Capture of Viable Circulating Tumor Cells in the Liver of Colorectal Cancer Patients

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BACKGROUND: The incidence and number of circulating tumor cells (CTCs) in the peripheral blood of colorectal cancer patients are lower than in other cancer types, which may point to a particular biology of colorectal cancer affecting CTC detection.

METHODS: We detected CTCs in the peripheral and mesenteric blood of colorectal cancer patients by use of 2 independent technologies on the basis of different biological properties of colon cancer cells. Seventy-five patients diagnosed with localized (M0, n = 60) and metastatic (M1, n = 15) colorectal cancer were included. Peripheral and mesenteric blood samples were collected before tumor resection. We performed CTC enumeration with an Epispot assay that detected only viable CK19-releasing EpCAM-independent enrichment method followed by the Epispot assay that detected only viable CK19-releasing CTCs. In parallel, we used the FDA-cleared EpCAM-dependent CellSearch® as the reference method.

RESULTS: The enumeration of CK19-releasing cells by the CK19–Epispot assay revealed viable CTCs in 27 of 41 (65.9%) and 41 of 74 (55.4%) (P = 0.04) patients in mesenteric and peripheral blood, respectively, whereas CellSearch detected CTCs in 19 of 34 (55.9%) and 20 of 69 (29.0%) (P = 0.0046) patients. In mesenteric blood, medians of 4 (range 0–247) and 2.7 CTCs (range 0–286) were found with Epispot and CellSearch (P = 0.2), respectively, whereas in peripheral blood, Epispot and CellSearch detected a median of 1.2 (range 0–92) and 0 CTCs (range 0–147) (P = 0.002).

CONCLUSIONS: A considerable portion of viable CTCs detectable by the Epispot assay are trapped in the liver as the first filter organ in CRC patients.

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Received January 6, 2013; accepted April 26, 2013.

Previously published online at DOI: 10.1373/clinchem.2013.202846

7 Nonstandard abbreviations: CRC, colorectal cancer; CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial–mesenchymal transition; RC, releasing cells.
EpCAM is a type I transmembrane molecule with an epidermal growth factor–like domain, followed by a thyroglobulin repeat domain, a cysteine-poor region, a transmembrane domain, and a short cytoplasmic tail (7). EpCAM was originally described as a homotypic cell adhesion molecule and later identified as a major player in the Wnt signaling pathway and a marker of CRC-initiating cells (8). Although EpCAM is expressed in most primary CRCs, it can be downregulated during tumor cell dissemination as a consequence of epithelial–mesenchymal transition (EMT) plasticity (1). Thus, CellSearch may miss CTCs that have undergone EMT and lack EpCAM expression. Moreover, many CTCs detected with CellSearch are not viable but rather apoptotic (9). More recently, we developed a new CTC test called the Epispot assay (epithelial immunospot), which allows the detection of only viable cells after CD45+ cell depletion. This test enables the detection of EpCAM+ and EpCAM− CTCs and is based on cultured viable cells releasing CK19 as marker for tumor cells, including colon cancer cells (10).

The liver is thought to be an important filter organ for colon cancer cells (11). However, there is little direct evidence obtained from measurements in patients with CRC. Two recent studies have shown a significantly higher amount of CTCs (detected by CellSearch) in mesenteric blood compared to peripheral blood in patients with CRC, suggesting that CTCs are captured in the liver (12, 13). Here, we investigated whether this difference was due to a specific capture of EpCAM+ tumor cells by the liver and whether the CTCs in the mesenteric and peripheral blood were viable. In addition to the EPCAM-dependent CellSearch system, we used the EpCAM-independent Epispot assay to specifically detect viable CTCs.

Materials and Methods

STUDY DESIGN
We enrolled 75 patients with newly diagnosed localized (stage M0, n = 60) and metastatic (stage M1, n = 15) CRC without preoperative chemo- or radiotherapy at the Gastrointestinal Surgery Department of the Saint-Eloi Hospital, University Medical Centre of Montpellier, France. Patients were treated according to international guidelines, and no major local complications (i.e., tumor perforation under chemotherapy or ureteral or nervous compression) were reported. The use of peripheral and mesenteric blood samples was approved by the multidisciplinary bioethics committee of oncology of Montpellier before the inclusion of patients into the study (biobank number DC2008830), and all patients provided written informed consent. Twenty healthy controls were also enrolled in this study at the blood transfusion Centre of Montpellier.

