

Improved Circulating Tumor Cell Detection by a Combined EpCAM and MCAM CellSearch Enrichment Approach in Patients with Breast Cancer Undergoing Neoadjuvant Chemotherapy

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Abstract

Circulating tumor cells (CTC) are detected by the CellSearch System in 20% to 25% of patients with primary breast cancer (pBC). To improve CTC detection, we investigated melanoma cell adhesion molecule (MCAM) as enrichment marker next to epithelial cell adhesion molecule (EpCAM) and tested the clinical relevance of MCAM-positive CTCs in patients with HER2-negative stage II/III pBC starting neoadjuvant chemotherapy (NAC) in the NEOZOTAC trial. Using the CellSearch System, EpCAM-positive and MCAM-positive CTCs were separately enriched from 7.5 mL blood, at baseline and after the first NAC cycle. Circulating endothelial cells (CEC) were measured using flow cytometry. Primary objective was to improve the CTC detection rate to $\geq 40\%$ combining EpCAM/MCAM. Correlations of CTC and CEC counts and pathologic complete response (pCR) were also

explored. At baseline, we detected EpCAM-positive and MCAM-positive CTCs in 12 of 68 (18%) and 8 of 68 (12%) patients, respectively. After one cycle, this was 7 of 44 (16%) and 7 of 44 (16%) patients, respectively. The detection rate improved from 18% at baseline and 16% after one cycle with EpCAM to 25% ($P = 0.08$) and 30% ($P = 0.02$), respectively, with EpCAM/MCAM. No patients with MCAM-positive CTCs versus 23% of patients without MCAM-positive CTCs at baseline achieved pCR ($P = 0.13$). EpCAM-positive CTCs and CEC counts were not correlated to pCR. Combined EpCAM/MCAM CellSearch enrichment thus increased the CTC detection rate in stage II/III pBC. We found no associations of CTC and CEC counts with pCR to NAC. The clinical relevance of MCAM-positive CTCs deserves further study. *Mol Cancer Ther*; 14(3); 821–7. ©2014 AACR.

Introduction

A circulating tumor cell (CTC) count from peripheral blood as measured by the FDA-approved CellSearch System (Janssen Diagnostics) is a strong prognostic factor in both primary and meta-

static breast cancers (1). Although 70% of patients with metastatic breast cancer (mBC) have ≥ 1 CTC/7.5 mL of blood, in primary breast cancer (pBC), this proportion is only as low as 20% to 25% (1–6). In both cases, the presence of CTCs is associated with poor prognosis. For mBC, patients with ≥ 5 CTCs/7.5 mL blood have significantly shorter median progression-free survival (PFS) and overall survival (OS) compared with patients with < 5 CTCs (1, 7, 8). For pBC, patients with ≥ 1 CTC do significantly worse concerning disease-free survival (DFS) and OS compared with patients without CTCs (1, 3, 5–7).

Improvements in the detection of CTCs can be made. The CellSearch System relies on the expression of the epithelial cell adhesion molecule (EpCAM; CD326) on CTCs and misses EpCAM-negative CTCs (8–11). We showed that particularly breast cancer cell lines with epithelial-to-mesenchymal transition (EMT) features lack expression of EpCAM and are therefore not detected by the CellSearch System (9, 12). Because cells that have undergone EMT probably represent an aggressive, clinically relevant subpopulation of CTCs (10), we aimed to detect EpCAM-negative CTCs by alternative approaches. We found melanoma cell adhesion molecule (MCAM; CD146) to be expressed on EpCAM-negative breast cancer cell lines and tested its use as enrichment marker next to EpCAM. In a small series of patients with mBC, MCAM-positive CTCs were detected in 9 of 20 patients

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(45%), suggesting that CTC detection can be improved using this dual enrichment approach (9).

Besides CTCs, circulating endothelial cells (CEC) have been proposed as prognostic marker in breast cancer (13). Being sloughed off the vessel wall, they are a putative marker of angiogenesis and vascular damage. Accordingly, increased CEC counts are found in patients with different solid malignancies, including breast cancer (13). However, the clinical value of CEC counts before start of and changes during treatment remains to be investigated.

