original article

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Clinical value of circulating endothelial cells and circulating tumor cells in metastatic breast cancer patients treated first line with bevacizumab and chemotherapy

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Background: We investigated whether circulating tumor cells (CTCs) and circulating endothelial cells (CECs) predict clinical outcome of first-line chemotherapy combined with bevacizumab in metastatic breast cancer patients.
Patients and methods: In a French substudy of the MO19391 trial, CTC and CEC counts (CellSearch system) at baseline and changes after two cycles of treatment were correlated with time to progression (TtP).
Results: CTC and CEC levels were not correlated in the 67 patients included. At baseline, CTC positivity was a significant prognostic marker for TtP at a threshold of 3 CTC/7.5 ml ($P < 0.05$) but not at 5 CTC/7.5 ml ($P = 0.09$). Baseline CEC levels (median 17 CEC/4 ml, range 1–769) were associated with age ≥45 years ($P = 0.01$), elevated lactate dehydrogenase ($P < 0.01$) and not with TtP at any threshold. Changes of CTC count during treatment were not a surrogate of TtP, with any of the model tested (threshold based or relative decrease in percent). However, increase in CEC count was associated with improved TtP, at the threshold of 20 CEC/4 ml ($P < 0.01$).
Conclusion: Bevacizumab combined with first-line chemotherapy may modify the predictive value of CTC during treatment possibly due to impaired tumor cells intravasation through vessels endothelium. Variations in CEC levels appear to be a promising early surrogate marker of TtP under antiangiogenic treatment.
Key words: bevacizumab, breast cancer, circulating endothelial cells, circulating tumor cells

background

Several methods for the detection of cancer cells disseminated in the bone marrow and/or circulating in the peripheral blood of patients have been developed recently for the most common epithelial tumors. Beyond biological characterization of the metastatic process, disseminated tumor cells (DTCs) and circulating tumor cells (CTCs) detection can be used in a few clinical settings as a prognostic factor and/or as an early surrogate marker of treatment response [1, 2]. In patients with metastatic breast cancer, it has been reported that DTC detection was not clinically relevant, whereas CTC detection was of prognostic significance [3]. Currently, the most commonly used detection system in this setting is the automated and standardized CellSearch® assay [4], which has a validated reproducibility [5]. In a pivotal study reported by Cristofanilli et al. in 2004 [6], CTCs were screened by this system in patients who received hormonal treatments or chemotherapy as first- or second-line treatment of metastatic breast cancer. In patients under standard chemotherapy, CTC positivity at baseline was an independent prognostic factor for progression-free survival (PFS; 2.7 versus 7.0 months; $P < 0.001$) and overall survival (OS; 10.1 versus >18 months; $P < 0.001$). CTC positivity was defined in a training set ($n = 102$ patients) and validated in another set of patients ($n = 75$ patients). Three weeks after the start of the treatment, CTC status was also independently associated with PFS (2.1 versus 7.0 months; $P < 0.001$) and OS (8.2 versus >18 months; $P < 0.001$). In the same series, other studies reported that CTC detection was poorly correlated to the CA 27-29 serum marker nor to the tumor burden [7, 8]. The threshold of 5 CTC/7.5 ml for CTC positivity and its association with clinical outcome have been confirmed in 80 patients treated with standard chemotherapy at the European Institute of Oncology (Milan, Italy) [9], and in another 74 American patients [10]. However,

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the clinical benefit of using CTC count as an early surrogate marker of response and/or time to progression (TtP) under treatment has not yet been demonstrated prospectively, explaining why American Society of Clinical Oncology has not recommended its use [11].

