Detection of Circulating Tumor Cells in Peripheral Blood of Patients with Metastatic Breast Cancer: A Validation Study of the CellSearch System

Sabine Riethdorf,[1] Herbert Fritsche,[4] Volkmar Müller,[3] Thomas Rau,[2] Christian Schindlbeck,[6] Brigitte Rack,[6] Wolfgang Janni,[6] Cornelia Coith,[1] Katrin Beck,[3] Fritz Jänicke,[3] Summer Jackson,[4] Terrie Gornet,[4] Massimo Cristofanilli,[5] and Klaus Pantel[1]

Abstract

Purpose: The CellSearch system (Veridex, Warren, NJ) is designed to enrich and enumerate circulating tumor cells (CTCs) from peripheral blood. Here, we validated the analytic performance of this system for clinical use in patients with metastatic breast cancer.

Experimental Design: This prospective multicenter study conducted at three independent laboratories involved samples from 92 patients with metastatic breast cancer. Intra- and inter-assay variability using controls containing defined numbers of cells (average, 50 and 1,000, respectively), cell stability based on varying storage and shipment conditions, recovery precision from samples spiked with 4 to 12 tumor cells, inter-instrument variability, and positivity of samples from metastatic breast cancer patients were tested.

Results: Intra- and inter-assay precision for two sites were high: All eight positive controls analyzed in the same run and >95% of the run to run control values (n = 299) were within the specified ranges. Recovery rate of spiked samples averaged between 80% and 82%. CTCs were detected in ~70% of metastatic breast cancer patients. CTC values of identical samples processed either immediately after blood drawing or after storage for 24, 48, or 72 h at room temperature or at 4°C did not differ significantly. Shipment of samples had no influence on CTC values. When analyzing identical samples in different centers, inter-instrument accordance was high.

Conclusions: The CellSearch system enables the reliable detection of CTCs in blood and is suitable for the routine assessment of metastatic breast cancer patients in the clinical laboratory. Blood samples should be shipped at room temperature and CTC counts are stable for at least 72 h.

Distant metastasis is the leading cause of breast carcinoma–related death. However, factors enabling cancer cells to move and grow outside of the primary site and the timing of tumor cell dissemination are still not well understood. Current models of metastasis support the detachment of cells from primary tumors and their movement to distant sites through the blood and lymphatic system (1). A large number of studies have documented disseminated tumor cells (DTCs) in bone marrow or circulating tumor cells (CTCs) in peripheral blood from patients with most types of epithelial cancers (for review, see refs. 1–3). Within the last 10 years, several studies have shown that detection of tumor cells in bone marrow of cancer patients is accompanied by a substantially worse prognosis for these patients. Particularly, Braun et al. have reported that ~30% of women with primary breast cancer have DTCs in bone marrow, and a 10-year follow-up of these patients revealed a significantly decreased disease-free survival and overall survival when compared with patients without DTCs (4, 5). However, aspiration of bone marrow is time consuming and, in many cases, uncomfortable for the patients precluding multiple samplings for therapy monitoring studies. Therefore, recent efforts have concentrated on the detection of CTCs in peripheral blood of cancer patients.

The clinical use of CTCs has not been implemented for routine clinical practice for several reasons. Most notably, the lack of standardization and automation of the technology has required the use of laborious sample preparation procedures with corresponding high intra- and inter-laboratory differences in results. Furthermore, different reagents and methods are used for the staining and evaluation of immunocytochemically prepared slides in search of these rare events in blood and bone marrow,

Authors' Affiliations: Institutes of [1]Tumor Biology and [2]Pharmacology and [3]Clinic of Gynecology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; Departments of [4]Laboratory Medicine and [5]Breast Medical Oncology, M. D. Anderson Cancer Center, Houston, Texas; and [6]Department of Obstetrics and Gynecology, Innenstadt, Klinikum of the Ludwig Maximilians University of Munich, Munich, Germany

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Requests for reprints: Klaus Pantel, Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany. Phone: 49-40-42803-3503; Fax: 49-40-42803-5379; E-mail: pantel@uke.uni-hamburg.de.

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which can lead to differences concerning specificity and sensitivity. Finally, although peripheral blood is an ideal source for the detection of CTCs because of the noninvasive sampling procedure, the clinical significance of CTCs in peripheral blood is less clear than that for DTCs in bone marrow.

Important progress in this field arose from the development of an automated enrichment and immunocytochemical detection system for CTCs (CellSearch; Veridex; ref. 6). This system consists of a CellSave sample tube (Immunicon, Huntingdon Valley, PA) for preserving and transporting blood samples, the CellSearch Epithelial cell kit (Veridex) containing all of the reagents and consumables for conducting the test, CellSearch control cells (Veridex) for assuring proper performance on a daily or run-to-run basis, an automated instrument for adding reagents and washing cells (AutoPrep; Veridex), and a semi-automated microscope for scanning and reading results (CellSpotter Analyzer; Veridex).

