disease. Fifteen percent, 21%, and 15% of patients with bone only, visceral, and other disease sites had an incidence of any doublet(s) or cluster(s), respectively ($P = 0.24$; Table 1C). More extensive data regarding CTC number and doublets and clusters by hormone receptor status and HER2 status of primary tumor as well as by disease site are further reported in Supplementary Tables S2B and S2C.

First follow-up. By definition, at first follow-up none of the 160 patients originally assigned to Arm B had ≥5 CTC/7.5 mL WB with the classic algorithm. Using the revised algorithm, 154 patients were assigned to the revised Arm B. Of these, at first follow-up, 89 (58%) had 0 and 65 (42%) had 1–4 single CTC/7.5 mL WB. None had any doublet(s) or clusters(s) in their first follow-up blood specimens (Supplementary Table S3A).

At first follow-up, using the revised algorithm, 112 patients were assigned to the revised Arm C. Of these, at first follow-up blood draw, in total, 35 (31%) patients in Arm C had CTC doublet (s) or cluster(s)(Supplementary Table S3A). There was a statistically significant difference in the incidence of CTC clusters at first follow-up between patients in Arm B versus Arm C (Arm B: 0%; Arm C: 31%; $P < 0.0001$).

The incidence of doublets or clusters was higher in patients who, using the classic algorithm, had ≥20 versus 5-19 CTC/7.5 mL WB at baseline (≥20 CTC/7.5 mL WB = 54%; 5–19 CTC/7.5 mL WB = 9%; $P < 0.0001$). Similar results were observed using the revised algorithm (≥20 revised CTC/7.5 mL WB = 55%; 5–19 revised CTC/7.5 mL WB = 10%; $P < 0.0001$; Table 2).

There were significant differences in the incidence of CTC doublet(s) or cluster(s) among different breast cancer subtypes at first follow-up in Arm B and C. Fourteen and 19% of patients with HR^+/$HER2^-$ or triple-negative disease, respectively, had doublets or clusters ($P = 0.31$). However, only eight patients with $HER2^+$ disease had ≥5 CTC/7.5 mL WB at first follow-up, and none of them had CTC clusters or doublets ($P = 0.005$ comparing HER2+ to other subsets; Supplementary Table S3B). There was no significant difference in the incidence of CTC doublet(s) and/or cluster(s) between different sites of disease. Twelve percent, 13%, and 14% of patients with bone only,

Table 2. Correlation between CTC cluster(s) and/or doublet(s) in patients with 5–19 versus ≥20 CTC/7.5 mL WB using the revised algorithm

		Single CTC (no doublets or clusters)	CTC with doublets or clusters present Any doublet(s) or cluster (s)			
	Baseline	≥5 CTC	Doublet(s) only	Cluster(s) only	Doublet(s) and cluster(s)	Any doublet(s) or cluster(s)
5–19 CTC	$N = 103$	93 (90%)	5 (5%)	2 (2%)	3 (3%)	10 (10%)
≥20 CTC	$N = 165$	74 (45%)	50 (30%)	9 (6%)	32 (19%)	91 (55%)

NOTE: P value of presence of doublets or clusters comparing 5–19 versus ≥20 CTC/7.5 mL WB: < 0.0001.

visceral, and other had doublet(s) or cluster(s), respectively ($P = 0.95$; Supplementary Table S3C).

Outcomes according to presence or absence of doublets or clusters

Benefit from randomization. We explored whether OS for patients in Arm C differed according to clusters or not between those who were assigned to stay on the original chemotherapy (C1) versus those who were assigned to switch to an alternative chemotherapy regimen (C2). For those without cluster(s) at first follow-up, we observed no difference in OS between Arm C1 and C2 (mOS: C1 = 13.3 months; C2 = 12.5 months; $P = 0.58$; Supplementary Fig. S3A). For patients with cluster(s), there was a nonsignificant trend (mOS: C1 = 3.5 months; C2 = 11.0 months; $P = 0.49$; Supplementary Fig. S3B). In addition, for those without clusters and doublets at first follow-up, we observed no difference in OS between Arm C1 and C2 ($P = 0.78$). Similar results were found for patients with doublets and clusters ($P = 0.63$; Supplementary Fig. S3C and S3D).

