Prognostic Relevance of Disseminated Tumor Cells in the Bone Marrow of Patients with Primary Breast Cancer – Results of a Standardized Follow-Up

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Abstract. *Background: The prognostic significance of disseminated tumor cells from bone marrow (BM-DTCs) of breast cancer patients has been demonstrated previously. In this study, data of a standardized long term follow-up of 829 patients with examination of BM-DTCs at primary diagnosis are presented. Patients and Methods: BM aspiration and immunocytochemical examination of DTCs was performed according to a standardized protocol. Follow-up data of all patients were adjusted with the cancer registries of southern Bavaria. Results: A total of 268 patients (32%) had BM-DTCs with a median of 2 (1-1223)/2×10^6 cells. Positive BM findings correlated with tumor size (p=0.032), but not with other histopathological parameters. After a median follow-up of 73 months, BM-DTCs were highly relevant for the development of distant metastases (p=0.006) and, beneath standard histological parameters, reduced overall survival (p=0.038). Conclusion: These results confirm the prognostic relevance of the detection of BM-DTCs. Newer methods, such as detection of circulating tumor cells in blood, will have to demonstrate comparable prognostic information in the future.*

The search for methods to visualize single disseminated tumor cells (DTCs) in blood or bone marrow (BM) to estimate the risk of distant metastasis has been going on for more than 130 years, but only since the 1980s sensitive immunocytochemical (ICC) methods have made it possible to discriminate rare cancer cells from regular blood cells (1).

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As has been known for many years, the BM is a homing organ for DTCs, here these can be found in much higher concentrations compared to blood (2). Many groups have worked on the detection of BM-DTCs in breast cancer patients, as well as in those with other solid tumors. The most common marker to identify DTCs derived from an epithelial tumor such as breast cancer in an otherwise mesenchymal compartment is cytokeratin (CK), the most important protein of epithelial cells' cytoskeleton, which hematopoetic cells do not express (3). If CK-positive cells can be detected in an otherwise CK-negative compartment such as the BM, dissemination from an epithelial tumor can be assumed. Detection was possible in patients who already had distant metastases, due to much higher concentrations of DTCs in such individuals. As was shown by Janni *et al*., the prognosis of patients who already had distant metastases was worse if a certain number of BM-DTCs were found (4).

However, the clinically most important application of BM-DTCs is at primary diagnosis. The importance of loco-regional therapy has been decreasing with the increase of systemic therapies over the past decades. Therefore, the search for prognostic markers which indicate the need for intensified therapy or otherwise cases of low risk in which such therapies are not necessary is of major importance. Many studies have shown the prognostic significance of BM-DTCs at primary diagnosis for the subsequent development of disease recurrence (5-7). As Braun *et al*. showed as early as 2000 in 552 cases with primary breast cancer, detection of BM-DTCs (36 % of cases) by ICC staining against CK significantly predicted the development of distant metastases and tumor associated death (8). In 2005, Braun *et al*. put together the data of 4703 patients of 9 centers with DTC analysis by the same method. Again, in this pooled analysis the presence of BM-DTCs was an independent prognostic factor for poor prognosis (9). Not only at primary diagnosis, but also when present during recurrence-free follow-up, BM-

DTCs significantly predicted the later development of metastases, as was shown by Janni *et al*. (10).

More recently, the focus has been put on the further characterization of these cells. Sensitive ICC or molecular methods allow the determination of factors of DTCs such as stem cell markers (11), or the identification of targets such as HER2 (12), which can be used for tailored therapies. However, BM aspiration is an invasive procedure which cannot be repeated with unlimited frequency. Therefore, the search for methods to detect circulating tumor cells (CTCs) in peripheral blood, which is easily accessible, was the aim of recent years' research, and technical developments have indicated the feasibility of these newer methods (13). Nevertheless, the ICC detection of BM-DTCs is the method which has been most standardized and for which most data are available. At least at primary diagnosis, this method is recommended for the detection of hematogenous tumor cell spread (14). In this study, databases of breast cancer patients with BM examination at primary diagnosis were re-evaluated and the follow up of these patients was adjusted with the data of the Cancer Registries of Southern Bavaria.