Using this system, Cristofanilli et al. showed in a prospective study that CTC detection provided significant prognostic information for patients with metastatic breast cancer (7, 8). Similar to the primary setting in bone marrow, detecting elevated numbers of CTCs in peripheral blood in the metastatic setting of breast cancer is accompanied by a decreased disease-free and overall survival. There are also several reports about the detection of CTCs in patients with primary breast cancer, however, mostly with lower frequencies and varying results concerning both the number of positive patients and the number of CTCs in individual patients (9–12). Thus, measurement of CTC levels might have considerable potential to influence therapeutic strategies for breast cancer patients. For this purpose, a reliable method that is easy to perform and reproducible in different laboratories is urgently needed. Furthermore, standardization and automation of this method are pivotal to ensure high-throughput analyses as a precondition for clinical application and multicenter studies.

The Cell Search system has been cleared by the U.S. Food and Drug Administration for routine clinical use in metastatic breast cancer patients, but independent validation of this new technology in the routine setting is lacking. Therefore, the aim of the present multicenter study was to validate the analytic performance of the CellSearch system for clinical use in patients with metastatic breast cancer. The main objectives of this study were to validate accuracy and precision of the test, determine if pre-analytic variables that might affect the reliability of the test result are adequately controlled, and to verify the clinical performance claims for the CellSearch system, including CellSave blood collection tubes, CellSearch controls, and CellSearch Epithelial cell kit reagents.

Validation data from three independent laboratories and high inter-instrument accordance confirm the reliability of this system for CTC measurements and show that the system is a suitable tool for the routine assessment of therapeutic efficacy in metastatic breast cancer patients. Furthermore, our data show that samples can be shipped at room temperature, and that CTC counts are stable for at least 72 h, which facilitates testing at central laboratories or remote sites requiring transportation.

Materials and Methods

Patients and sample collection. In this study, three testing centers participated: (*a*) The Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (UKE); (*b*) The M. D. Anderson Cancer Center, Houston, TX (MDA); and (*c*) The Department of Obstetrics and Gynecology, Innenstadt, Klinikum of the Ludwig Maximilians University Munich, Munich, Germany (LMU).

Blood was drawn from 92 metastatic breast cancer patients treated at UKE ($n = 49$) or MDA ($n = 43$). Patients who contributed blood for this study were not limited to any type or line of therapy. Blood was also drawn from healthy control subjects ($n = 7$) who had no known illness at the time of draw and no history of malignant disease. All enrolled patients and healthy subjects gave their informed consent for study inclusion and were enrolled using institutional review board–approved protocols. The examination of blood samples was approved by the local ethics review.

Blood (10 mL) was collected from each donor into CellSave blood collection tubes (Immunicon, Inc., Huntingdon Valley, PA), which are evacuated blood draw tubes containing EDTA and a cellular preservative. To avoid contamination of blood samples for CTC testing with epithelial skin cells, one site (UKE) collected the Cell Save tube after a first blood sample was collected. Blood samples were maintained at room temperature for different time intervals and processed within a maximum of 72 h after blood drawing.

Cell culture. At UKE, the breast cancer cell line SK-BR-3 originally obtained from the Central Cell Service Unit of the Imperial Cancer Research Fund (London, United Kingdom; ref. 13) was grown as monolayer in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA Laboratories GmbH, Pasching, Austria) and 2 mmol/L L-glutamine (Invitrogen) in T75 flasks at 37°C in a humidified 10% CO_2 atmosphere and subcultured twice a week using 0.25% trypsin/1 mmol/L EDTA to detach cells.

At MDA, human SK-BR-3 cell line was obtained from the American Type Tissue Culture Collection (Rockville, MD) and grown at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with fetal cow serum (Life Technologies, Grand Island, NY), 100 μg/mL streptomycin (Life Technologies), 10% glucose, and 1 mmol/L sodium pyruvate (Life Technologies). The stock culture was maintained in a T25 tissue culture flask until confluence before subculture. The cells were dislodged with 1% trypsin.

Sample processing. The CellSearch Epithelial Cell kit (Veridex, Warren, NJ) contains ferrofluid particles coated with anti-EpCAM antibodies (14), two phycoerythrin-conjugated anti-cytokeratin antibodies recognizing cytokeratins (predominantly cytokeratins 8, 18, and 19) to specifically identify epithelial cells, an antibody against CD45 conjugated with allophycocyanin to identify WBC, a nuclear dye [4′,6-diamidino-2-phenylindole (DAPI)] to fluorescently label the cell nuclei, and a permeabilization buffer to allow cytokeratin antibodies entry into epithelial cells. Sample processing and evaluation were done as described by Allard et al. (15). In brief, 7.5 mL of blood were gently mixed with 6.5 mL of dilution buffer, centrifuged for 10 min at $800 \times g$ at room temperature, and transferred into the CellTracks AutoPrep system. The remaining steps are done automatically by the instrument, including all reagent addition, mixing, incubation, and aspiration steps. Following aspiration of the plasma and dilution buffer layer, anti-EpCAM antibody–coated ferrofluids and capture enhancement reagent are added. After incubation and magnetic separation, unbound cells and remaining plasma are removed, and ferrofluid-labeled cells are re-suspended in buffer, permeabilized, and fluorescently labeled using the two antibodies described above. After incubation and repeated magnetic separation, unbound staining reagents are aspirated, and a cell fixative is added. During sample processing, 7.5 mL of blood is reduced to ~300 μL containing enriched CTCs.