CTC-clusters and overall survival. Because S0500 and this study differ by only 14/549 patients (2.6%), the study population is almost identical to the original for the primary endpoint: OS [original cohort: mOS Arm A, B, and C = 34.8, 22.9, and 13.1 months, respectively; revised CTC cohort: mOS Arm A, B, and C = 34.2 (95% CI, 29.0–37.3), 22.9 (95% CI, 18.6–28.1), and 12.4 (95% CI, 9.1–14.1) months, respectively] (18).

Baseline. Arm A. There was a non statistically significant trend toward longer OS for patient with 0 versus 1–4 CTC/7.5 mL WB (mOS: 0 CTC/7.5 mL WB = 35.8 months; 1–4 CTC/7.5 mL WB = 31.2 months; $P = 0.20$; Fig. 2A). Only three patients had clusters so no statistically meaningful conclusions could be drawn.

Arm B/C. In Arm B/C, patients with doublets and clusters at baseline had statistically significantly worse OS (11.7 months) compared with doublets only (16.8 months) or clusters only (22.9 months) or no doublets or clusters (19.9 months; $P = 0.008$ comparing the 4 groups; Fig. 2B). Pairwise comparison between no doublets and no clusters versus doublets and clusters was highly statistically significant ($P = 0.002$).

In patients ultimately assigned to Arm B, there was no statistically significant difference in OS between patients who had doublets and clusters (23.6 months) compared with patients with only doublets (18.6 months) or patients with no doublets or clusters (23.9 months; $P = 0.51$ comparing the 4 groups; Fig. 2C). The group with clusters only was too small to conduct any analysis. In Arm C, patients with doublets and clusters had statistically significantly worse OS (9.3 months) compared with those with only doublets (12.1 months) or only clusters (22.9 months) or no doublets or clusters (14.8 months; $P = 0.008$ comparing the 4 groups; Fig. 2D). Pairwise comparisons between doublets and clusters versus doublets only, clusters only, and no doublets or clusters was highly statistically significant ($P = 0.02$, $P = 0.006$, and $P = 0.0008$, respectively). See Supplementary Figs. S4–S6 for additional KM curves of OS.

First follow-up. Arm B. There was a nonstatistically significant difference in OS between patients with 0 versus those with 1–4 CTC/7.5 mL WB at first follow-up (mOS: 0 CTC/7.5 mL WB = 25.4 months; 1–4 CTC/7.5 mL WB = 21.3 months; $P = 0.15$; Fig. 3A). No patients in Arm B had doublets or clusters at first follow-up.

Arm C. Patients with doublets and clusters at first follow-up had statistically significantly worse OS (7.3 months) compared with those with only doublets (8.4 months) or no doublets or clusters (14.4 months; $P = 0.01$; Fig. 3B). Pairwise comparison between no doublets or clusters versus doublets and clusters was highly statistically significant ($P = 0.004$). Additional KM curves are shown in Supplementary Fig. S7A–S7C.