In this study, we used an EpCAM/MCAM CellSearch enrichment approach to improve CTC detection in patients with stage II/III breast cancer starting neoadjuvant chemotherapy (NAC). Primary objective was to improve the CTC detection rate from approximately 20% to 40% of patients. Secondary objectives were to determine baseline CEC counts and changes of CTCs and CECs during NAC, and to investigate associations between the presence and dynamics of EpCAM-positive and MCAM-positive CTCs and CECs with pathologic complete response (pCR) to NAC.

Patients and Methods

Patients

As a side-study to the NEOZOTAC trial—a multicenter, randomized phase III trial initiated by the Dutch Breast Cancer Research Group (BOOG; ref. 14)—patients with HER2-negative stage II/III breast cancer who provided additional informed consent for CTC blood sampling were enrolled. Patients were treated with neoadjuvant docetaxel/doxorubicin/cyclophosphamide (TAC) ± zoledronic acid (ZA) and underwent surgery afterwards. Pathologic responses on primary tumors and lymph nodes were scored by a pathologist at the Leiden University Medical Center, Leiden, the Netherlands. The definition for pCR was a total absence of invasive tumor cells. This side-study was approved by the Erasmus MC and local Institutional Review Boards (METC 10-229).

Blood draws and sample processing

Before start of and after the first NAC cycle, 2 × 10 mL blood was drawn into CellSave preservative tubes (Janssen Diagnostics). All samples were processed within 96 hours at the central laboratory, Erasmus MC Cancer Institute, Rotterdam, the Netherlands. Two CTC enumerations, both from 7.5 mL of blood, were done using the CellSearch System as described before (9). In brief, EpCAM-positive and MCAM-positive CTCs were enumerated in two separate runs using the CellSearch Epithelial Cell Kit (Janssen Diagnostics). For the MCAM enrichment, anti-MCAM ferrofluid-bound antibodies from the CellSearch Circulating Endothelial Cell Kit (Janssen Diagnostics) were used and FITC-conjugated CD34 (BD Biosciences; clone 8G12) was added as extra marker to exclude a subset of cytokeratin (CK)-18-expressing CECs (9). Nucleated, EpCAM, or MCAM-enriched cells, positive for CK8/18/19, and negative for CD45 and CD34 for MCAM-positive cells, were considered CTCs. To enable distinction between EpCAM-positive and MCAM-positive CTCs, separate EpCAM- and MCAM enrichments were run. Combined EpCAM/MCAM CTC counts were calculated afterwards, using the sum of both separate enrichments.

The enumeration of CECs was done from 4 mL of blood using a flowcytometric assay with CD34⁺/DNA⁺/CD146⁺/CD45⁻ as CEC phenotype, as described in full detail before (15).

Immunohistochemistry on primary tumor tissue

Expression of EpCAM and MCAM was evaluated on diagnostic core needle biopsies of primary tumors taken before NAC. Slides were incubated with anti-MCAM (1:100, clone N1238; Abcam) or anti-EpCAM (1:500, clone VU1D9; Cell Signaling Technologies), followed by the Envision System (DAKO) and counterstaining with hematoxylin. Scoring of staining intensity (negative/weak/moderate/strong) and estimation of the percentage of positive tumor cells were done by a well-trained technician and pathologist.

Statistical analysis

Primary objective of this study was to improve the CTC detection rate in patients with pBC using the EpCAM/MCAM