The sample is transferred automatically to a cartridge in a MagNest and is then placed inside the MagNest cell presentation device. Here, during an incubation of at least 20 min in the dark at room temperature, the immunomagnetically labeled cells move to the surface of the cartridge caused by the strong magnetic field of the MagNest device, whereby they are oriented for analysis using the CellSpotter Analyzer.

Sample evaluation. Evaluation of the samples was done with the CellSpotter Analyzer, a semiautomated fluorescence microscope used to enumerate fluorescently labeled cells that are immunomagnetically selected and aligned. After placing the Magnests on the microscope, the entire surface of the cartridge is scanned four times, automatically changing fluorescence filters between each scan before images of the four filters are compiled. Captured images that contain objects fulfilling the predetermined criteria are automatically presented in a gallery format from which final classification of cells was done independently by two operators in each center.

From the images presented in a gallery, a cell is classified as epithelial cell (CTC) when its morphologic features (nearly round or oval morphology with a visible nucleus within the cytoplasm) and staining patterns are consistent with that of an epithelial cell (cytokeratin-phycoerythrin positive/DAPI positive/CD45-allophycocyanin negative). CTCs must have a size of at least 4 μm but present with a large heterogeneity regarding both CTC size and morphology.

Each sample was independently analyzed by two trained readers. Questionable interpretations were evaluated again until consent was reached.

Analysis of intra-assay variability. The maximum number of samples to be processed in one batch is eight. CellSearch controls intended to monitor system operation and operator technique contain single-use vials with defined numbers of fixed, fluorescently labeled SK-BR-3 cells. Each vial contains two populations of cells at different concentrations for a high level and a low level control. The CellTracks AutoPrep system will check for a match when a control sample is loaded. To analyze intra-assay variability, eight CellSearch controls were processed in one run both at UKE and MDA. Subsequently, SD and CV values for both high and low control cell counts were determined.

Testing of inter-assay variability. Inter-assay variability was tested by two methods in two centers (UKE and MDA). The first method was used to analyze performance during the entire course of the study. Therefore, one CellSearch control vial was run every day during the study. The second method tested precision over at least 20 consecutive days. To do this, on consecutive testing days, two controls were run in the morning, and two controls were run in the afternoon of the same day.

Testing of cell stability. In center 1 (UKE), a total of 20 patients with confirmed metastatic breast cancer were enrolled, and two to four CellSave tubes were drawn from each patient from the same vein puncture. From all patients, one sample was processed immediately after drawing. The other sample was stored either for 24, 48, or 72 h at either room temperature or at 4°C until processing.

In center 2 (MDA), four CellSave tubes were drawn from the same vein puncture from nine patients with confirmed metastatic breast cancer. From each of five patients, one sample was analyzed immediately, whereas the second, third, and fourth samples were subsequently processed after 24, 48, or 72 h of storage at room temperature, respectively. The same procedure was followed for the other four patients, with the difference that the samples were stored at 4°C until processing.

To additionally test the influence of shipment conditions, duplicate samples of the same patients from center 1 (UKE) were analyzed in parallel: one after storage for 24 to 96 h at room temperature and the other one after shipment for 24 to 96 h.

Testing of inter-instrument variability. To test whether CTC values are influenced by different CellSearch instruments, duplicate drawings from the same patients were taken at the same time point in center 1 (Clinic of Gynecology, UKE). One of these samples was shipped to center 3 (LMU), and the other sample was stored in center 1 for the same time period. Within 24 to 48 h, both samples were analyzed in parallel in both centers (LMU and Institute of Tumor Biology, UKE). Image galleries were exchanged between the two centers, and discordant interpretations were discussed until consent was reached.

Testing of reproducibility/recovery. Because it is very difficult to accurately spike low numbers of cells, we chose two commonly used spiking methods to better assess recoverability.

At UKE, blood samples from healthy donors were drawn into CellSave tubes. By micromanipulation controlled by phase-contrast microscopy, a defined low number of SK-BR-3 cells (4-12 in 10 μL PBS) were spiked into each of two tubes from healthy donors.

At MDA, additional spiking experiments were done with DAPI-labeled SK-BR-3 cells (provided by Immunicon). Cells contained in a small drop of PBS (5-10 in 10 μL PBS) that was added to a tube were counted under a fluorescence microscope in the DAPI channel and in the bright-field channel for confirmation. The blood samples from healthy donors that were to be spiked were then added to the cells and processed in the CellSearch system.

Statistical analyses. Statistical analyses were done with the help of the SPSS software for PC (version 11 for Windows). *P*s < 0.05 were considered statistically significant.

Results

Detection of CTCs in metastatic breast cancer patients. In this study, 71.4% (35 of 49) of metastatic breast cancer patients in center 1 (UKE) and 65.1% (28 of 43) in center 2 (MDA) had at least one detectable CTC in 7.5 mL of blood. Conversely, across both centers, ~30% to 35% of metastatic breast cancer patients did not have CTCs in their blood when tested for this study (Table 1).