ISOLATION AND DETECTION OF CTCs
Peripheral and mesenteric blood was collected for CTC evaluation before the surgery (before any mobilization of the primary tumor, to avoid a passive release of tumor cells) for the patients with CRC, and only peripheral blood was collected and analyzed for the control group. Blood samples were drawn into 10-mL CellSave tubes (Immunicon) for CellSearch and EDTA tubes for Epispot. Samples (10–20 mL of blood) were maintained at room temperature and processed within 24 h of collection. We used CellSearch (6) and the CK19-Epispot assay (10) for CTC detection. For CellSearch, CTCs were enriched via EpCAM expression and defined as EpCAM-isolated intact cells staining positive for cytokeratins (CK8, 18, 19) and negative for CD45 (the specific marker of hematopoietic cells). For Epispot, viable CTCs were first enriched via a depletion of hematopoietic CD45+ cells (RosetteSep, StemCell Technology) (14) and defined as CK19-releasing cells (CK19-RCs).

STATISTICAL ANALYSIS
Patients’ characteristics are presented by use of median and range (or mean and SD) for continuous variables and frequencies and proportions for categorical variables. The numbers of CTCs were compared between sites (peripheral vs mesenteric) and methods (Epispot vs CellSearch) by use of the Wilcoxon rank test. We used the χ² test or the Fisher exact test to compare proportions.

We assessed the agreement between the two methods after dichotomization (0 vs >0 CTCs) with the κ coefficient (values close to 1 indicating strong agreement). It should be noted that this index expresses the level of agreement between 2 techniques that measure different types of cells. An ROC curve was created for each method by plotting the diagnostic sensitivity against (1 – the diagnostic specificity) for each value. The diagnostic sensitivity was measured in the population deceased before time t, and the diagnostic specificity was measured in the population alive at time t.

The CTC threshold at each follow-up time was chosen to minimize the number of misclassifications (or to maximize the Youden index). We observed that at all times of interest (1, 2, 3, and >3 years), the threshold was almost the same. We then estimated the area under the curve (AUC) at each time, selecting the time of interest to be that time where the AUC was the greatest. The CTC threshold used across all times of follow-up was that value that minimized misclassifications at the follow-up time with the greatest AUC. We compared the survival curves between patients with fewer CTCs than this defined threshold vs those with CTCs exceeding the threshold. Kaplan–
Meier curves and the log-rank test were used for statistical evaluation of survival data. The threshold for statistical significance was set at 5%. Statistical analysis was performed with SAS software 9.1 (SAS Institute).

Results

PATIENT CHARACTERISTICS
Between 2006 and 2008, a total of 75 patients with CRC (median age 75 years, range 38–95 years) were enrolled in this study where we detected viable CTCs with the Epispot technology. We compared these results with those obtained by use of the CellSearch system. Twenty-six patients had a laparoscopy; the remaining forty-nine patients were operated by laparotomy. The primary tumors were 34 right-sided, 26 left-sided, 8 rectum, 4 transverse, 1 left/transverse, 1 right- and left-sided, and 1 rectum/right-sided (Table 1). The moderate predominance of right-sided colon cancer in our cohort was a chance finding and not due to any preselection criteria. Samplings done by laparoscopy (34.7%) were more difficult to obtain than those done by laparotomy (65.3%). Sixteen patients operated by laparotomy and 18 by laparoscopy did not have mesenteric blood collected. We noted that there were no significant differences in CTC detection depending on type of surgery [laparoscopy vs laparotomy for both technologies; $P = 0.77$ (Epispot) and $P = 0.19$ (CellSearch)]. All patient characteristics are shown in Table 1. Patients were compared on the basis of Epispot and CellSearch results. Sixty patients had no detectable overt metastasis (stage M0), and 15 patients presented with existing synchronous metastases in various organs (stage M1), particularly in the liver (13 of 15, 86.7% (Table 1). Among patients with metastatic cancer, 3 and 5 patients had liver surgery synchronously and metachronously, respectively.