Patients and Methods

Patients. All patients who attend the Department of Obstetrics and Gynecology, Innenstadt Campus, Ludwig Maximilians University, Munich, Germany, for primary treatment of breast cancer are offered BM aspiration for screening of hematogenous tumor cell dissemination. BM aspiration is carried out either under general anesthesia during surgery, or under local anesthesia. The experimental nature of this method and the potential side-effects are explained to the patients. Futhermore, a written informed consent must have been completed according to the local Ethics Committee guidelines. Histologic examination of the breast tumors was performed either at the Laboratory for Histopathology of the Department of Obstetrics and Gynaecology (until 2002) or at the Institute of Pathology of the University of Munich. Nearly one quarter of all BM samples were gathered at the Department of Obstetrics and Gynaecology, Zentralklinikum Augsburg, Germany, in the same manner as carried out in Munich. Samples were stored in heparinised syringes at room temperature and sent to Munich immediately for further processing. Previous tests had shown that BM samples are stable for 72 h at room temperature. For comparison of results, the original patients' files and pathology reports were taken into account.

Preparation of BM and ICC. The procedure for BM aspiration and preparation used at the Laboratory of Tumor Biology, Department of Obstetrics and Gynecology, Innenstadt Campus, Ludwig-Maximilians-University, Munich, Germany, has been described previously (23). BM aspiration was performed of both anterior iliac crests (2-8 ml each side). Samples were collected in heparinized syringes. After centrifugation at 900 ×g for 30 min with Ficoll-Hypaque (Pharmacia, Freiburg, Germany) density gradient (1.077 g/mol), mononuclear cells were washed, and 2×10^6 cells centrifuged onto a glass slide at 150 ×g for 5 min. The cytospin slides were dried overnight and then stained by ICC or frozen at –80˚C. The detection of BM-DTCs was achieved by staining with the monoclonal antibody A45-B/B3 (Micomet, Munich, Germany), which is directed against common cytokeratin epitopes, including the CK heterodimers 8/18 and 8/19. The concentration used to detect CK-positive cells in bone marrow cytospins was 2.0 μg/ml. The specific reaction of the primary antibody used the alkaline phosphatase anti-alkaline phosphatase technique (APAAP), combined with new fuchsin staining. For each patient, 2×10^6 cells were screened manually by bright field microscopy. Because of the absence of any background staining, we obtained no indeterminate results. Stained cells were classified as BM-DTCs only if they matched the criteria which have been defined in the consensus recommendations. All positive results were evaluated by two independent observers. The breast cancer cell line BT-20A served as a positive control for CK immunostaining. The specificity of the antibody reaction was tested using an unrelated mouse-myeloma antibody (Sigma, Deisenhofen, Germany) for isotype control on the patients' BM samples, with an identical number of cells as negative control. To date, BM samples of more than 200 patients without malignant disease have been examined, with a false-positive rate of 1% (25).

Statistics. For an update of the follow-up of our patients, we contacted the Cancer Registries of Munich and Augsburg, Germany. Data of all cancer cases in Southern Bavaria have been gathered there for decades, and external institutions or practitioners performing follow-up examinations or treatment of recurrences are required to report these to the Cancer Registry. In order to register all cases of cancer-related deaths, the Cancer Registries adjust their data regularly with those of the legal registration offices.

For data security, legal requirements have been taken into account. A compilation of patients' and tumor data, the results of BM examinations and follow-up data were stored in a Microsoft Excel database. Statistical evaluation was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). For comparison of ICC, immunohistochemistry and patients' characteristics, χ^2 test and correlation analysis were used. Disease- free- (DFS), distant disease-free- (DDFS) and overall survival (OS) in dependence of the examined factors was calculated by Kaplan-Meier analysis (log-rank test, univariate), and cox regression analysis (multivariate, inclusion stepwise forward).

Results

Patients' characteristics and histological parameters. Looking at patients with primary breast cancer whose histopathological reports and follow-up data were available, 829 cases with BM aspiration between 1994 and 2006 were found in our database. Of those, 614 patients had BM aspiration in Munich and 215 at the Augsburg hospital. Median patients age was 59 (23-85) years with 173 (28%) being premenopausal and 439 (72%) postmenopausal (menopausal status missing in 217 cases). Following former recommendations, most patients had complete axillary dissection to evaluate nodal status. A median of 16 lymph nodes (0-54) were removed; 454 patients (55%) were nodal negative and 375 positive (45%). Nodal-positive patients had a median of 4 involved lymph nodes (0-54). Overall, 58 tumors were grade 1 (7%), 432 (56%) grade 2, and 283 (37%) grade 3 (56 missing). All of the patients' characteristics and histopathological details are summarized in Table I.