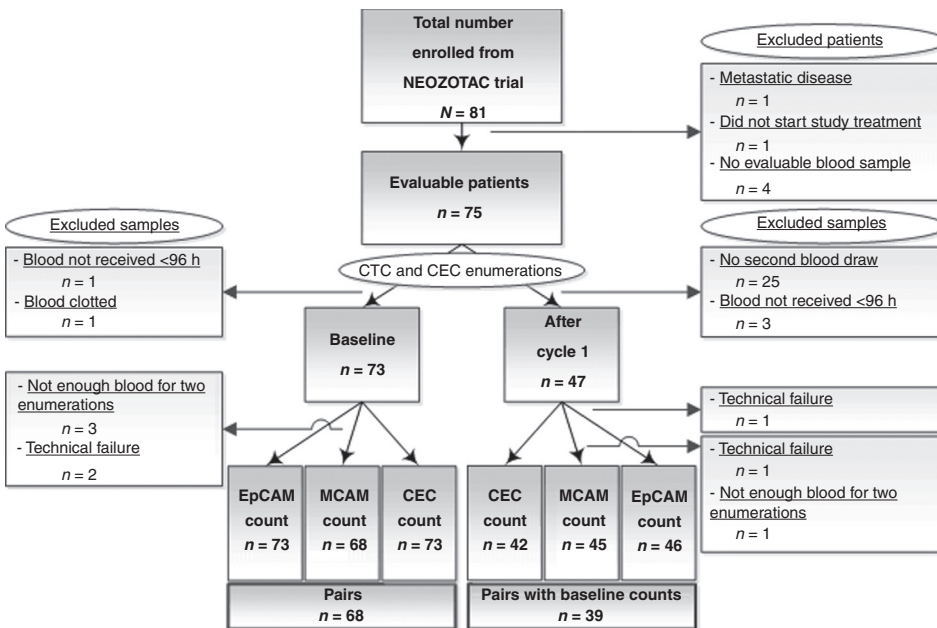


Figure 1. Study flow chart showing all included and evaluable patients and blood samples.

Table 1. Patient characteristics and comparison of characteristics and outcome to NAC between patients with and without EpCAM-positive CTC(s) and patients with and without MCAM-positive CTC(s)

Clinicopathologic variable at diagnosis	All patients	EpCAM-positive CTCs at baseline			MCAM-positive CTCs at baseline			EpCAM- and/or MCAM-positive CTCs at baseline		
		No CTCs	≥1 CTC	P value	No CTCs	≥1 CTC	P value	No CTCs	≥1 CTC	P value
<i>n</i>	68	56	12		60	8		51	17	
Age at diagnosis (years ± SD)	51.2 ± 7.7	51.4 ± 7.9	49.9 ± 6.8	0.54	51.4 ± 7.8	49.8 ± 7.6	0.59	51.5 ± 8.0	50.2 ± 7.0	0.55
Clinical tumor classification before NAC										
cT2	44 (65%)	36 (64%)	8 (67%)	0.88	40 (67%)	4 (50%)	0.35	35 (69%)	9 (53%)	0.24
cT3 or cT4	24 (35%)	20 (36%)	4 (33%)		20 (33%)	4 (50%)		16 (31%)	8 (47%)	
Clinical lymph node classification before NAC										
cN0	39 (57%)	33 (59%)	6 (50%)	0.57	35 (58%)	4 (50%)	0.65	31 (61%)	8 (47%)	0.32
cN+	29 (43%)	23 (41%)	6 (50%)		25 (42%)	4 (50%)		20 (39%)	9 (53%)	
Histological subtype										
Ductal	37 (54%)	28 (50%)	9 (75%)	0.57	30 (50%)	7 (88%)	0.38	24 (47%)	13 (76%)	0.26
Lobular	13 (19%)	12 (21%)	1 (8%)		13 (22%)	0		12 (23%)	1 (6%)	
Other	7 (10%)	6 (11%)	1 (8%)		6 (10%)	1 (12%)		5 (10%)	2 (12%)	
Unknown ^a	11 (16%)	10 (18%)	1 (8%)		11 (18%)	0		10 (20%)	1 (6%)	
Hormone receptor expression										
Estrogen receptor										
Positive	57 (84%)	46 (82%)	11 (92%)	0.42	50 (83%)	7 (88%)	0.76	42 (82%)	15 (88%)	0.57
Progesterone receptor										
Positive	45 (66%)	36 (64%)	9 (75%)	0.48	38 (63%)	7 (88%)	0.18	32 (63%)	13 (77%)	0.30
Menopausal status										
Pre/perimenopausal	35 (52%)	29 (52%)	6 (50%)	0.88	31 (52%)	4 (50%)	0.93	27 (53%)	8 (47%)	0.75
Postmenopausal	32 (47%)	26 (46%)	6 (50%)		28 (47%)	4 (50%)		23 (45%)	9 (53%)	
Unknown	1 (1%)	1 (2%)	0		1 (2%)	0		1 (2%)	0	
Treatment received										
TAC	33 (49%)	27 (48%)	6 (50%)	0.91	30 (50%)	3 (38%)	0.51	25 (49%)	8 (47%)	0.89
TAC + ZA	35 (51%)	29 (52%)	6 (50%)		30 (50%)	5 (62%)		26 (51%)	9 (53%)	
Pathologic complete response to NAC										
Yes	14 (21%)	12 (21%)	2 (17%)	0.71	14 (23%)	0	0.13	12 (24%)	2 (12%)	0.30
No	54 (79%)	44 (79%)	10 (83%)		46 (77%)	8 (100%)		39 (76%)	15 (88%)	