Prognosis according to number of CTC versus CTC clusters

We hypothesized that perhaps the worse OS observed with clusters was due to the presence of more CTC/7.5 mL WB, in clusters or not, because the number of CTC would be increased by virtue of the revised algorithm. OS was longer in patients who had 5–19 versus ≥20 CTC/7.5 mL WB at baseline, regardless of which algorithm was used (classic: mOS in patients with 5–19 CTC/7.5 mL WB = 24.6 months; ≥20 CTC/7.5 mL WB = 14.2 months; $P < 0.0001$; revised: mOS in patients with 5–19 CTC/7.5 mL WB = 26.6 months; ≥20 CTC/7.5 mL WB = 14.4 months; $P < 0.0001$; Fig. 4A; Supplementary Table S4). However, within each of these subgroups using the revised algorithm, mOS was not statistically different for presence of clusters and doublets compared with no clusters or doublets (revised: mOS in patients with 5–19 CTC/7.5 mL WB with doublets or clusters = 25.2 months; without doublets or clusters = 26.6 months; $P = 0.92$; Fig. 4B); (revised: mOS in patients with ≥20 CTC/7.5 mL WB with doublets or clusters = 13.7 months; without doublets or clusters = 14.9 months; $P = 0.45$; Fig. 4C). Taken together, these data suggest the worse prognosis in patients with clusters may be a function of the number of CTC/7.5 mL WB, which is increased with clusters using the revised algorithm.

Discussion

In this prospective–retrospective translational medicine study, we observed that using the CellSearch system, CTC-doublet(s) and cluster(s) were commonly identified in patients with MBC starting first-line chemotherapy and who had elevated CTC at baseline. As expected, CTC clusters were associated with a worse prognosis, regardless of whether they were detected at baseline or at first follow-up.

However, unexpectedly, CTC clusters were not independent of the number of CTC enumerated using the revised CellSearch algorithm. Rather, the negative association of clusters with OS was likely due to the number of CTC present, which is increased by the high presence of doublets or clusters using the revised