The number of samples reaching the prognostically relevant cutoff level of ≥5 CTCs/7.5 mL blood (7) was similar between both sites (38.8% at UKE and 34.9% at MDA; Table 1). More than 50 CTCs per 7.5 mL blood were identified in five and eight patients from UKE and MDA, respectively. The highest number of CTCs was 1,491 per 7.5 mL blood. In Fig. 1A, a part of a gallery of images is shown showing 3 CTCs (marked in composite image), which are positive in the DAPI and cytokeratin-phycoerythrin channel but negative for CD45-APC (allophycocyanin) and in the FITC (fluorescein) channel. Figure 1B and C show pictures that were directly taken from a CellSpotter cartridge after sample processing. Three cells are cytokeratin positive (*C*), whereas the other seven cells although captured by EpCAM-coated ferrofluids are cytokeratin negative (*B*).

Specificity of the assay was tested by analyzing each 7.5 mL of blood from seven healthy volunteers. We did not find any CTCs in these control samples, confirming results from Allard et al. (15) who found only 8 of 145 samples from healthy women with 1 CTC/7.5 mL blood and no sample with ≥2 CTCs.

Analysis of assay variability and recovery rate. To assess intra-assay variability, eight control samples with a defined number of high and low control cells were analyzed in the same run. Because the expected number of cells depends on the particular control cell kit used in the run, observed differences in the exact

Table 1. Number of CTCs in metastatic breast cancer patients

No. CTCs in 7.5 mL blood	No. patients	% Patients
UKE (*n* = 49)		
0	14	28.6
1-4	16	32.6
≥5	19	38.8
MDA (*n* = 43)		
0	15	34.9
1-4	13	30.2
≥5	15	34.9

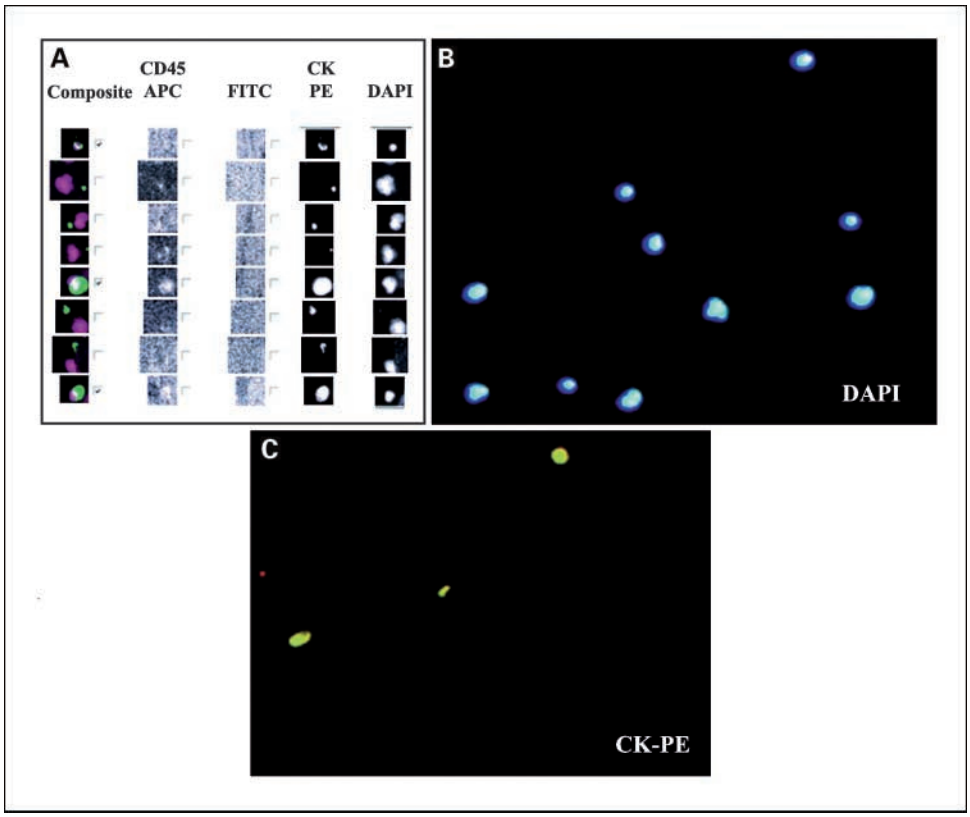

Fig. 1. *A,* gallery of images that has to be screened for CTCs. Three CTCs are marked in the composite image. CK, cytokeratin; PE, phycoerythrin. Pictures taken from the cartridge (*B*) DAPI staining of nuclei and (*C*) cytokeratin-positive CTCs.

numbers and in the SD and CV values are not unexpected. However, in both centers, the obtained numbers of high and low control cells from all eight samples reached the expected values (21-80 for low controls and 791-1,157 for high controls; Table 2).

Inter-assay variability was also low at both centers (Table 2). When analyzing one control sample per day on each day of testing (either a.m. or p.m.), very few samples had control cell results outside the ranges predefined by the manufacturer (Table 2). At UKE for example, in 3 and 4 of 25 control samples, low and high level control cell numbers, respectively, were below the predefined ranges. Altogether, inter-assay variability was below 5% for 299 analyzed control samples.

The mean recovery rate of tumor cells spiked into normal blood at the 4 to 12 cell level was 82% for UKE (N = 20) and 80% for MDA (N = 12) samples. Recovery data from single samples ranged from 30% to 200% (UKE) and 60% to 90% (MDA) because of the inherent variation in spiking of low numbers of cells. However, all spiked samples, regardless of the low number added, had detectable cells.