In this specific setting of first-line treatment of metastatic breast cancer patients, it has been demonstrated that the antiangiogenic agent bevacizumab, when given in combination with first-line chemotherapy, significantly improves PFS and response rates as compared with chemotherapy alone [12–14]. The impairment of vascular endothelial growth factor-A actions (endothelial cell proliferation and migration, endothelial cell apoptosis inhibition, extracellular matrix remodelling, vasodilatation, increase of vascular permeability) [15] explains bevacizumab antitumoral effects together with its main side-effects. There is currently no validated prognostic or predictive marker of response to the combination of bevacizumab and standard chemotherapy. In a French substudy of the phase IIIb international multicentric MO19391 trial evaluating the safety of bevacizumab with first-line taxane-based chemotherapy in patients with metastatic breast cancer, we investigated whether CTC and circulating endothelial cell (CECs) counts were associated with the outcome (TtP, as defined in the MO19391 trial).

patients and methods

This article has been written in accordance with the REporting of tumor MARKer studies criteria [16].

patients and treatments

The single-arm, prospective, multinational, observational MO19391 study enrolled >2000 patients with metastatic breast cancer to better understand the safety profile of first-line bevacizumab in combination with taxane-based therapy. Briefly, MO19391 inclusion criteria were age ≥18 years, metastatic (mBC) or nonresectable locoregional recurrence (LR) of breast cancer adenocarcinoma, HER2-negative tumors (or HER2 positive if they have progressed after previous adjuvant trastuzumab), Eastern Cooperative Oncology Group performance status of zero to two and adequate liver, kidney and hematopoietic functions. Exclusion criteria included prior chemotherapy for LR or mBC and evidence of central nervous system metastasis. Bevacizumab regimen was 10 mg/kg every 2 weeks or 15 mg/kg every 3 weeks, combined with the physician's choice of taxane regimen (or investigator's standard of care, excluding anthracyclines). Treatment with bevacizumab was continued until disease progression, unacceptable toxicity or patient withdrawal. Our ethically approved companion study was prospectively conducted in 10 centers in France between June 2007 and June 2008. All samples were obtained with the patient's written informed consent, after approval by the regional ethics committee. Neither patients nor clinicians were informed of the results of CTC and CEC analysis. Lactate dehydrogenase (LDH) and CA 15-3 levels were also investigated before the first treatment cycle (baseline) and before cycle 3 (C3). There was no prior publication on this cohort of patients.

CTC and CEC counts

Briefly, two CellSave® tubes of blood (one tube for CTC and the other for CEC analysis) were drawn at baseline and before C3, i.e. 6 weeks after the start of the treatment, at the time of physician consultations. Samples were maintained at room temperature and processed within 96 (CTC samples) or 72 (CEC samples) h after collection. All evaluations were carried out with no knowledge of the patient's clinical status. The standardized CellSearch® technique has been reported previously for CTC [4] and CEC [17] detection. Briefly, CTCs expressing the epithelial cell adhesion molecule were immunomagnetically enriched and stained with 4,2-diamidino-2-phenylindole dihydrochloride (DAPI) (+), cytokeratin 8,18,19 (+) and CD45 (−). CECs expressing CD146 were immunomagnetically enriched and stained with DAPI (+), CD105 (+) and CD45 (−). CTC and CEC morphology was confirmed in all cases. Quantitative results were expressed as per 7.5 ml blood and per 4 ml blood for CTCs and CECs, respectively.

criteria and statistical analyses

CTC and CEC detection was carried out whenever possible, without any target statistical power. According to MO19391 study end points, TtP was measured from the time treatment began to the time documented progression occurred (i.e. not taking into account deaths occurring before tumor progression). Tumor response to treatment was evaluated as per standard of care and included complete and partial responses. Initially, the ≥5-CTC/7.5 ml threshold was used to define CTC positivity (for Table 1). The chi-square test or Fisher's exact test was applied for dichotomous and categorical data, and the Student's *t*-test or analysis of variance was used to compare continuous variables between groups. For comparison of quantitative parameters, if the normality hypothesis was not fulfilled, nonparametric tests were used as Wilcoxon test or Kruskall–Wallis test. Signed rank test was used to compare quantitative parameter between baseline and before C3. Correlation between CTCs and CECs was studied using linear regression. Kaplan–Meier survival curves were used to analyze TtP for each group of interest defined by CEC or CTC thresholds, and Cox regression model was used to provide the hazard ratio and its 95% confidence interval (CI). To correlate CTC and CEC changes under treatment with tumor response, two models were used: threshold-based and relative change-based models, the latter considering CTCs and CECs as continuous variables. Threshold-based models divided patients into four classes (positive at baseline/positive before C3 (+/+), +/−, −/+ and −/−). For TtP analysis, in the threshold-based models, Wald test (provided by Cox model regression) compared TtP of patients classified according to their positivity status before C3, this analysis being stratified on patient's baseline status. Relative change-based model divided patients into two classes: patients with relative change (between baseline and before C3) above a threshold and patients with relative change below this threshold. For TtP prediction by CTC/CEC counts, this article reports every test made. Two-tailed P values <0.05 were regarded as statistically significant. Statistical analyses were carried out using SAS® 9.1.3 software.