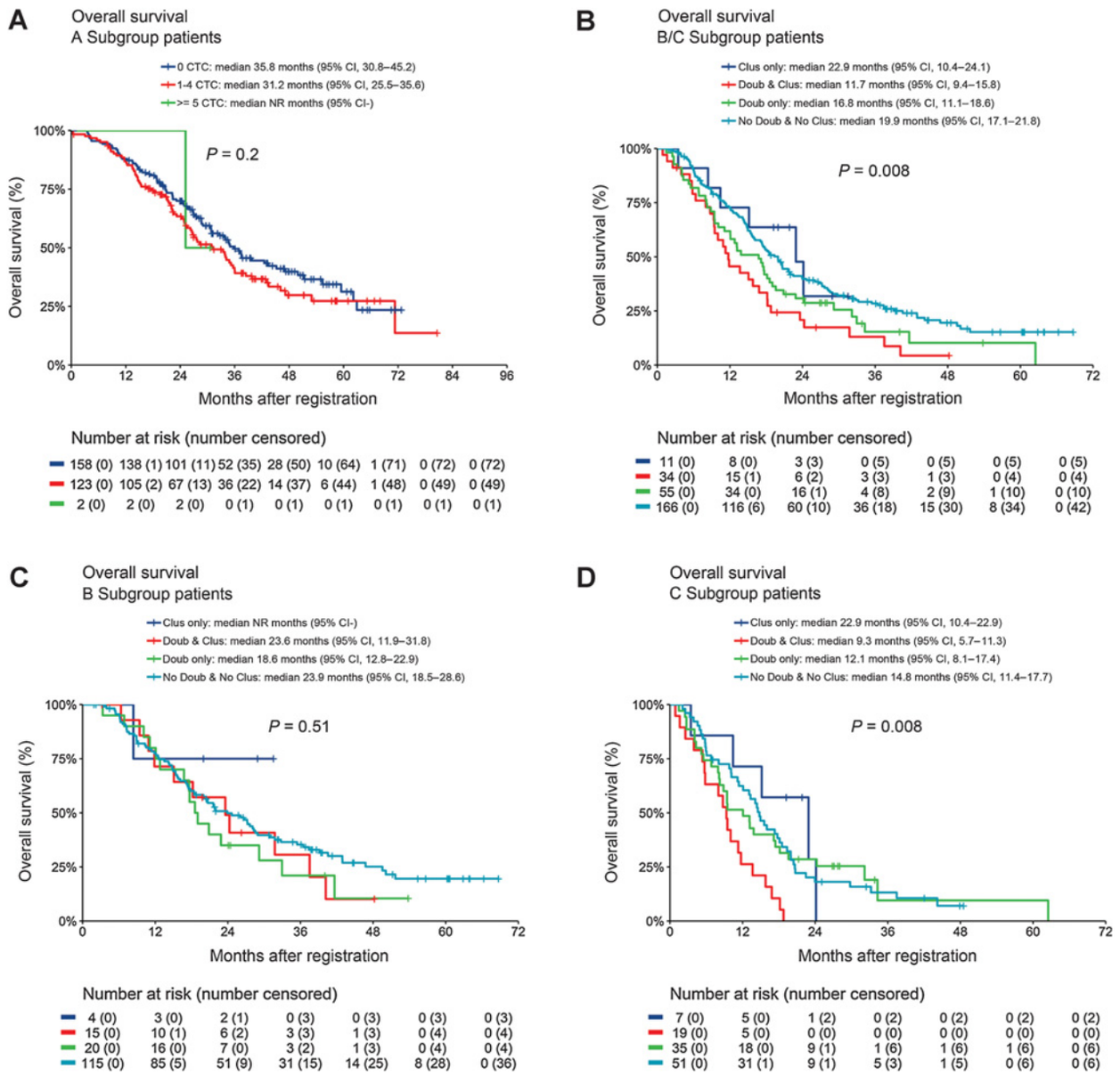

Figure 2.

Overall survival according to CTC enumeration or doublets and clusters at baseline. **A,** Arm A: OS for patients with 0 versus 1–4 CTC/7.5 mL WB using revised algorithm. **B,** Arm B/C: OS according to presence or absence of clusters. **C,** Arm B: OS according to presence or absence of clusters. **D,** Arm C: OS according to presence or absence of clusters (See text for definition of Arm A, B, and C).

algorithm, because the latter permits counting every cell within a single thumbnail composite image. Even though patients with ≥20 CTC/7.5 mL WB at baseline had a shorter OS than those with 5–19 CTC/7.5 mL WB ($P < 0.0001$), when we revisited the importance of doublets and clusters within these two groups there was no difference in outcomes according to the presence of doublets or clusters. Even though CTC counts are higher by using the revised algorithm, the observation that doublets and clusters are more common in specimens with high CTC counts using the classic algorithm (Supplementary Fig. S2) further suggests that it is the number of cells, regardless of whether they are singlets or in clusters, that drives a worse prognosis in patients with MBC starting first-line chemotherapy. Our results are consistent with a recently reported study that suggested that CTC clusters were not significantly prognostic when examined in patients with MBC who had the cutoff of ≥20 CTC/7.5 mL WB (21).

Our results are dissimilar with those of other investigators, who have suggested that, in contrast to individual CTC, CTC clusters in breast as well as lung cancers have distinct biological properties and mediate the metastatic process (2, 7, 11). These discrepancies could be due to the use of different assays. One concern might be that the CellSearch system is not ideal for identifying or