We analyzed 74 and 69 peripheral blood samples, as well as 41 and 34 mesenteric blood samples, by the CK19 Epispot assay and the CellSearch system, respectively (Table 2).

DETECTION OF VIABLE CTCs WITH THE EpCAM-INDEPENDENT EPISPOT ASSAY
The enumeration of CK19-RCs by Epispot allowed the detection of viable CTCs in 65.9% and 55.4% of patients in mesenteric and peripheral blood, respectively. Medians of 4 (range 0–247) or 1.2 (range 0–92) CTCs were found in the mesenteric and peripheral blood ($P = 0.04$) (Table 2). In comparison, no CK19-RCs were found in the peripheral blood of the 20 healthy volunteers analyzed in this study as controls (data not shown).

CTC DETECTION WITH THE EpCAM-DEPENDENT CELLSEARCH SYSTEM
The enumeration of EpCAM$^{+}$CK$^{+}$DAPI$^{-}$CD45$^{-}$ cells by the CellSearch system allowed the detection of CTCs in 55.9% and 29% of patients in the mesenteric and peripheral blood, respectively. Medians of 2.7 (range 0–286) or 0 (range 0–147) CTCs were detected in the mesenteric and peripheral blood ($P = 0.0046$) (Table 2). No CTCs were found in the peripheral blood of the healthy controls (data not shown).

COMPARISON OF CELLSEARCH VS EPISPOT RESULTS FOR CTC DETECTION
We first compared the detection rates for both CTC assays. When the enumeration of CTCs was performed in the mesenteric blood, no significant difference in the percentage of CTC-positive patients was found between Epispot and CellSearch ($P = 0.2$) (Table 2). In contrast, a significantly higher number of CTCs were detected in the peripheral blood with Epispot compared with CellSearch ($P = 0.002$) (Table 2).

We then investigated whether the 2 assays classified the same samples as CTC positive. For a direct comparison of the results obtained with both assays, we focused on the patients who were analyzed with both Epispot and CellSearch. There was no concordance between the 2 technologies, in either mesenteric blood ($n = 32$, $\kappa = -0.27$, $P = 0.1$) or peripheral blood ($n = 68$, $\kappa = 0.08$, $P = 0.45$) (Table 3), showing that most CTC results obtained with either Epispot or CellSearch in individual patients were not overlapping. If both technologies were taken into account, 30 of 32 patients were positive in the mesenteric vein (93.8%) and 44 of 68 patients in peripheral blood (64.7%).

CORRELATION OF CTC DETECTION TO CLINICO-HISTOPATHOLOGIC RISK FACTORS AND PROGNOSIS
To investigate whether the detection of CTCs was associated with a higher risk to develop metastasis, we first analyzed the correlation to known risk factors in CRC. As shown in Table 1, the results of both CTC assays were inversely correlated to the presence of lymphatic emboli in the primary tumor ($P = 0.008$). Lymphatic emboli were defined by the pathologist as the presence of tumor cells in the lymphatic vessels in the primary tumor. For all other factors, no significant correlations were found except for a correlation of $P = 0.045$ for the Epispot results and the differentiation grade of the primary CRC (Table 1).

We subsequently analyzed our CTC results in the context of the clinical follow-up of the patients with CRC. It should be noted that the follow-up period was rather short (median 36 months; mean 31 months; range 0–52) and the number of patients analyzed was quite small. Among the 60 stage M0 patients, 10 devel-
<table>
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<th>Table 1. Characteristics of colorectal cancer patients (N = 75).</th>
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<sup>a</sup>Epispota CellSearch<sup>b</sup> Continued on page XX
oped tumor recurrence, including 7 metastatic relapses
in the liver and other organs (3). Our preliminary
Kaplan–Meier analysis indicated that M0 patients with
a high CTC count (≥27 CTCs) measured by Epispot
had a worse overall survival than patients with a CTC
count below this threshold (P = 0.046) (Fig. 1). Other
CTC thresholds were also analyzed for the Epispot re-
sults, but no other statistically significant correlations
were found.