NOTE: Reported *P* values are from Student *t* tests for age and Pearson χ^2 for all other categorical variables.^aNot enough tumor cells left after NAC for reliable histological subtyping.

enrichment approach. The overall relapse rate in breast cancer approximates 40% (16). Because this study included patients with a poor prognosis, we deemed an improvement in CTC detection rate from 20% to 25% with EpCAM (2, 3, 6) to ≥40% using the extended approach clinically relevant. In patients with stage II/III pBC starting NAC, EpCAM-positive CTCs have before been detected in 46 of 213 (22%; ref. 2) and 22 of 97 (23%; ref. 5). Using these numbers, with an alpha of 0.05 and power 0.80, we had to enroll 57 patients. Secondary objectives included (i) assessment of CEC counts at baseline and changes thereof during NAC, and (ii) associations of CTCs and CEC numbers and changes thereof with pCR to NAC.

Standard statistical tests were applied: binomial tests for percentages, Pearson χ^2 tests for categorical variables, and Student *t* or Mann-Whitney *U* tests for continuous variables. Changes in CEC counts were tested by the Wilcoxon signed rank test. Binomial tests were one-sided, and all other tests were two-sided. We considered a *P* < 0.05 as statistically significant. Analyses were performed using SPSS 21 (IBM).

Results

From December 2010 until May 2012, 81 patients were recruited from the NEOZOTAC trial (14). Only patients with both EpCAM and MCAM enumerations available were used in the analyses, leaving 68 evaluable patients at baseline—thus meeting our power calculations—and 39 patients after one NAC cycle (Fig. 1). The characteristics of the 68 patients are summarized in Table 1 and specified per patient in Supplementary Table S1.

CTC counts

At baseline, 12 patients (18%) had ≥1 EpCAM-positive CTC(s) (median, 1; range, 1–4) and 8 (12%) had ≥1 MCAM-positive CTC(s) (median, 1; range, 1–5). Five patients (7%) had MCAM-positive CTCs only. The CTC detection rate increased from 18% with EpCAM alone to 25% when considering all EpCAM-positive and/or MCAM-positive CTCs (*P* = 0.08). The comparison between EpCAM-positive and MCAM-positive CTCs is shown in Table 2.

After the first NAC cycle, we detected EpCAM-positive CTCs in 6 patients (15%; median, 1; range, 1–7) and MCAM-positive CTCs in 6 patients (15%; median, 1; range, 1–4). Only one patient had both five EpCAM-positive CTCs and one MCAM-positive CTC. The CTC detection rate at this time point significantly increased from 16% with EpCAM only to 30% using EpCAM/MCAM combined (*P* = 0.02; Table 2).

Table 2. Observed CTC counts after EpCAM versus MCAM enrichment in patients with both enumerations available at baseline and after the first cycle of NAC

MCAM	EpCAM		
	Negative	Positive	
Baseline			
Negative	51	9	60
Positive	5	3	8
	56	12	68
After cycle 1			
Negative	31	6	37
Positive	6	1	7
	37	7	44

NOTE: Both enrichments were done from 7.5 mL of blood in separate runs and compared afterwards. A positive CTC count means ≥1 CTC/7.5 mL.