results

patients

Sixty-seven patients have been included, of whom 63 patients (94%) had a metastatic disease and 4 patients (6%) a nonoperable locoregional relapse. Fifty-five patients (82%) received prior chemotherapy for localized breast cancer. The patient characteristics are shown in Table 1. Only two patients had a HER2-positive breast cancer, as targeted anti-HER2 treatment was not authorized in this study. Main metastatic sites were bone (n = 37, 55%), liver (n = 24, 36%) and lungs (n = 21, 31%). Chemotherapy regimens associated with bevacizumab, were mostly docetaxel and paclitaxel as single agents (n = 27 and 16 patients, respectively). The median number of bevacizumab cycles received was 10 (range 1–27); however, 11 patients (19%) discontinued bevacizumab before the fourth cycle. After a median duration of follow-up of 8.8

Table 1. Patients characteristics and CTC/CEC detection at baseline ($N = 67$)

	No. of	CTC positivity[a]		CEC count		Tumor Response		TtP	
	patients (N)	n/N assessed	P value	Median (range)	P value	n/N assessed	P value	Hazard ratio (95% CI)	P value
Age, years									
<45	11	5/11 (45%)	0.54[c]	8 (1–68)	**0.01**[b]	3/7 (43%)	0.68[c]	1.1 (0.2–4.8)	0.92[d]
≥45	56	30/54 (56%)		19.5 (5–769)		25/43 (58%)		Ref	
Missing		$n = 2/67$		$n = 8/67$		$n = 17/67$		$n = 0/67$	
Disease-free interval, months									
<24	27	12/26 (46%)	0.31[e]	17 (1–769)	0.84[b]	12/19 (63%)	0.42[e]	Ref	0.60[d]
≥24	40	23/39 (59%)		17 (4–205)		16/31 (52%)		1.3 (0.5–3.3)	
Missing		$n = 2/67$		$n = 8/67$		$n = 17/67$		$n = 0/67$	
HR status									
Negative	17	7/17 (41%)	0.19[e]	15 (4–90)	0.59[b]	6/11 (55%)	1.0[c]	1.9 (0.5–7.1)	0.31[d]
Positive	49	28/47 (60%)		17 (1–769)		21/38 (55%)		Ref	
Missing		$n = 3/67$		$n = 9/67$		$n = 18/67$		$n = 1/67$	
Number of metastatic sites									
<3	20	7/19 (37%)	**0.03**[e]	15 (3–68)	0.33[b]	8/14 (57%)	0.79[e]	1.2 (0.43.3)	0.75[d]
≥3	43	28/42 (67%)		18 (1–769)		18/34 (53%)		Ref	
Missing		$n = 6/67$		$n = 10/67$		$n = 19/67$		$n = 4/67$	
Performance status									
0	38	18/36 (50%)	0.49[e]	17 (3–769)	0.77[b]	20/31 (65%)	0.12[e]	Ref	0.07[d]
1–2	29	17/29 (59%)		15.5 (1–205)		8/19 (42%)		2.4 (0.9–6.3)	
Missing		$n = 2/67$		$n = 8/67$		$n = 17/67$		$n = 0/67$	
LDH levels									
≤UNL	34	16/34 (47%)	**0.04**[c]	12 (1–65)	**<0.01**[b]	12/24 (50%)	0.67[e]	Ref	0.27[d]
>UNL	19	14/18 (78%)		26 (7–205)		8/14 (57%)		1.(0.7–4.9)	
Missing		$n = 15/67$		$n = 21/67$		$n = 29/67$		$n = 13/67$	
CA 15-3 levels									
≤UNL	17	3/16 (19%)	**<0.001**[c]	12 (6–769)	0.33[b]	9/14 (64%)	0.52[e]	Ref	0.09[d]
>UNL	37	26/37 (70%)		19.5 (1–90)		14/26 (54%)		3.7 (0.8–16.7)	
Missing		$n = 14/67$		$n = 20/67$		$n = 27/67$		$n = 12/67$	
CTC									
<5	30			13.5 (4–205)	0.22[b]	16/24 (67%)	0.11[e]	Ref	0.09[d]
≥5	35			19.5 (1–769)		11/25 (44%)		2.5 (0.9–7.1)	
Missing		$n = 2/67$		$n = 10/67$		$n = 18/67$		$n = 2/67$	