Testing of CTC stability. To evaluate stability of CTCs during storage, blood from patients with metastatic breast cancer was either immediately processed or stored at room temperature or 4°C for 24, 48, or 72 h. CTC-negative samples at T_0 were excluded from further analysis.

The number of detected CTCs was stable for at least 72 h at both temperatures because there were no statistically significant changes observed both for the UKE data and for MDA data alone (Wilcoxon Signed-rank test, Table 3; Kruskal-Wallis test, Fig. 2A and B). This was also true if all patients from both centers were taken together. However, variation in the number of detected CTCs was higher during storage at 4°C (Table 3).

Considering individual cases, three MDA patients and one UKE patient changed from a CTC value of ≥5 to <5 per 7.5 mL blood after storage (Table 3). This decrease was more frequently observed during storage at 4°C than at room temperature. For three UKE patients and one MDA patient, CTCs were detected in the samples that were immediately processed but were not found in the samples stored for 48 or 72 h. However, this loss was exclusively found after storage at 4°C. For four UKE patients, a change to a higher category (<5 to ≥5 CTCs/7.5 mL) was observed (Table 3).

Shipment conditions, such as temperature variations or shaking, did not have a significant influence on CTC values. As shown in Table 4, there were no statistically significant differences between CTC values of two samples drawn from the same patient at the same time point: one analyzed after storage at room temperature and the other one after shipment for 24 to 96 h (Wilcoxon Signed-rank test, P = 0.209; Table 4). Both values correlated significantly (regression analysis: correlation coefficient, R^2 = 0.98, slope = 1.07; Spearman's rank correlation: P = 0.0028, Rho = 0.742).

Testing of inter-instrument variability. As also shown in Table 4, there were no statistically significant differences in the number of detected CTCs, when duplicate samples from the same patients were drawn, one of which was shipped from center 1 (UKE) to center 2 (LMU), and both samples were analyzed with different CellSearch instruments in parallel (Wilcoxon Signed-rank test, P = 0.119). Four of 14 cases revealed to be CTC negative, and 7 of 14 cases were positive for 1 to 4 CTCs in both centers. In the remaining three cases, CTCs

(2-4) were only detected in center 2 (LMU). Thus, 78.6% of cases were concordant regarding the classification into CTC negative and CTC positive for 1 to 4 CTCs/7.5 mL blood (Table 4).

Unassigned events. After CellSpotter analysis, all areas containing cytokeratin-phycoerythrin– and DAPI-positive images are displayed in a gallery. Besides CTCs, these images also include artifacts, cytokeratin-phycoerythrin and CD45-APC double-positive cells, cytokeratin-negative leukocytes with a phycoerythrin-labeled object in the same image area, or enucleated cells (as determined by cytokeratin positivity but without DAPI staining within the cytoplasm). All images that are not classified as CTCs are designated as "unassigned events." The higher the number of unassigned events, the longer the time required for the evaluation of an individual sample by the reader. For most patient samples, the number of unassigned events that had to be evaluated after CellSpotter analysis increased during storage both at room temperature and at 4°C (Fig. 2C and D). Taken all values obtained in both centers together, changes in the unassigned event values significantly increased with storage time (Spearman's rank correlation: room temperature, $P = 0.0019$, Rho = 0.84; 4°C, $P = 0.0033$, Rho = 0.89).

For MDA data, this increase over the entire storage time (0-72 h) was continuous and statistically significant both for room temperature (Kruskal-Wallis test, $P = 0.0047$) and for 4°C (Kruskal-Wallis test, $P = 0.0118$, Fig. 2C and D). At UKE, there also was a tendency to a stronger increase in the number of unassigned events after storage at 4°C compared with room temperature, which became statistically significant after 72 h (Mann-Whitney *U* test, $P = 0.0143$). Furthermore, absolute values of unassigned events were extraordinarily high in some cases, in particular after storage for 48 and 72 h at 4°C (Fig. 2C and D).

Comparing numbers of CTCs and unassigned events at T_0 from the same patients, a statistically significant correlation was observed in that the higher the number of detected CTCs, the higher the number of unassigned events (Spearman's rank correlation: $P = 0.0005$, Rho = 0.643).

Table 2. Precision data for cell line controls

	N	Observed range	Mean	SD	CV (%)
Within run UKE					
Low	8	45-84	56.5	14.7	26
High	8	961-1014	987.0	21.3	2.1
Within run MDA					
Low	8	35-60	44.8	8.3	18.4
High	8	743-1005	875.4	101.5	11.6
Day to day UKE control kit 1					
Low	39	34-65	48.9	8.3	17
High	39	874-1,142	1,010.0	70.6	7
Day to day UKE control kit 2					
Low	25*	9-74	39.6	17.0	42.9
High	25†	436-1,100	825.1	130.3	15.8
Day to day MDA					
Low	59	29-65	46.9	8.0	17.0
High	59	893-1,250	1,008.0	116.4	11.5
a.m. UKE					
Low	40*	7-81	48.3	16.2	33.5
High	40†	220-1,142	922.0	180.5	19.6
p.m. UKE					
Low	40	16-68	46.5	10.6	22.8
High	40	772-1,121	946.7	100.3	10.6
a.m. MDA					
Low	40	31-61	49.2	6.9	14.0
High	40	816-1,114	981.5	57.9	5.9
p.m. MDA					
Low	40	29-60	48.0	7.5	15.6
High	40*	667-1,104	948.5	93.0	9.8

*Three samples outside range.
†Four samples outside range.