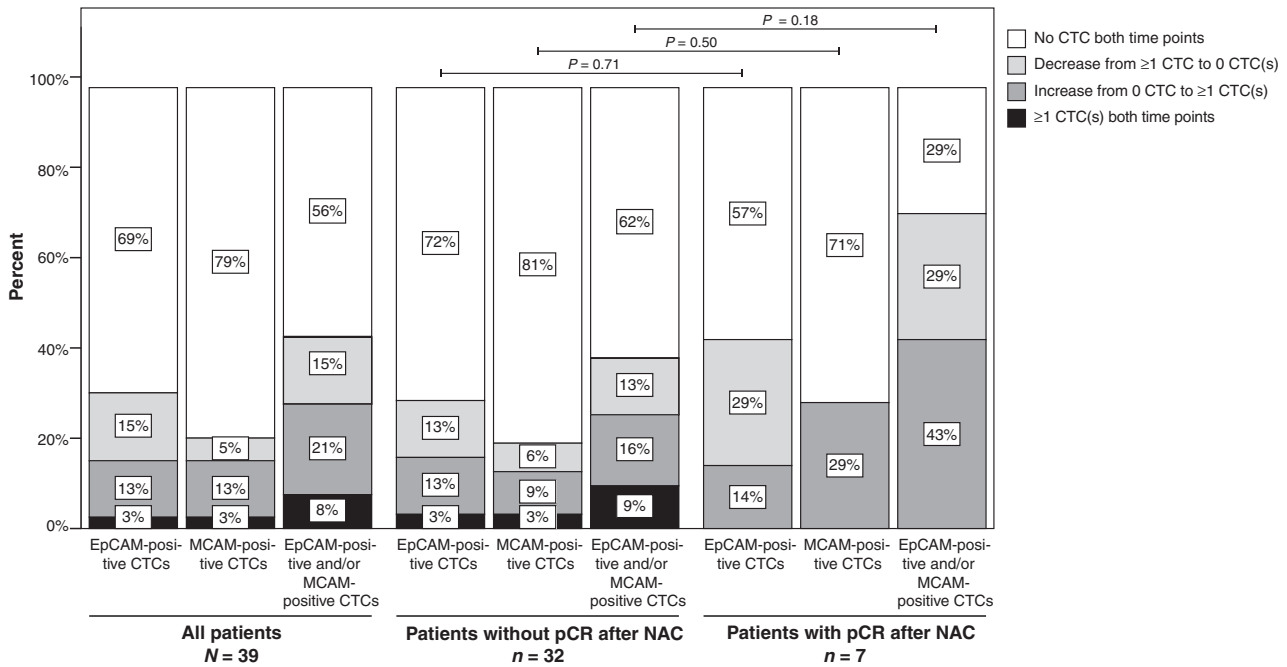


Figure 2. Changes in CTC counts between baseline and after the first cycle of NAC with respect to the cutoff of ≥ 1 CTC/7.5 mL blood. Left three bars, all evaluable patients with blood drawn at both time points. Middle three bars, patients in whom pCR to NAC was not achieved. Right three bars, patients with pCR to NAC. No differences in CTC changes were found between patients with and without pCR. Reported P values are from χ^2 tests.

Comparing CTC counts at baseline and after the first cycle, 5 patients (13%) switched from CTC negative to positive when considering EpCAM-positive CTCs. Three of these patients did not have any MCAM-positive CTCs at both time points, whereas two had one MCAM-positive CTC after the first NAC cycle, of whom one had no MCAM-positive CTCs at baseline. In five other patients, we detected MCAM-positive CTCs after the first NAC cycle, whereas there were none detectable at baseline. None of these patients had EpCAM-positive CTCs at baseline or after the first cycle. One patient (3%) had MCAM-positive CTCs at both time points and also turned positive for EpCAM-positive CTCs during NAC. Figure 2 (left three bars) shows the observed directions of changes in CTC counts during NAC.

Endothelial cell counts

At baseline and after the first cycle, CECs were enumerated in 68 and 42 patients, respectively (Fig. 1). Median CEC counts were 44.5/4 mL blood (range, 3–1,475) at baseline and 144.5/4 mL blood (range, 9–807) after the first cycle. In the 42 patients with CEC counts at both time points available, we observed a significant median increase during the first NAC cycle from 31.5 to 144.5 CECs ($P < 0.001$; Fig. 3). In 10 patients (24%), CECs decreased during treatment.

Associations with clinical parameters

We found no associations between the presence of EpCAM-positive and/or MCAM-positive CTC(s) at baseline and clinical characteristics (Table 1). Fourteen of 68 patients (21%) achieved pCR after NAC. The presence of EpCAM-positive CTC(s) at baseline was not correlated with pCR. Interestingly, none of the 8 patients with ≥ 1 MCAM-positive CTC(s) at baseline achieved pCR compared with 14 of 60 patients (23%) without MCAM-

positive CTCs ($P = 0.13$). Changes of either EpCAM-positive or MCAM-positive CTCs during NAC were not associated with pCR (Fig. 2, middle and right bars).