Tumor grade was assessed on the primary tumor. HR status was considered positive when either progesterone and/or estrogen receptor were positive. All but two patients were HER2 negative. 'Missing' patients indicates the number of patients not included in the statistical analysis for lack of data. Significant values are in bold.

[a]The ≥5 CTC/7.5 ml threshold was used to define CTC positivity.

[b]Wilcoxon test.

[c]Fisher's exact test.

[d]Wald test.

[e]Chi-square test.

CEC, circulating endothelial cells; CI, confidence interval; CTC, circulating tumor cells; HR, hormone receptor; LDH, lactate dehydrogenase; Ref, reference; TtP, time to progression; UNL: upper normal limit.

months (range 0.4–14.7), the median TtP was 11.9 months (95% CI 8.3 to not reached). Twenty-eight of 50 patients (56%) patients achieved an objective response and 10 patients (17%) had died at the time of analysis.

CTC and CEC detection

The median CTC count was 7 CTC/7.5 ml (range 0–496) at baseline ($n = 65$ patients assessed), dropping to 0 (range 0–71) before C3 ($n = 42$ patients assessed). CTC count change between baseline and before C3 was statistically significant ($P < 0.001$). A decrease in CTC count was observed in 32 patients (76%), in 9 patients (22%) values remained stable (at 0 CTC/7.5 ml) whereas an increase was seen in 1 patient (2%). According to the ≥5 CTC/7.5 ml threshold previously defined by Cristofanilli et al. [6], 35 patients (54%) were CTC positive at baseline and only 5 patients (12%) were CTC positive before C3. Detection of CTC at baseline was correlated with high tumor burden (more than 3 metastatic sites; $P = 0.03$), elevated LDH ($P = 0.04$) and elevated serum marker CA 15-3 ($P < 0.001$) (Table 1). Also, CTC levels ≥5 CTC/7.5 ml were highly associated with bone ($P < 0.001$) and liver ($P = 0.003$) metastatic sites.

The median CEC count was 17 CEC/4 ml (range 1–769) at baseline ($n = 59$ patients assessed) and 26 CEC/4 ml (range

2–335) before C3 ($n = 42$ patients assessed). CEC count change between baseline and before C3 was statistically significant ($P = 0.013$). CEC levels at baseline were associated with age ≥45 years ($P = 0.01$) and elevated LDH ($P < 0.01$) (Table 1). No association was seen between CEC count and metastatic sites (type and number). We found no association between CEC levels and CTC levels or positivity (according to the ≥5 CTC/7.5 ml threshold).

outcome prediction

The patient characteristics at baseline were not associated with tumor response or TtP (Table 1). At baseline, CTC prognostic value was assessed using different thresholds to define CTC positivity (Table 2). In this serial, using the ≥5 CTC/7.5 ml threshold to define CTC positivity, CTC status was not predictive of tumor response ($P = 0.11$) nor of TtP ($P = 0.09$). However, among the different thresholds tested (Table 2), a threshold of ≥3 CTC/7.5 ml was a significant predictor of both tumor response and TtP (Figure 1; $P < 0.05$). At baseline, CEC count was not associated with tumor response or TtP, at any threshold (Table 2).