Discussion

The CellSearch system represents the first automated, standardized, and regulatory-approved system for the detection and quantification of CTCs in peripheral blood (7, 8). Furthermore, it is the first system to fully validate a clinical use of CTCs in the metastatic setting of cancer. To our knowledge, our study is the first multicenter validation study of the CellSearch system for this purpose, and the protocol described here could serve as a template for individual laboratories looking to validate this system or any rare cell detection system. Our results show that the system is a suitable tool for the assessment of CTCs in these patients, enabling the reliable detection of CTCs in whole blood.

Approaches available today for the isolation of CTCs in a research setting range from enrichment of tumor cells using density-gradient centrifugation (9, 16, 17) to filtration approaches (11, 18, 19) and flow cytometry (20, 21). Although most methods described in the literature use immunocytochemical identification of CTCs, the reagents are not standardized and vary depending on the method. Additionally, nucleic acid–based techniques have also been introduced into the research setting, which further complicates comparison of results between laboratories (for review, see refs. 2, 3). One advantage of immunocytochemical methods is the opportunity to include morphologic criteria and to further characterize individual cells by additional antibodies, fluorescence *in situ* hybridization technology, or molecular methods after detection (22, 23).

The precision, intra- and inter-assay agreement, and recovery rates at low cell level given by the CellSearch system in two centers (UKE and MDA) were high. The similarity of the data generated from all participating institutions shows the robustness of the CellSearch system. The high reproducibility of the results observed with the CellSearch system was also underlined by a high accordance of CTC values after processing duplicate samples from the same metastatic breast cancer patients with different instruments at different centers. Only 3 of 14 duplicate measurements (21.4%) were discrepant between the two centers regarding the classification into CTC-negative and CTC-positive samples (Table 4). Because blood samples were not pooled before shipment, and because these discrepancies occurred at the analytic low level of 2 or 3 cells/7.5 mL blood, they might be due to an uneven distribution of CTCs in the two blood drawings. CTC counts were stable for at least 72 h, and blood samples can be shipped at room temperature. Because the test recognizes epithelial cells, it is suitable for use in different cancers of epithelial origin, the major form of malignant tumors in the United States and Europe.

Table 3. Comparison of the numbers of CTCs in 7.5 mL blood between T_0 and 24, 48, or 72 h after storage at room temperature or 4°C

Room temperature						4°C					
UKE			MDA			UKE			MDA		
Patent ID	T_0	24 h	Patent ID	T_0	24 h	Patent ID	T_0	24 h	Patent ID	T_0	24 h
1*†	33	48	1*	12	16	4	5	8	6	11	1
2	79	129	2	44	25	1†	1,491	1,055	7	7	9
3	3	1	3	127	104	5	1	3	8	43	68
4	5	6	4	42	107	8	1	5	9	495	87
5	1	4	5	7	9	9	25	31			
Patent ID	**T_0**	**48 h**	**Patent ID**	**T_0**	**48 h**	**Patent ID**	**T_0**	**48 h**	**Patent ID**	**T_0**	**48 h**
2	79	109	1	12	23	1	1,491	1,173	6	11	1
6	1	5	2	44	61	13	2	7	7	7	11
3	3	3	3	127	103	14	3	0	8	43	33
7	12	12	4	42	123	15	2	0	9	495	39
8	1	1	5	7	4	16	1	0			
9	25	27									
Patent ID	**T_0**	**72 h**	**Patent ID**	**T_0**	**72 h**	**Patent ID**	**T_0**	**72 h**	**Patent ID**	**T_0**	**72 h**
2	79	84	1	12	24	17	10	14	6	11	0
6	1	2	2	44	80	18	2	18	7	7	1
10	6	8	3	127	125	19	9	1	8	43	46
11	1	1	4	42	98	16	1	1	9	495	60
7	12	28	5	7	10	20	4	3			
12	4	3									

NOTE: All observed differences between CTC values at T_0 and after storage were not statistically significant (Wilcoxon's signed-rank test).
*Different patients at UKE and MDA.
†CTC values from the same patient, but second blood drawing was 4 wks later.

We chose to analyze the sensitivity of the system by determining the recovery rate of SK-BR-3 cells spiked directly into blood of healthy control subjects at the low cell level of 5 cells (the clinical decision point) and 10 cells/7.5 mL of blood. Common limitations of such model systems are described by Ring et al. (2). Mean recovery rates of 82% (UKE) and 80% (MDA) were high in both centers. Similar results were obtained by Witzig et al. (12) and Allard et al. (15), also using immunomagnetic separation techniques, whereas Ficoll gradient centrifugation sometimes is associated with a higher loss of spiked tumor cells as shown for bone marrow (24). The variation of the recovery rate was mainly due to the difficulty to spike low numbers of intact cells accurately into blood samples. Our recovery data show precision of duplicate measurements, another supporting point for reliability of measurements.