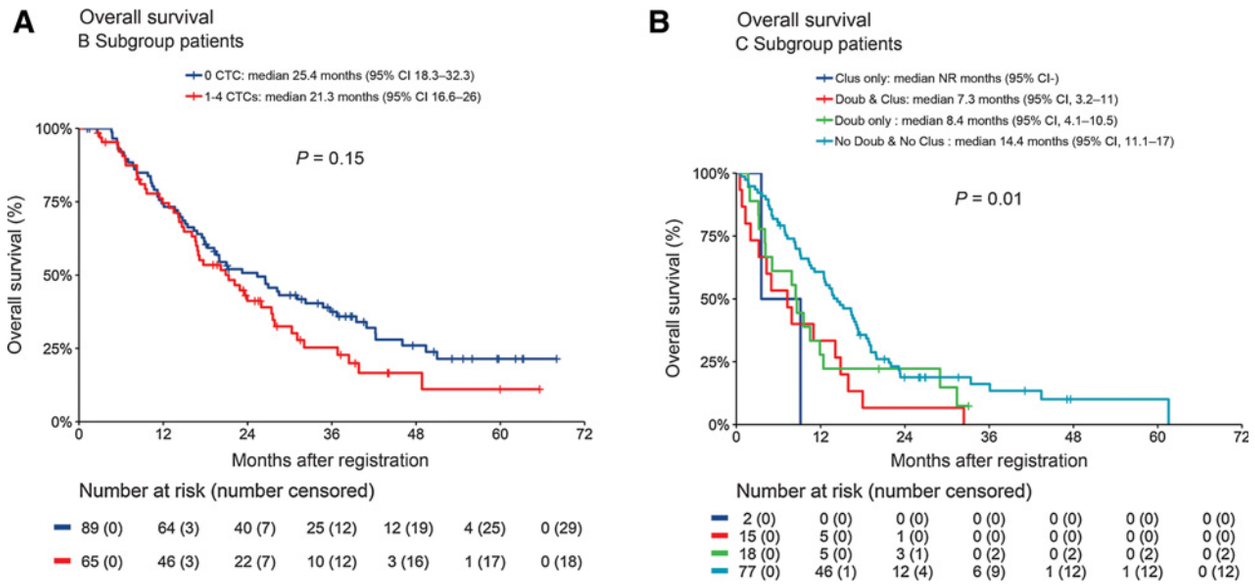

Figure 3.

Overall survival according to CTC enumeration or doublets and clusters at first follow-up**. A,** Arm B: OS for patients with 0 versus 1–4 CTC/7.5 mL WB using revised algorithm. **B,** Arm C: OS according to presence or absence of clusters (See text for definition of Arm A, B, and C).

enumerating doublets or clusters, because the blood specimens are subjected to multiple manipulations prior to CTC analyses. The CellSearch platform might potentially disrupt CTC doublets and clusters, underestimating their biological/prognostic effect compared with other assays. However, the incidence of CTC doublets and clusters in MBC in our study (~19%) is similar to that previously reported in MBC detected either by the CellSearch (10–12) or other assays (2, 22). Similar to other studies, we found that CTC doublets and/or clusters were more likely to be associated with ≥5 CTC/7.5 mL WB (10, 11). However, new technologies specifically designed to isolate CTC clusters have been developed (23–25) and might provide further insight into this issue.

Another concern is that other investigators have reported that CTC clusters are prognostic when identified in longitudinally sampled specimens (12, 21). Because our study focuses on CTC enumeration and evaluation of CTC doublets and clusters at baseline and first follow-up, we cannot exclude that longitudinal evaluation of CTC doublets and clusters could provide additional prognostic information for patients with MBC.

Our study was exclusively conducted in patients with MBC starting first-line chemotherapy (18). It is possible that clusters are important biologically in different types of breast cancer, such as inflammatory breast cancer (11) or in completely different cancers, such as small-cell lung cancer (7). Whereas it is possible that clusters do indeed play a critical role in inflammatory breast and small-cell lung cancer, we are confident from this blinded prospective–retrospective study that CTC clusters do not provide substantial prognostic information over CTC enumeration, either at baseline or first follow-up, in patients starting first-line chemotherapy for MBC.