We further studied whether CTC and CEC count changes under treatment may be correlated with tumor response and used as an early surrogate of TtP. Results are shown in Table 3. Strikingly, focusing on CTC count, all threshold-based and relative change-based models failed to accurately predict tumor response or TtP. On the contrary, TtP was significantly associated with CEC changes, using the threshold-based model, at the threshold of ≥20 CEC/4 ml ($P < 0.01$; Table 3; see also Figure 2A). More simply, TtP was also associated with the CEC status before C3, using the ≥20-CEC/4 ml threshold (Figure 2B; $P = 0.003$). Among relative change-based models, a relative increase of 50% of CEC count during treatment was trended toward significance ($P = 0.06$). In comparison, neither LDH and CA 15-3 serum levels before C3 nor their changes between baseline and C3 were associated with TtP or to tumor response (data not shown). We checked that these results, based on patients assessed twice, were not biased by a significant difference in TtP between patients assessed only at inclusion or twice ($P = 0.59$ and $P = 0.85$ for CTCs and CECs, respectively).

conclusion

This exploratory prospective substudy is the first to report both CTC and CEC counts in metastatic breast cancer patients who were homogeneously treated by first-line chemotherapy (mostly taxanes) combined with the antiangiogenic agent bevacizumab.

circulating tumor cells

Our analysis does not confirm the clinical relevance of CTC counts reported previously by Cristofanilli et al. [18] and Nole et al. [9], using the same ≥5 CTC/7.5 ml threshold as a threshold for poor outcome. In line with these results, we observed that (i) at baseline CTC was of prognostic significance for TtP and (ii) was also predictive of tumor response, using a lower threshold of ≥3 CTC/7.5 ml. However, the most striking result was that 6 weeks after the start of the treatment, irrespective of the model tested, CTC count was not associated with TtP or tumor response.

Concerning these finding for CTC counts, a lack of power should be discussed at first, as our analysis included 67 patients. By comparison, the successful validation set of the study of Cristofanilli et al. [6] included 75 patients, of whom about a third were treated only by hormone therapy, a clinical setting

Table 2. Baseline CTC and CEC as prognostic markers ($N = 67$)

	Number of patients in each group	Tumor response, *P* value	TtP, *P* value
Baseline CTC threshold (CTC/7.5 ml), $N = 65$			
0 versus ≥1	18 versus 47	0.10[a]	0.99[b]
<3 versus ≥3	27 versus 38	**<0.05**[c]	**<0.05**[b]
<5 versus ≥5	30 versus 35	0.11[c]	0.09[b]
<10 versus ≥10	36 versus 29	0.39[c]	0.40[b]
<15 versus ≥15	37 versus 28	0.58[c]	0.52[b]
<20 versus ≥20	42 versus 23	0.27[c]	0.16[b]
<30 versus ≥30	46 versus 19	0.28[c]	0.10[b]
Baseline CEC threshold (CEC/4 ml), $N = 59$			
<10 versus ≥10	15 versus 44	0.44[c]	0.69[b]
<15 versus ≥15	24 versus 35	0.86[c]	0.24[b]
<20 versus ≥20	32 versus 27	0.45[c]	0.28[b]
<25 versus ≥25	38 versus 21	0.27[c]	0.27[b]
<30 versus ≥30	43 versus 16	0.49[a]	0.42[b]
<40 versus ≥40	48 versus 11	0.41[a]	0.32[b]
<50 versus ≥50	50 versus 9	0.66[a]	0.81[b]

Univariate analysis results are shown, since no other characteristic was predictive of tumor response or TtP. Significant values are in bold.
[a]Fisher's exact test.
[b]Wald test.
[c]Chi-square test.
CEC, circulating endothelial cells; CTC, circulating tumor cells; TtP, time to progression.

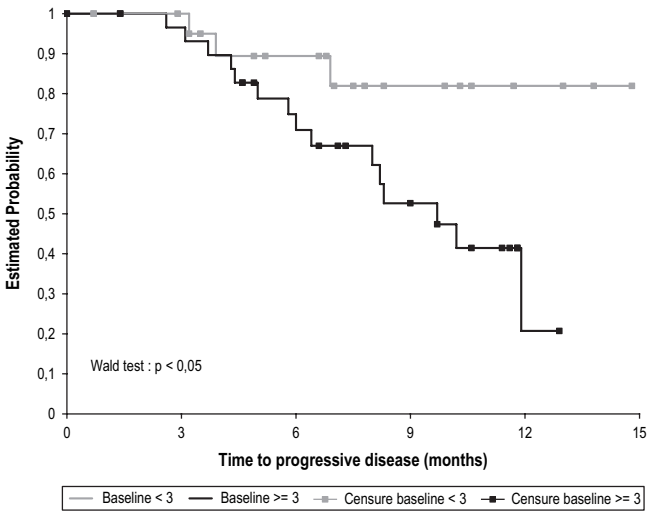

Figure 1. Time to progression according to circulating tumor cell detection at baseline (in black, patients with ≥3 CTC/7.5 ml).