A reliable method to identify CTCs should be characterized by both high sensitivity and high clinical and analytic specificity, requirements that are strongly influenced by the choice of antibodies in immunocytochemical assays. One possible limitation regarding sensitivity of the CellSearch method is the use of EpCAM expression as the primary mechanism for positive selection of CTCs. Consequently, CTCs that do not express EpCAM will not be detected by the assay. Although the vast majority of epithelial tumors are characterized by EpCAM expression (25), heterogeneity of expression has been reported in mammary carcinomas (25, 26). Down-regulation of EpCAM expression has also been reported for DTCs in bone marrow (27) and CTCs in peripheral blood (26). The CellSearch reagents incorporate avidin-biotin chemistry that increases the magnetic load of EpCAM expressing cells to compensate for low antigen density. This not only reduces variation in the selection efficiency (28) of the system but could also increase the number of captured non-epithelial cells. A direct comparison of positively selected and negatively selected metastatic breast cancer samples might help to determine if the current EpCAM ferrofluid configuration is optimal.

Theoretically, it is also possible that CTCs that express EpCAM will not express cytokeratins and thus be undetected by the CellSearch system. The down-regulation of particular cytokeratins is a characteristic feature of epithelial-mesenchymal transition that is necessary for tumor cell invasion and migration and has been observed in breast cancer (29–31). However, this potential limitation is minimized in the CellSearch system by the use of a pan-anti-cytokeratin antibody that recognizes multiple cytokeratins.

Conversely, low nonspecific expression of cytokeratins (e.g., cytokeratin 8, cytokeratin 19, and cytokeratin 20) has been detected in blood and bone marrow from normal volunteers, patients with malignant hematologic disorders (32–36), or can be induced in peripheral blood mononuclear cells by cytokines and growth factors (37), leading to illegitimate transcription of, for example, cytokeratin 19, which could undermine the clinical specificity of the assay. However, in our study, we did not detect CTCs in any of the normal volunteers tested, which confirms the findings of Cristofanilli et al. (8) that in the metastatic setting using 7.5 mL of blood, the CellSearch test is highly specific for CTCs. The highest detected CTC value was 1,491/7.5 mL blood in a patient who died days after blood

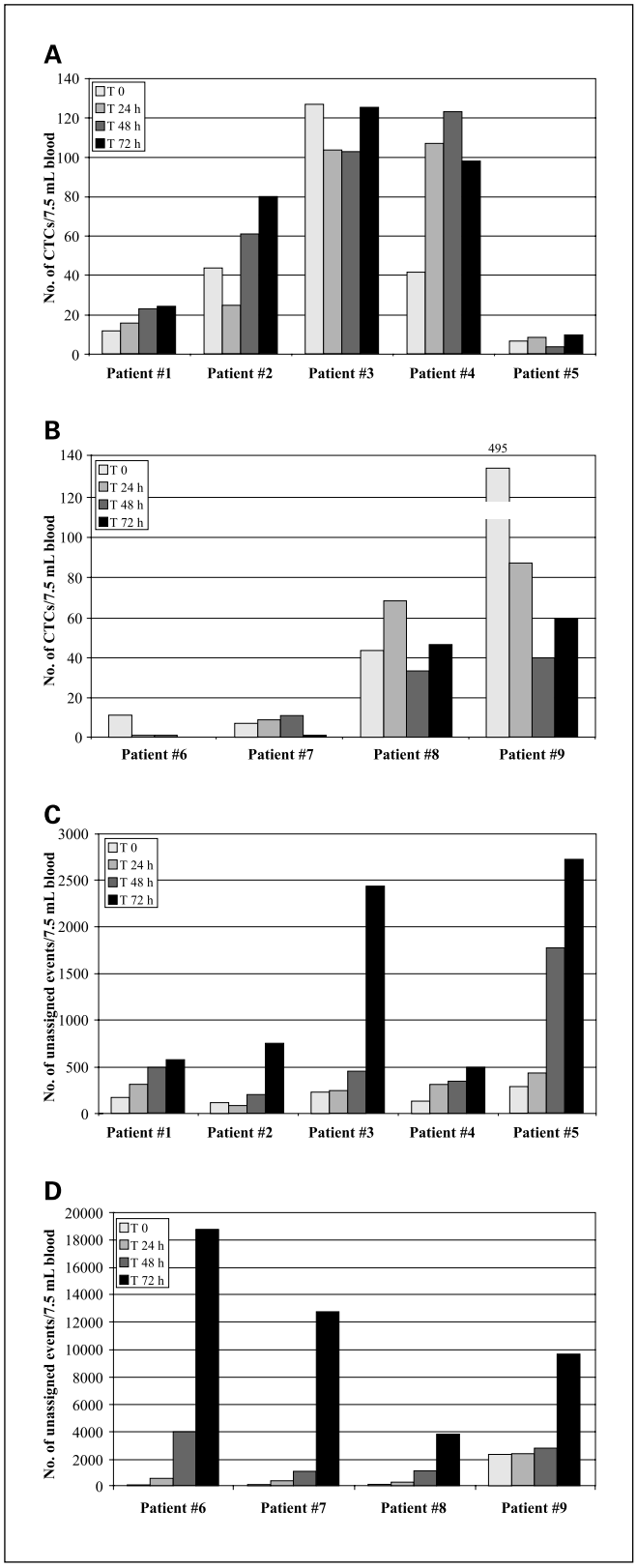

Fig. 2. Identical samples of each 7.5 mL blood from metastatic breast cancer patients stored at room temperature (*A* and *C*) and at 4°C (*B* and *D*) and analyzed at MDA. The number of CTCs did not significantly change during storage at room temperature (*A*, Kruskal-Wallis test, $P = 0.96$) or 4°C (*B*, Kruskal-Wallis test, $P = 0.799$). The number of unassigned events significantly increased during storage at room temperature (*C*, Kruskal-Wallis test, $P = 0.0047$) and 4°C (*D*, Kruskal-Wallis test, $P = 0.0118$).

evaluation. The same patient was found positive 4 weeks earlier in a first CellSearch analysis, with 48 CTCs/7.5 mL blood. Previously published data indicate that the absence or presence of CTCs in peripheral blood of metastatic cancer patients is a direct reflection of the patient's response, or lack of response, respectively, to therapy (7, 8, 38).