Table I. *Patients' characteristics and histological parameters in correlation with the presence of BM-DTCs (χ^2 test, p-value).*

	No. of pts	%	No. of pts with BM-DTCs	%	p=correl. with BM status
Menopause					
No	235	28	86	37	0.47
Yes	592	72	201	34	
Tumor size					
<2 cm	472	58	138	29	0.032
≥2 cm	344	42	125	36	
Lymph node involv.					
No	446	55	149	33	0.43
Yes	370	45	114	31	
Grading					
1, 2	480	64	150	31	0.24
3	274	36	97	35	
ER					
Neg	222	29	76	34	0.33
Pos	532	71	163	31	
Lymphangiosis					
No	645	81	206	32	0.44
Yes	150	19	43	29	
Hemangiosis					
No	767	97	237	31	0.15
Yes	22	3	10	46	

Table II. *Correlation of histological factors (χ^2, p-value).*

χ^2, p	T size	LNpos	G=3	ER	LA	HA	BM
T size	/	<0.001	<0.001	0.016	<0.001	0.118	0.048
LN pos	<0.001	/	<0.001	0.010	<0.001	<0.001	0.28
G=3	<0.001	<0.001	/	<0.001	<0.001	0.506	0.42
ER	0.016	<0.010	<0.001	/	0.141	0.230	0.40
LA	<0.001	<0.001	0.001	0.141	/	<0.001	0.001
HA	0.118	<0.001	0.506	0.230	<0.001	/	0.001
BM	0.048	0.28	0.42	0.40	0.001	0.001	/

T size=Tumor size (>2 cm); LNpos=lymph node positivity; G=3=grade 3; ER=estrogen receptor positivity; LA=lymphangiosis; HA=hemangiosis; BM=bone marrow.

Table III. *Prognostic significance of the examined factors for overall survival (OS), disease-free survival (DFS), and distant disease-free survival (DDFS), on univariate analysis (log-rank-test, p-value).*

	BM-DTCs	Tsize	LN pos	G3	ERpos	LA	HA
DFS	0.32	<0.001	<0.001	0.006	0.26	<0.001	<0.001
DDFS	0.006	<0.001	<0.001	<0.001	0.49	<0.001	<0.001
OS	0.038	<0.001	<0.001	0.001	0.62	<0.001	<0.001

BM-DTCs=Disseminated tumor cells in bone marrow; T size=tumor size (>2 cm); LNpos=lymph node positivity; G=3=grade 3; ER=estrogen receptor positivity; LA=lymphangiosis; HA=hemangiosis; BM=bone marrow.

Following the recommendations of that time, 278 low-risk patients (34%) had no adjuvant therapy at all, 222 patients (27.2%) received hormonal therapy only, and 307 patients (37.6%) had chemotherapy (with/without hormonal therapy).

BM analysis and correlations. Of all patients, 268 (32.3%) had detection of BM-DTCs with a median of 2 BM-DTCs/2×10^6 screened cells (1-1223). In 561 samples (67.7%), no DTCs were detected.

Looking at correlations between BM-DTCs and histopathological parameters, a positive BM finding was significantly related to larger tumors (≥T2, p=0.032), but not to nodal positivity, grading or estrogen receptor expression (Table I). All the other histopathologic parameters (tumor size, lymph node status, grading, estrogen receptor positivity, lymphangiosis) were correlated significantly among each other (Table II).

DFS. Complete follow-up data of 816 patients were finally gathered from the Cancer Registries. Median follow-up time was 73 (1-199) months. A total of 567 patients (67.5%) were free of local or distant recurrence. For 249 patients (30.5%) recurrences have been reported: 58 patients had local recurrences only, and 191 local and/or distant recurrence. Considering those patients with recurrence, significant prognostic parameters were tumor size, nodal positivity (Figure 1), lymph- and hemangiosis (p<0.001 each) and grade 3 tumor (p=0.006), but not BM positivity (p=0.32). In multivariate analysis, significant independent prognostic parameters were nodal positivity (p<0.001, relative risk (RR)=1.72, 95% confidence interval (CI)=1.29-2.29), grade 3 tumor (p<0.001, RR=1.66, 95% CI=1.26-2.18) and lymphangiosis (p=0.001, RR=1.66, 95% CI=1.22-2.25).