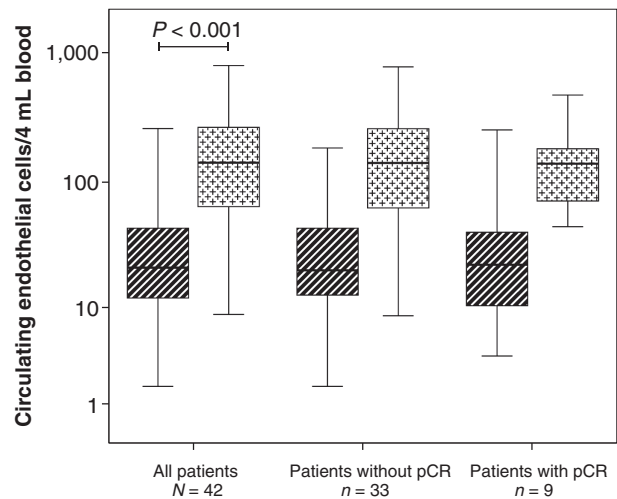


Figure 3. Box-and-whisker plots showing observed CEC counts in 4 mL blood at baseline (black-striped boxes) and after the first cycle of NAC (white-crossed boxes) in all evaluable patients with blood drawn at both time points ($N = 42$). All 42 patients are shown in left two boxes, patients who did not achieve pCR are shown in the middle two boxes, and those who did achieve pCR are shown in the right two boxes. A significant median increase in CECs during NAC was found, which was not different for patients with or without pCR. Boxes show the medians (middle line) and interquartile ranges (IQR), whiskers extend from the median $+ 1.5 \times$ IQR to median $- 1.5 \times$ IQR. Reported P value is from a Wilcoxon signed ranks test.

Median CEC counts at baseline were 61.5/4 mL in the 14 patients with pCR compared with 40.5 in the 54 patients without pCR ($P = 0.37$). In the 42 patients with both CEC counts available, comparable median increases were observed between patients with and without pCR to NAC (Fig. 3). The pCR rate in patients with decreasing CEC counts was 2/10 (20%), which was not different from the 7 of 32 patients (22%; $P = 0.90$) with pCR and increasing CEC counts.

Expression of EpCAM and MCAM in primary tumors

Core needle biopsies taken before NAC were collected from 65 patients. In 5 patients, no invasive tumor or too few tumor cells were present for reliable evaluation, leaving 60 tumors for the evaluation of EpCAM. All tumors were positive for EpCAM, but seven tumors showed an EpCAM-negative focus and six had an EpCAM-weak focus. Expression of MCAM could be assessed in 59 tumors and was found positive in 11 (19%; Supplementary Fig. S1; Supplementary Table S1).

Expression of EpCAM/MCAM in primary tumors was not correlated to the presence of MCAM-positive CTCs at baseline. No MCAM-positive CTCs were detected in patients with MCAM-positive tumors. We detected MCAM-positive CTCs in 14% of the patients with an EpCAM-negative focus in the primary tumor compared with 33% of the patients with an EpCAM-weak focus and 6% of the patients with homogeneously EpCAM-positive tumors.

Discussion

In this study, we investigated MCAM as additional CellSearch enrichment marker next to EpCAM to improve the CTC capture rate in stage II/III breast cancer. At baseline, the CTC detection rate increased from 18% using EpCAM only to 25% using both MCAM and EpCAM. After one NAC cycle, we observed a significant increase from 16% to 30%. Nevertheless, the primary goal to improve the detection rate to $\geq 40\%$, at beforehand defined as clinically relevant, was not met.

Neither the presence of EpCAM-positive or MCAM-positive CTCs at baseline, nor changes of CTCs after the first NAC cycle correlated with clinicopathologic parameters. Interestingly, none of the patients with MCAM-positive CTCs at baseline achieved pCR compared with 23% of patients without MCAM-positive CTCs. Although not statistically significant, this difference may point to a prognostic unfavorable value of MCAM-positive CTCs and deserves further study. The pCR rate between patients with

and without EpCAM-positive CTCs was similar. Baseline CEC counts and changes of either CECs or CTCs during NAC were not associated with pCR in our patient group. Associations with clinical outcome in terms of DFS and OS will have to be awaited.