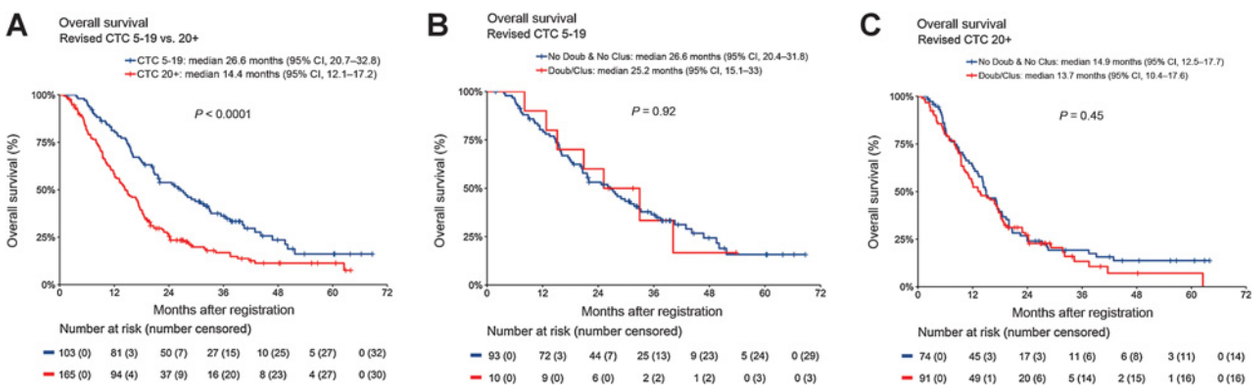

Figure 4.

Overall survival according to CTC enumeration in patients with 5–19 versus ≥20 CTC/7.5 mL WB at baseline using the revised algorithm. **A,** OS for patients with 5–19 versus ≥20 CTC/7.5 mL WB; **B,** OS of patients with 5–19 CTC/7.5 mL WB divided by doublets or clusters versus no doublets or clusters; **C,** OS of patients with ≥20 CTC/7.5 mL WB divided by doublets or clusters versus no doublets or cluster.

Other investigators have reported that heterotypic clusters consisting of CTC and leukocytes might be more prognostic than homotypic clusters of CTC alone (3, 4). However, the CellSearch system is not designed to analyze heterotypic clusters, and therefore we cannot address this issue.

Finally, patients enrolled in S0500 might not have been typical or representative of other studies. However, S0500 was a pragmatic trial, in which patients with MBC regardless of intrinsic subtype (ER and HER2 status) were enrolled, and the treatment was the choice of standard-of-care single-agent chemotherapy at the treating doctor's discretion. The incidence of having elevated CTC ($\geq$5/7.5 mL WB) at baseline in S0500 was approximately 50%, in line with several other studies of CTC in patients with MBC (15). Moreover, there was no difference in the incidence of CTC doublets or clusters according to clinical or pathologic subtypes or site of disease (Table 1B and C). Indeed, a strength of this study is that it was conducted using images from 97% of participants in the S0500 clinical trial ($N = 595$). Prespecified definitions of clusters and doublets were used to reread the images without knowledge of clinical outcomes and clinical correlation was conducted in the SWOG statistical center.

Nonetheless, although we interrogated data from 97% of the more than 500 patients enrolled in S0500, subgroup size limits the power of our analyses. However, results obtained in Fig. 4B and C, suggest that it is very unlikely that our results would be substantially different with larger numbers. In addition, although all the patients entered in the trial had received single-agent first-line chemotherapy, chemotherapy regimens were not assigned, rather they were chosen by the treating physician limiting the power to attempt subgroup analyses on the effect of doublets and clusters versus not according to treatment type.

Taken together, these considerations suggest that S0500 is very representative of the average patient starting first-line chemotherapy in MBC and an ideal setting in which to test the biological and clinical role of CTC-clusters.

Perhaps the only suggestion that doublets or clusters might have some biological or clinical effect was raised by the observation that they were present more frequently at baseline in patients ultimately assigned to Arm C compared with Arm B. By definition, Arm B represents patients who had a "CTC response," while Arm C contains those who did not. Taken together, these data might suggest that CTC doublets or clusters mediate resistance to first-line chemotherapy in MBC. However, within Arm C, there was no statistically significant difference in OS between patients with or without doublets or clusters for those assigned to stay on initial chemotherapy (C1) versus switching to an alternative chemotherapy (C2).