Table 3. CTC (n = 42 patients) and CEC (n = 37 patients) changes as early surrogates of tumor response and TtP

Changes between baseline and C3	Number of patients in each group	Tumor response, P value	TtP, P value
CTC: threshold model			
0 versus ≥1	−/+ (n = 1), +/+ (n = 11) −/− (n = 9), +/− (n = 21)	0.85[a]	0.36[b]
<3 versus ≥3	−/+ (n = 0), +/+ (n = 6) −/− (n = 18), +/− (n = 18)	0.72[a]	0.36[b]
<5 versus ≥5	−/+ (n = 0), +/+ (n = 5) −/− (n = 20), +/− (n = 17)	1.00[a]	0.60[b]
<10 versus ≥10	−/+ (n = 0), +/+ (n = 5) −/− (n = 22), +/− (n = 15)	1.00[a]	0.55[b]
<15 versus ≥15	−/+ (n = 0), +/+ (n = 5) −/− (n = 23), +/− (n= 14)	1.00[a]	0.55[b]
<20 versus ≥20	−/+ (n = 0), +/+ (n= 3) −/− (n = 26), +/− (n = 13)	0.87[a]	0.94[b]
CTC: relative change model[c]			
≥ −50% decrease	10/42	0.69[a]	0.99[b]
≥ −60% decrease	11/42	1.00[a]	0.15[b]
≥ −70% decrease	12/42	1.00[a]	0.18[b]
≥ −80% decrease	14/42	0.89[d]	0.25[b]
≥ −90% decrease	15/42	0.62[d]	0.39[b]
≥ −99% decrease	21/42	0.85[d]	0.83[b]
CEC: threshold model			
<10 versus ≥10	−/+ (n = 8), +/+ (n = 25) −/− (n = 2), +/− (n = 2)	0.10[a]	0.52[b]
<15 versus ≥15	−/+ (n = 14), +/+ (n = 16) −/− (n= 4), +/− (n = 3)	0.53[a]	0.07[b]
<20 versus ≥20	−/+ (n = 16), +/+ (n = 9) −/− (n = 9), +/− (n = 3)	0.35[a]	< 0.01[b]
<25 versus ≥25	−/+ (n = 15), +/+ (n = 7) −/− (n = 11), +/− (n = 4)	0.66[a]	< 0.05[b]
<30 versus ≥30	−/+ (n = 11), +/+ (n = 5) −/− (n = 17), +/− (n = 4)	1.00[a]	0.28[b]
<40 versus ≥40	−/+ (n = 11), +/+ (n = 2) −/− (n = 21), +/− (n = 3)	1.00[a]	0.67[b]
<50 versus ≥50	−/+ (n = 9), +/+ (n = 1) −/− (n = 24), +/− (n = 3)	1.00[a]	0.60[b]
CEC: relative change model			
≥ +80% increase	17/37	0.70[d]	0.22[b]
≥ +50% increase	23/37	1.00[a]	0.06[b]
≥ +25% increase	24/37	1.00[a]	0.12[b]
≥ −25% decrease	28/37	1.00[a]	0.23[b]
≥ −50% decrease	31/37	1.00[a]	0.34[b]
≥ −80% decrease	35/37	1.00[a]	0.45[b]

[a]Fisher's exact test.
[b]Wald test.
[c]Results shown were obtained on the whole population, including patients without any CTC detected at baseline. Excluding these patients from the analysis did not reveal significant results (data not shown).
[d]Chi-square test (significant values are in bold).
C3, cycle 3; CEC, circulating endothelial cells; CTC, circulating tumor cells; TtP, time to progression.