DDFS. A total of 191 patients developed distant metastases during the follow-up period. This was significantly predicted by tumor size, nodal positivity, grade 3 tumor, lymph- and hemangiosis (p<0.001 each). Estrogen receptor status was not significant in this term (p=.49). In contrast, presence of DTC-BM highly significantly predicted the later occurrence of distant metastases (p=0.006, Figure 2). Of 263 patients with BM-DTCs, 82 (31.2%) developed distant metastases, whereas this occurred in 109 out of 533 patients (19.7%) without BM-DTCs (Figure 2). This independent prognostic value was also confirmed in multivariate analysis. Independent prognostic factors were nodal positivity, grade 3 tumor, lymphangiosis, and presence of BM-DTCs. The relative risk for patients with BM-DTCs was 1.47 (95% CI=1.09-1.99, p=0.012).

Looking at subgroups, for patients with adjuvant chemotherapy (n=307) tumor size (p=0.01), grade (p=0.002), lymphangiosis (p<0.001) and hemangiosis (p=0.001) as well as detection of BM-DTCs (p=0.04) significantly predicted development of distant metastases. Similar findings were made for the patients with hormonal therapy (n=222), but detection of BM-DTCs was not statistically significant for this group.

OS. Overall, 166 patients (20.3 %) died of their disease by the end of follow-up after a median of 73 (1-199) months. Tumor size (Figure 3), nodal positivity, grade 3 tumor, lymph- and hemangiosis (p<0.001 each) predicted tumor-associated death, as did the presence of BM-DTCs (p=0.038, Figure 4). Of the 650 patients that were alive after the follow-up period, 193 had BM-DTCs (29.7%), whereas of the 166 patients that had died 70 (42.2%) had BM-DTCs. In multivariate analysis, significant factors were nodal positivity (p<0.001, RR=1.98, 95% CI=1.39-2.82), grade 3 tumor (p=0.001, RR=1.74, 95% CI=1.25-2.42) and lymphangiosis (p=0.016, RR=1.57, 95% CI=1.09-2.26), but not BM-DTCs (p=0.079). In the subgroup of patients with chemotherapy, again tumor size (p=0.04), grading (p=0.001), lymphangiosis (p=0.001) and hemangiosis (p=0.002) were significant for OS, as was the presence of BM-DTCs (p=0.034). In the subgroup of patients with hormonal therapy, detection of BM-DTCs, just as for DDFS, was not significant for OS (Table III).

Discussion

In the past two decades, many single institutional studies have shown the prognostic relevance of the ICC detection of CK-positive DTCs in BM aspirates of breast cancer patients at primary diagnosis (5-7). Although the applied methods varied in some aspects such as the detected antigens of tumor cells or the antibodies used for ICC staining, consistent detection rates between 13.4% (15) and 42% have been reported (5). The study by Braun *et al*. in 2000 already reported on a subgroup of patients from our study (522 cases) with a shorter follow-up time of 38 months (8), and the BM positivity rate was 36%. Not surprisingly, here we also found BM-DTCs in 32.4% of patients. In the pooled analyses performed by Braun *et al*. in 2005 (9), including data of 4703 patients from nine different centers, BM positivity was correlated with larger tumor size, lymph node positivity, grade 3 tumor (p<0.001 each) and negative hormone receptor status (p=0.003). In our study, only larger tumor size (p=0.032) was significantly related to the detection of BM-DTCs. One reason for this finding is the lower number of cases. However, it is known from tumor biology that hematogenous tumor cell spread occurs independently from known histopathological factors (16), indicating the complex interactions between genetic, immunologic, and environmental factors. Considering prognostic information, the data by Braun *et al*. (8) were confirmed in our larger cohort, with longer follow-up time and standardized follow-up provided by the Bavarian Cancer Registries. In our study, the presence of BM-DTCs was not a significant prognostic factor for local recurrence, but for the development of distant metastases (p=0.006) and tumor-associated death (p=0.038). However, significance was not as high as in the former study (p<0.001 both for DDFS and OS (8)), and in the multivariate analysis the presence of BM-DTCs was an independent factor only for DDFS (p=0.012, RR=1.47), but not for OS (p=0.079). This difference might partly be due to the follow-up information provided by the Cancer Registries, which themselves obtain their data heterogeneously from different external hospitals and practitioners. In addition, advances in modern systemic therapies for metastatic breast cancer which have taken place during the last decade, such as antibody based treatments, may have reduced the prognostic impact of BM-DTCs for OS during longer follow-up. Looking at subgroups of patients, in those without any adjuvant therapy BM-DTCs were not of prognostic relevance for DDFS and OS. This is in contrast to former reports where the presence of BM-DTCs increased the risk of metastasis two-fold and that of death by a factor of 3.65 (9) in that collective. One reason might be that many cases of adjuvant or secondary adjuvant therapy, mainly hormonal treatment, were not reported to the Cancer Registries and thus this group was not homogeneous. Only in the group of patients with chemotherapy, representing the high-risk group at that time, BM-DTCs did significantly predict reduced DDFS and OS. Looking at the biology of DTC-BM, this finding is not surprising. As was shown by Pantel *et al*., the majority of these cells are in a dormant state, as shown by low Ki 67 expression, explaining the sometimes long latency between primary diagnosis and late reactivation of these cells (17). By this, antiproliferative chemotherapy has no or little effect on the elimination of these non-mitotic cells (18). The majority of these cells also seem to have stem cell markers, making them possibly resistant towards standard adjuvant treatment (11, 19).