Regarding the results obtained with the Cell-
Search, we also tested various cutoffs for the respective
CTC counts but observed no statistically significant
correlations to the prognosis of our small CRC patient
cohort (data not shown).

Discussion

The present results showed a significant mesenterico-
peripheral gradient of CTCs and therefore support the
view that the liver is a filter for CTCs (12, 15). Using 2
independent assays, we detected significantly more
CTCs in the mesenteric blood compared with the pe-
ripheral blood. This finding is also consistent with the
previous report of Thorsteinsson et al. (16), who re-
ported low CTC counts in nonmetastatic colon cancer
with the CellSearch system. Using the Epispot assay, we
further showed, for the first time, that CTCs released by
the primary CRC into the mesenteric blood are viable.
Interestingly, Epispot detected more CTCs than Cell-
Search in both mesenteric and peripheral blood. This
lower analytical sensitivity of the CellSearch system
might be explained, at least in part, by the positive
EpCAM selection used for the enrichment of CTCs; the
Epispot assay uses CD45 depletion as an unbiased en-
richment method including EpCAM+/EPCAM−CTCs (1).
Thus, different subsets of CTCs are detected
by these 2 different technologies (Fig. 2), which can
also explain the lack of concordance that we found in

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\begin{array}{|c|c|c|c|}
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\text{Characteristics of colorectal cancer patients (N = 75). (Continued from page XX)}
\hline
\hline
\text{Epispot}^a & \text{CellSearch}^b & \text{Epispot}^a & \text{CellSearch}^b \\
\text{All} & \text{Positive, n/total (%)} & \text{Positive, n/total (%)} & \text{P} \\
\hline
\text{Differentiated} & 8 & 2/8 (25) & 2/7 (28.6) \quad \text{P} \\
\text{Clear cells} & 1 & 1/1 (100) & 0/1 (0) \quad \text{P} \\
\text{Adjuvant treatment} & 0.44 & 0.31 \quad \text{P} \\
\text{No} & 60 & 34/59 (57.6) & 18/56 (32.1) \quad \text{P} \\
\text{Yes} & 15 & 7/15 (46.7) & 2/13 (15.4) \quad \text{P} \\
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* Missing data in 1 case.

\text{Table 2. CTC detection in the mesenteric and peripheral blood of colorectal patients using Epispot and CellSearch.}

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\text{CTC detection in the mesenteric and peripheral blood of colorectal patients using Epispot and CellSearch.} & \text{Epispot} & \text{CellSearch} \quad \text{P (Wilcoxon)} \\
\hline
\text{Mesenteric blood CTCs} & 4 & 2.7 \quad \text{P} \\
\text{Median} & 0–247 & 0–286 \quad \text{P} \\
\text{Range} & 27/41 & 19/34 \quad \text{P} \\
\text{Patients, n/total} & 65.9 & 55.9 \quad \text{P} \\
\text{Patients, %} & 0.002 & \text{P} \quad \text{P} \\
\text{Peripheral blood CTCs} & 1.2 & 0 \quad \text{P} \\
\text{Median} & 0–92 & 0–147 \quad \text{P} \\
\text{Range} & 41/74 & 20/69 \quad \text{P} \\
\text{Patients, n/total} & 55.4 & 29 \quad \text{P} \\
\text{Patients, %} & 0.04 & 0.0046 \quad \text{P} \\
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\]

Table 3. Concordance between Epispot and CellSearch in peripheral and mesenteric blood.

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\begin{array}{|c|c|c|c|}
\hline
\text{CTC detection in the mesenteric and peripheral blood of colorectal patients using Epispot and CellSearch.} & \text{Epispot} & \text{CellSearch} & \text{P (Wilcoxon)} \\
\hline
\text{Peripheral blood CTCs} & 4 & 2.7 \quad \text{P} \\
\text{Median} & 0–247 & 0–286 \quad \text{P} \\
\text{Range} & 27/41 & 19/34 \quad \text{P} \\
\text{Patients, n/total} & 65.9 & 55.9 \quad \text{P} \\
\text{Patients, %} & 0.002 & \text{P} \quad \text{P} \\
\text{Peripheral blood CTCs} & 1.2 & 0 \quad \text{P} \\
\text{Median} & 0–92 & 0–147 \quad \text{P} \\
\text{Range} & 41/74 & 20/69 \quad \text{P} \\
\text{Patients, n/total} & 55.4 & 29 \quad \text{P} \\
\text{Patients, %} & 0.04 & 0.0046 \quad \text{P} \\
\hline
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\]