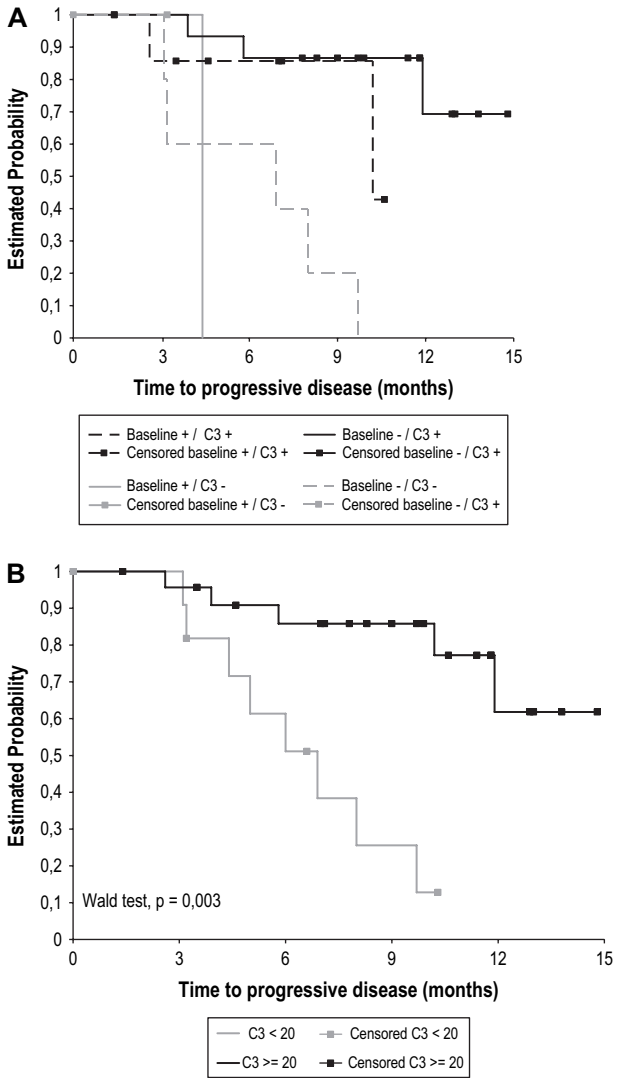

Figure 2. Time to progression according to (A) circulating endothelial cell (CEC) change during chemotherapy, using the ≥20 CEC/4 ml threshold (plus symbol means ≥20 CEC/4 ml at the defined time point), and to (B) CEC status at C3, using the ≥20 CEC/4 ml threshold.

in which CTC count seemed to have less significance [6]. The study of Nole et al. [9], which was also successful, included 80 patients: 70 patients received chemotherapy (in combination with trastuzumab for 27 patients), but only 33 patients had not received previous chemotherapy for their metastatic disease [9]. The sample size in our study is therefore of similar size to that of Cristofanilli's group [19] (validation set) and Nole et al. [9] and also showed a similar CTC detection rate at baseline (≥5 CTC/7.5 ml). Moreover, the fact that we do describe in our series the significant correlations of CTC positivity (≥5 CTC/7.5 ml) with high CA 15-3 serum marker and with bone metastatic site, which have been also reported by Nole et al. [9] and De Giorgi et al. [19], does not support a lack of power of our study. The lack of clinical relevance of CTC changes during treatment might also be due to the difference in the CTC count schedule, as it was before C2 (i.e. theoretically at day 21) in the study by Cristofanilli et al. [6], at day 30 in the study of Nole et al. [9] and before C3 (i.e. theoretically at day 42) in our study. However, a recent retrospective study of the series by Cristofanilli's group showed that later CTC count (i.e. between day 56 and day 91) retained a strong

prognostic relevance in patients treated by standard chemotherapy alone [20].