Three other studies investigated the predictive and/or prognostic values of CTCs in the neoadjuvant setting (Table 3; refs. 2, 5–7, 17). The 11% to 23% of patients found CTC positive by EpCAM enrichment in these trials compares well with the 18% we found using the EpCAM enrichment only. Also in agreement with our findings, neither the presence of CTCs before or after NAC, nor changes during treatment correlated with pCR (2, 5, 17). Importantly, in the REMAGUS02 trial, pCR was no prognostic factor for distant metastasis-free survival and OS, whereas the baseline CTC count was (6, 7). The presence of CTCs thus might outperform pCR as prognostic factor in patients treated with NAC, possibly as indicator of the presence of micrometastases.

Increasing the CTC capture rate from peripheral blood will probably improve the prognostic and predictive value of CTC enumeration. Because MCAM is an EMT-inducer (18, 19), it might be a valuable enrichment marker for mesenchymal CTCs. Epithelial and mesenchymal CTCs were found to co-occur in patients with mBC, but mesenchymal cells showed to be better capable in predicting treatment failure (10). Previously, we showed that the CellSearch System misses EpCAM-negative breast cancer cell lines with EMT features and that recovery of these cell lines improves using MCAM, which is frequently expressed on these cell lines (9, 12). We investigated the dual EpCAM/MCAM enrichment approach in patients with mBC and detected MCAM-positive CTCs in 9 of 20 patients (45%; ref. 9). Although associations with clinical outcome were not investigated, we hypothesized that MCAM-positive CTCs represent the mesenchymal, more aggressive subtype of CTCs. An upregulation of EMT-related transcription factors in CTCs during NAC has also been reported, possibly as survival mechanism for CTCs during chemotherapy (20). More insight into the process of EMT and the phenotype of mesenchymal CTCs will be required to investigate the clinical relevance of mesenchymal CTCs. Besides a loss of EpCAM, we found a down-regulation of cytokeratins. Instead we found CD49f to be up-regulated. Combining cytokeratin staining with CD49f in the CellSearch System resulted in improved recovery of cell lines with EMT features (21). The value of CD49f on the recovery of MCAM-positive CTCs and the clinical value thereof will be subject in a future study.

Table 3. Overview of relevant literature concerning the prognostic value of CTCs in patients with pBC treated with NAC

Reference	Trial	Detection platform	Blood volume (mL)	n	% CTC-positive patients		Correlation with pCR	DMFS	OS
					Pre NAC	Post NAC			
Pierga et al. (5)	REMGUS02	CellSearch	7.5	115	23%	17%	No	HR 5.0 (95% CI, 1.4–17; $P = 0.01$) after 36 months FU	HR 9.0 (95% CI, 1.8–45; $P = 0.007$) after 36 months FU
Bidard et al. (6,7)								HR 2.4 (95% CI, 0.9–6; $P = 0.06$) after 70 months FU	HR 3.0 (95% CI, 1.0–9.5; $P = 0.05$) after 70 months FU
Riethdorf et al. (2)	GeparQuattro	CellSearch	7.5	287	22%	11%	No	NR	NR
Azim et al. (18)	NeoALLTO	CellSearch after Ficoll density gradient separation	22.5	51	11%	13%	No	NR	NR

Abbreviations: HR, hazard ratio; CI, confidence interval; FU, follow-up; DMFS, distant metastasis-free survival; NR, not reported.

Little is known about the prognostic value of CECs in breast cancer. Research in this field is greatly hampered by the lack of consensus on CEC phenotype. Consequently, different CEC definitions are handled and observed CEC counts using the different techniques are a 1,000-fold apart. Nonvalidated methods also showed to count macroparticles and large platelets as CECs, leading to incomparable results (13, 22). Technical obstacles have to be taken before concluding on the clinical value of CEC counts. Using a thoroughly validated flowcytometric method to measure CECs in 4 mL of peripheral blood (15), we found increasing CEC numbers during NAC, but no associations with pCR to NAC. The increase in CECs probably represents vascular damage due to NAC (13). Whether this is associated with long-term vascular complications warrants additional studies.

In conclusion, using MCAM as additional enrichment marker next to EpCAM in the CellSearch System might improve the detection of CTCs in stage II/III breast cancer. Whether the detection of MCAM-positive CTCs and changes thereof during treatment of localized or metastatic breast cancer are of clinical relevance in terms of clinical outcome deserves further investigation.

Disclosure of Potential Conflicts of Interest

J.W.M. Martens and S. Sleijfer report receiving a commercial research grant from Janssen Diagnostics LLC. J.A. Foekens reports receiving other commercial research support from Janssen Diagnostics LLC. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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