The inter-patient variability seen in Table 3 seems to be high, reflecting variability seen in previous publications (7, 15). However, the purpose of the present study was rather to validate the analytic performance of the system than a clinical validation. Independent of the stage of the disease as a primary factor influencing the number of CTCs in an individual patient, there is a need to determine other pre-analytic patient factors, such as mobility, age, etc., that also might have an effect on the number of CTCs.

CTCs are fragile and require preservation for valid analysis. Therefore, blood for the CellSearch system is drawn into special preservation tubes. Samples collected in CellSave tubes showed CTC stability for at least 3 days. Variations in the number of CTCs might predominantly be caused by sampling errors rather than by a destruction of CTCs because there were also some samples with higher CTC numbers after storage compared with T_0 values (Table 3). Refrigeration caused a loss of cells in some samples, and although this effect did not reach statistical significance, in some cases, CTC-positive samples became negative and this effect was exclusively observed after storage of the blood at 4°C. Thus, as recommended by the manufacturer, the blood has to be shipped and stored at room temperature.

Although there were no significant differences in CTC numbers before and after storing the blood for 72 h, the number of unassigned events that had to be evaluated on the CellSpotter increased substantially during storage. Evaluation of these images is not only time consuming but can also become a source of false-positive CTCs when not analyzed carefully. The increase in the number of unassigned events after 72 h of storage was significantly higher at 4°C, at least for MDA data, but was not observed in blood from a small number of primary breast cancer patients (data not shown). The manufacturer warns against the storage of blood at refrigerated temperatures for this reason, and all studies and validations on the CellSearch system to date have been conducted on samples stored only at room temperature. The presence of unassigned events, although complicating the readings, does not detract from the accuracy of the measurements. The manufacturer has recently improved the CellSearch kit by lowering the concentration of cytokeratin 19 antibody, and our own unpublished results show that this leads to a decreased number of unassigned events without affecting the number of detected control cells.

Interestingly, the number of unassigned events correlated with the number of CTCs in a given sample. Because there is a prognostic relevance of the number of CTCs in metastatic breast cancer patients (7, 8), there may be significance of the apoptotic cells as well. These unassigned events are very heterogeneous, including artifacts, but also nucleated cells that are frequently double positive for cytokeratin and CD45. It is tempting to speculate that some of these cells have eliminated CTCs by phagocytosis, but the strong increase in their number, whereas CTC number remains stable, could not be explained. As previously described by Allard et al. (15), apoptotic

Table 4. Influence of shipment on CTC values and comparison of CTC values after processing duplicate samples with different CellSearch instruments

Sample ID	No. CTCs in 7.5 mL blood		Sample ID	No. CTCs in 7.5 mL blood	
	Storage at room temperature, UKE, Instrument 1	After shipment, UKE, Instrument 1		UKE Instrument 1	LMU Instrument 2
1	0	0	21	1	4
2	0	0	22	0	2
3	27	29	23	2	1
4	0	0	24	2	4
5	0	0	25	0	0
6	1	1	26	3	2
7	0	0	27	3	4
8*	110	140	28	0	2
9*	0	0	29	0	0
10	0	2	30	0	0
11	136	129	31	1	1
12	0	1	32	0	0
13	0	0	33	4	2
14	4	2	34	0	3
15	0	2			
16	1	4			
17	0	1			
18	0	0			
19	0	1			
20*	1	0			

*Analysis after 96 h, all other samples were analyzed after 24 to 48 h of storage at room temperature or shipment.

enucleated cells could be observed but are not counted as CTCs. As a precondition for a high sample throughput, new generations of CellSpotter instruments should have the ability to exclude more unassigned event images from the gallery automatically.

The next step will be the analysis of primary breast cancer patients for CTCs and the clinical validation of the predictive and prognostic claim for CellSearch in this setting. Analyses directed to further characterize the CTCs concerning the expression of therapeutically important targets are currently under investigation. Because the majority of CTCs in peripheral blood (9) or DTCs in bone marrow (39) of breast cancer patients seem to be in a non-proliferating state, new therapeutic approaches have to be developed targeting dormant cells. Thus, detection of Her-2 overexpression in CTCs may assist in identifying high-risk breast cancer patients who might benefit from therapeutic strategies targeting Her-2 (22, 40).

In conclusion, our study shows that the examination of blood samples for CTCs with the CellSearch system is possible in a multicenter setting in clinical trials. Furthermore, the system is a robust tool for the routine assessment of metastatic breast cancer patients in the clinical laboratory.

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