We therefore favor the hypothesis that the lack of CTC significance during treatment is directly related to the systematic use of bevacizumab in our homogeneously treated series of patients. In our study, CTC counts were in line with previous studies at baseline but unusually low after two cycles of treatment compared with previous studies (without bevacizumab): 12% of patients had ≥5 CTC/7.5 ml here (day 42) versus 30% (day 30) [9] or 22% (day 42–56) [21]. Besides a hypothetical direct anti-CTC effect of bevacizumab, two possible explanations should be discussed. First, bevacizumab gained its worldwide regulatory approval after the demonstration of a significant prolongation in PFS, leading to its use as a standard for first-line treatment [2]. Importantly, it is highly probable that the intrinsic properties of CTC positivity, defined by a precise threshold (here, ≥5 CTC/7.5 ml was initially tested) and used as an early surrogate test of PFS, will strongly depend on the length of the PFS obtained by treatments, which are continuously improved. In other words, the ≥5 CTC/7.5 ml threshold which successfully separated early (median: 2.1 months) from late (7.0 months) progressive disease under standard chemotherapy will necessarily be challenged by the improvement of PFS due to new treatments such as bevacizumab (or trastuzumab, not used here and not reported in the series by Cristofanilli et al.). Accordingly, our results indicate that the CTC positivity threshold should be ≥3 CTC/7.5 ml rather than ≥5 CTC/7.5 ml, when used for prognostic purpose at baseline in our setting. Secondly, we also suggest that the use of bevacizumab may change the intravasation abilities of cancer cells, due to its effect on the vessels endothelium. For comparison purposes, in colorectal cancer studies, 56% and 100% of patients were treated with bevacizumab in the studies by Cohen et al. (n = 430 patients) [22] and Tol et al. (n = 467 patients) [23] studies, respectively. These two studies exhibited a similar baseline CTC positivity rate. However, CTC positivity rates reported under treatment were rather different (12% versus 5% at 5–6 weeks), inversely correlated with bevacizumab use. Intravasation impairment is a hypothesis to explain why CTC counts dropped off under treatment. This hypothesis may explain how CTC count (i) retains a significant prognostic value at baseline, as already described in metastatic colorectal cancer patients treated with bevacizumab combined with chemotherapy [24]; but (ii) has less individual significance as an early surrogate marker of TtP when assessed under treatment. The impact of bevacizumab on tumoral vessels has been reported in many ways since the original report of bevacizumab use in rectal cancers [25] but our analysis is the first, to our knowledge, to indicate that it may impair tumor cell intravasation in patients.

circulating endothelial cells

Published data about CECs are highly heterogeneous, obtained by various CEC-sorting techniques, in many different tumor types, and commonly reported in a small sample size [26–28]. To further complicate the survey on CECs, these cells can be divided into mature CECs and progenitor CECs, whereas some chemotherapy regimens (metronomic chemotherapy) are believed to exert antiangiogenic effects beyond their cytotoxic effects. More recently, it has also been shown that some standard chemotherapies (especially taxanes) have a stimulating effect on an endothelial progenitor which supports their combined use with an antiangiogenic agent [29]. CEC sorting by the CellSearch® system is based on a CD146(+), CD105(+), DAPI (+) and CD45 (−) phenotypes of CECs [17]. It has been also validated through gene expression profiling which verified that expression of endothelial genes such as vascular endothelial cadherin was present in the population of sorted cells [30]. Our analysis is the first to report that CEC count, by the CellSearch® automated system, could be a significant early surrogate marker of TtP for breast cancer patients treated by bevacizumab combined with standard chemotherapy. Among many models tested, three could be compared in a validation study: (i) a threshold-based model (≥20 CEC/4 ml); (ii) a relative variation model (+50%), both using the two CEC counts (baseline and day 42); and (iii) a threshold-based model at day 42 (≥20 CEC/4 ml). Interestingly, the barely lower statistical significance of the nearby thresholds of ≥25 CEC/4 ml and ≥15 CEC/4 ml ($P < 0.05$ and $P = 0.07$, respectively) strongly indicated that the statistical significance of the ≥20 CEC/4 ml threshold was not due to a random multiple testing artifact. Our results are in line with previous reports (using other CEC-sorting techniques) which indicated that tumor progression under antiangiogenic treatment combined with chemotherapy was associated with a significant CEC decrease [28]. In this exploratory, hypothesis-generating study, we did not attempt to establish a combinatory model using both CTC and CEC levels.

Finally, if our results are confirmed by another independent prospective study, CTC counts by the Food and Drug Administration-approved CellSearch® system should be interpreted carefully by clinicians when treating metastatic breast cancer patients with first-line chemotherapy combined with bevacizumab: clinically relevant baseline prognostic threshold may be different in this setting, and CTC changes during treatment may not be an early surrogate marker of survival. Contrarily, CECs are a promising early surrogate marker of TtP in such patients, and a validation study is required to further investigate the different CEC threshold proposals.

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