These findings demonstrate the need for further characterization of DTCs in order to use this method for prognostic estimation. Several biological factors on DTCs and CTCs have been indentified. As it is, also, a target for antibody-based therapy, HER2 is the factor examined most frequently. Detection of HER2-postive BM-DTCs might indicate even worse prognosis (20), but could also justify implementation of anti-HER2-targeted therapy, even in patients with HER2-negative primary tumors, as approximately 30% of cases change HER2 status during the course of time (21).

One major limitation for a more detailed characterization of DTCs is the low number of detected cells, which was 2 BM-DTCs per 2×10^6 screened cells in our study. Many attempts to

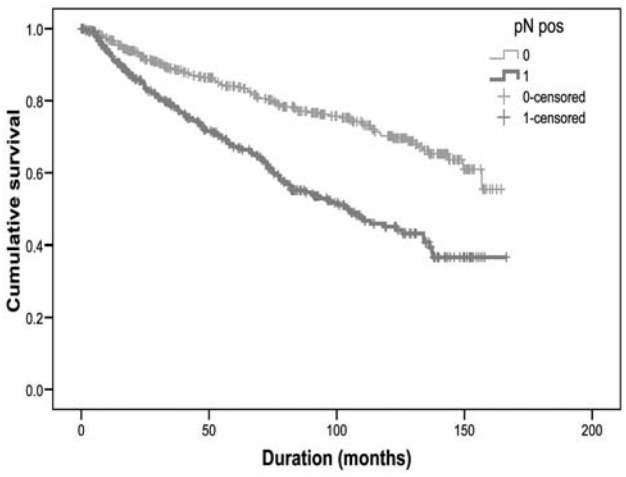

Figure 1. *Disease-free survival in dependence of nodal status (p<0.001).*

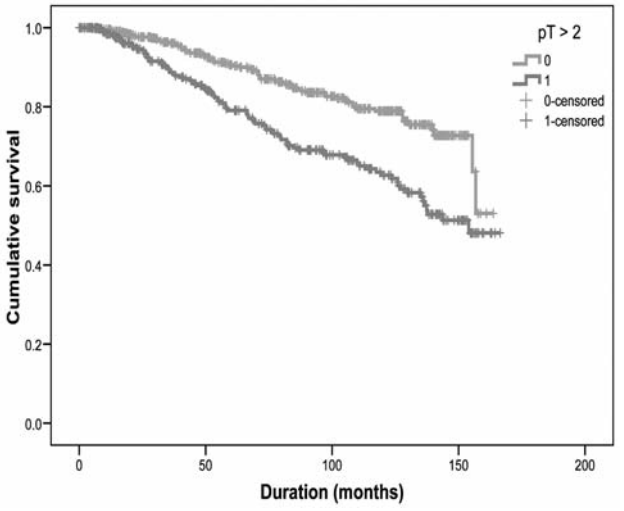

Figure 3. *Overall survival in dependence of tumor size >2 cm (p<0.001).*

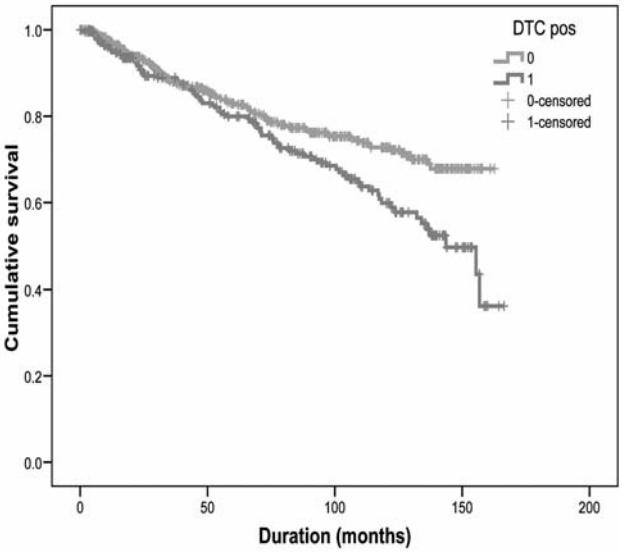

Figure 2. *Distant disease-free survival in dependence of the presence of disseminated tumor cells in bone marrow (p=0.006).*