In a series of secondary analyses, we also interrogated whether the classic cutoff ($\geq$5 CTC/7.5 mL WB) chosen for determination of elevated versus not elevated CTC in prior studies is optimal (15). As noted, the prognosis for patients in Arm A (those with <5 CTC/7.5 mL WB), was quite favorable using the classic algorithm, with a median OS = 36 months (18). In this regard, OS using the revised algorithm was essentially no different for patients in Arm A who had 0 versus 1–4 CTC/7.5 mL WB. Furthermore, there was no difference in OS in Arm B at first follow-up between patients with 0 CTC/7.5 mL WB versus 1–4 CTC/7.5 mL WB (mOS 0 CTC/7.5 mL WB = 25.4 months; 1–4 CTC/7.5 mL WB = 21.3 months $P = 0.15$). These data further validate the cutoff of $\geq$5 CTC/7.5 mL WB as the correct absolute cutoff for CellSearch assay in MBC (15). Incidentally, OS did not differ according to presence or absence of doublets or clusters in Arm A at baseline, or in Arm B at first follow-up, although the incidence of doublets or clusters in these two subgroups was quite low.

In summary, the results of this translational medicine study suggest that neither doublets nor clusters plays a major role in the outcome of patients with MBC starting first-line chemotherapy, raising questions regarding their role in progression of metastatic disease. Rather, our data suggest that absolute number of CTC may be more important than the physical presence of clusters. Therefore, analysis of CTC doublets or clusters is unlikely to provide additional information to direct patient care in standard or investigational clinical settings in patients with MBC starting first-line chemotherapy. Additional trials in patients with nonmetastatic breast cancer, or using different CTC enrichment assays specifically designed to capture clusters, warrant further investigation. In addition, our data confirm the classic clinical CellSearch algorithm and cutoff of $\geq$5 CTC/7.5 mL WB, as determined in prior studies, is appropriate.

Disclosure of Potential Conflicts of Interest

C. Paoletti reports her institution receiving commercial research grants from AstraZeneca and Pfizer, and received travel paid by Menarini Silicon Biosystems. J.R. Gralow is a consultant/advisory board member for Roche, Novartis, Sandoz, Immunomedics, Puma, AstraZeneca, Genomic Health, Pfizer, and Merck. D.F. Hayes reports receiving commercial research grants and commercial research support from Menarini Silicon Biosystems, and his institution has a patent for phenotypic analysis of CTC, which is licensed to MSB, and for which Hayes and his institution receive annual royalties. M. Repollet is an employee of Menarini Silicon Biosystems and is listed as a co-inventor on patents US 8,940,493 B2, "Circulating Tumor Cell Assay," and US 7,863,012 B, "Analysis of Circulating Tumor cells, fragments and debris," both owned by Menarini Silicon Biosystems. No potential conflicts of interest were disclosed by the other authors.

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Authors' Contributions

Conception and design: C. Paoletti, M.I. Repollet, G.V. Doyle, G.N. Hortobagyi, W.E. Barlow, D.F. Hayes

Development of methodology: C. Paoletti, E.M. Dolce, E.P. Darga, M.I. Repollet, G.V. Doyle, D.F. Hayes

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Paoletti, M.I. Repollet, G.N. Hortobagyi, J.B. Smerage, W.E. Barlow, D.F. Hayes

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Paoletti, J. Miao, J.R. Gralow, G.N. Hortobagyi, J.B. Smerage, W.E. Barlow, D.F. Hayes

Writing, review, and/or revision of the manuscript: C. Paoletti, J. Miao, E.M. Dolce, E.P. Darga, M.I. Repollet, J.R. Gralow, G.N. Hortobagyi, J.B. Smerage, W.E. Barlow, D.F. Hayes

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Paoletti, E.M. Dolce, E.P. Darga, J.B. Smerage, D.F. Hayes