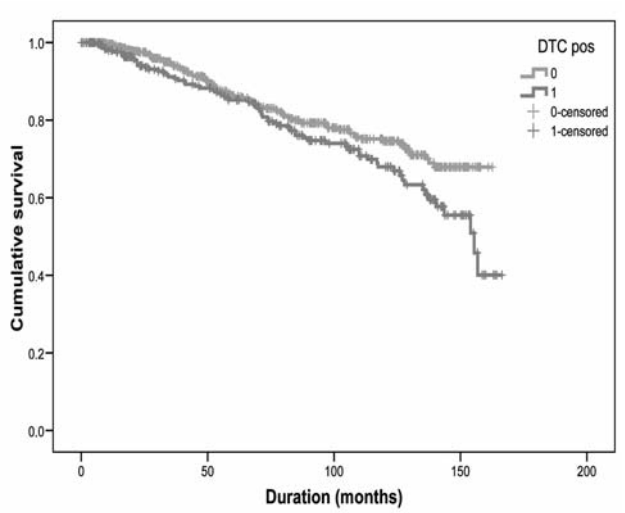

Figure 4. *Overall survival in dependence of the presence of disseminated tumor cells in bone marrow (p<0.038).*

increase the number of BM-DTCs have been made. Enrichment of DTCs by centrifugation media (22), filtration techniques (23), and immunomagnetic sorting (24-25) increased the percentage of positive cases up to 88% (26). However, the prognostic value has not been improved by enrichment techniques compared to standard methods (27). A second limitation of ICC or immunofluorescence for the characterization of DTCs is the fact that only two to three factors can be examined on the same cells. Therefore, in recent years, focus has been placed on alternative methods such as Polymerase Chain Reaction (PCR). After enrichment and separation of DTCs or CTCs and isolation and transcription of RNA sequences, in principle, all known genetic markers can be determined by this method (28). As was shown by Becker *et al*., there was good correlation between ICC and PCR for the detection of BM-DTCs (29), and prognostic information provided by PCR was even better compared to that obtained from ICC in the study by Benoy *et al*. (30). For prognostic estimation, as well as for the monitoring of therapies, repeated examinations during the course of the disease would be

desirable. The prognostic value of DTCs was demonstrated by BM aspirations performed years after primary diagnosis (10), and the influence of therapies such as bisphosphonates on BM-DTCs was seen (31). However, BM aspiration is an invasive method and thus cannot be repeated unlimitedly. For decades, laboratories have tried to enumerate CTCs in peripheral blood, but only the combination of effective enrichment and sensitive detection techniques have allowed the reliable analysis of peripheral blood in recent years. CTC analysis by semi-automated enrichment and computed immunofluorescence microscopy by the CellSearch device (Veridex, Warren, NJ, USA) has demonstrated considerable reliability (32) and showed prognostic value in the metastatic setting (13). Using laser scanning microscopy, Pachmann *et al*. found CTCs in 92% of breast cancer patients (33), with up to 100,000 detected cells per ml blood. With this method, prognostic information and the influence of therapies was demonstrated, at least in a single institutional setting (34). Additionally, the search for CTCs by PCR seems promising. As Stathopoulou *et al*. showed, detection of CK19-positive CTCs also predicted disease recurrence (35), and there was excellent correlation between detection of BM-DTCs and CTCs by PCR (36).

After nearly 30 years of research, there is no doubt that BM-DTCs and CTCs represent hematogenuously disseminated cells derived from epithelial tumors. These can be the seed of later distant metastases or at least indicate the risk for them. Many advances in the methods of detection and characterization of such cells have been made, and future opportunities are promising. Nevertheless, most data have been gathered by the method used in our study, and ICC analysis of BM aspirates can still be called the gold-standard of DTC detection. Data of our study add to these results, and newer methods need to demonstrate their reliability with comparable case numbers and follow-up times.

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