Study supervision: C. Paoletti, G.N. Hortobagyi, J.B. Smerage, D.F. Hayes

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
2. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 2014;158:1110–22.
3. Duda DG, Duyverman AM, Kohno M, Snuderl M, Steller EJ, Fukumura D, et al. Malignant cells facilitate lung metastasis by bringing their own soil. Proc Natl Acad Sci U S A 2010;107:21677–82.
4. Szczerba BM, Castro-Giner F, Vetter M, Krol I, Gkountela S, Landin J, et al. Neutrophils escort circulating tumour cells to enable cell cycle progression. Nature 2019;566:553–7.
5. Gkountela S, Castro-Giner F, Szczerba BM, Vetter M, Landin J, Scherrer R, et al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell 2019;176:98–112.
6. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 2012;30:525–32.
7. Hou JM, Krebs M, Ward T, Sloane R, Priest L, Hughes A, et al. Circulating tumor cells as a window on metastasis biology in lung cancer. Am J Pathol 2011;178:989–96.
8. Kats-Ugurlu G, Roodink I, de Weijert M, Tiemessen D, Maass C, Verrijp K, et al. Circulating tumour tissue fragments in patients with pulmonary metastasis of clear cell renal cell carcinoma. J Pathol 2009;219:287–93.
9. Molnar B, Ladanyi A, Tanko L, Sreter L, Tulassay Z. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. Clin Cancer Res 2001;7:4080–5.
10. Paoletti C, Li Y, Muniz MC, Kidwell KM, Aung K, Thomas DG, et al. Significance of Circulating Tumor Cells in metastatic triple negative breast cancer patients within a randomized, phase II trial: TBCRC 019. Clin Cancer Res 2015;21:2771–9.
11. Mu Z, Wang C, Ye Z, Austin L, Civan J, Hyslop T, et al. Prospective assessment of the prognostic value of circulating tumor cells and their clusters in patients with advanced-stage breast cancer. Breast Cancer Res Treat 2015;154:563–71.
12. Wang C, Mu Z, Chervoneva I, Austin L, Ye Z, Rossi G, et al. Longitudinally collected CTCs and CTC-clusters and clinical outcomes of metastatic breast cancer. Breast Cancer Res Treat 2017;161:83–94.
13. Paoletti C, Hayes DF. Circulating tumor cells. Adv Exp Med Biol 2016;882:235–58.
14. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10:6897–904.
15. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
16. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008;14:6302–9.
17. Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:3213–21.
18. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014;32:3483–9.
19. Krebs MG, Sloane R, Priest L, Lancashire L, Hou JM, Greystoke A, et al. Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. J Clin Oncol 2011;29:1556–63.
20. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406–14.
21. Larsson AM, Jansson S, Bendahl PO, Levin Tykjaer Jorgensen C, Loman N, Graffman C, et al. Longitudinal enumeration and cluster evaluation of circulating tumor cells improve prognostication for patients with newly diagnosed metastatic breast cancer in a prospective observational trial. Breast Cancer Res 2018;20:48.
22. Mu Z, Benali-Furet N, Uzan G, Znaty A, Ye Z, Paolillo C, et al. Detection and characterization of circulating tumor associated cells in metastatic breast cancer. Int J Mol Sci 2016;17:pii: E1665.
23. Sarioglu AF, Aceto N, Kojic N, Donaldson MC, Zeinali M, Hamza B, et al. A microfluidic device for label-free, physical capture of circulating tumor cell clusters. Nat Methods 2015;12:685–91.
24. Au SH, Edd J, Stoddard AE, Wong KHK, Fachin F, Maheswaran S, et al. Microfluidic isolation of circulating tumor cell clusters by size and asymmetry. Sci Rep 2017;7:2433.
25. Cheng SB, Xie M, Chen Y, Xiong J, Liu Y, Chen Z, et al. Three-dimensional scaffold chip with thermosensitive coating for capture and reversible release of individual and cluster of circulating tumor cells. Anal Chem 2017;89:7924–32.