\[
\begin{array}{|c|c|c|}
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\text{Peripheral blood} & \text{Mesenteric blood} & \text{P (Wilcoxon)} \\
\text{Negative} & 24 & 8 \quad \text{P} \quad \text{P} \\
\text{Positive} & 24 & 12 \quad \text{P} \\
\text{Mesenteric blood} & \text{Negative} & 2 & 8 \quad \text{P} \\
\text{Positive} & 11 & 11 \quad \text{P} \\
\hline
\end{array}
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* Peripheral blood missing data in 7 cases; χ² (0.08) not significantly different from 0 (P = 0.45). Mesenteric blood missing data in 43 cases; χ² (−0.27) not significantly different from 0 (P = 0.1).
the present study between the results obtained with these 2 CTC assays. However, we admit that other factors besides the different pre-enrichment strategies may contribute to this discordance of the 2 assays, including the dilution of CTCs from the mesenteric bloodstream in the peripheral circulation, additional trapping of CTCs in the lungs, higher analytical sensitivity of the Epispot assay for EpCAM$^+$ cells, and higher mechanical vulnerability of CTCs in the peripheral blood, resulting in a more pronounced destruction of EpCAM$^+$ CTCs by the ferrofluids used in the CellSearch system. Nevertheless, we envisage that EpCAM$^+$ CTCs with an epithelial phenotype might be less flexible and stiffer than the mesenchymal EpCAM$^-$ CTCs and, owing to the mechanical blockage by small capillaries in the liver, EpCAM$^+$ CTCs could be more readily trapped in the liver than EpCAM$^-$ CTCs.

Although the analytical specificity of the CellSearch and Epispot assays have been proven in healthy control subjects (5), we recently found that patients with benign inflammatory bowel diseases can harbor substantial amounts of epithelial cells in their blood, which cannot always be discriminated from CTCs (17). The CTC markers used in these assays (and all other assays published so far) are not tumor specific but simply discriminate epithelial cells from the surrounding mesenchymal blood cells. Morphological evaluation of CTCs helps to avoid the detection of normal epithelial cells, and the patients admitted to the present study had no obvious concomitant inflammatory bowel diseases. Thus, we are quite confident that our present CTC counts represent the amount of malignant cells in the respective blood samples. However, we admit that future CTC assays should include genetic markers specific for tumor cells, and the considerable genetic heterogeneity of CRC cells suggests a multi-marker approach (18).

What is the fate of the CTC subset trapped in the liver? Different groups have investigated the critical role of the mesenteric vein in the formation of liver metastases in patients with colorectal cancer (15, 19–21). Moreover, the intraportal injection of CRC cells represents a biologically relevant and adequate method for the induction of hepatic metastasis in mice (22). However, CTCs homed in the liver might also enter apoptosis or an extended period of latency called cancer dormancy (23). One way to determine the fate of these tumor cells in patients with cancer is analysis of the correlation between tumor cell detection in the liver and metachronous development of hepatic metastases in patients who underwent surgery of the primary tumor and were initially staged M$_0$. Disseminated tumor cells have been found in hepatic biopsies in 10% of the patients operated for CRC (stage I–III), but these cells were not typed for EpCAM expression and no significant correlation was observed between the presence of tumor cells and the survival of the patients (24).

Another approach is to determine the prognostic relevance of CTCs in the mesenteric blood for the subsequent development of liver metastasis. Katsuno et al. (25) reported a meta-analysis showing the potential importance of CTC detection in the venous drainage of CRC as a prognostic indicator and a mode of staging colorectal cancers. Moreover, EpCAM has been suggested as marker for CRC-initiating cells, and it is therefore conceivable that EpCAM$^+$ CRC cells may contribute to metastatic relapse in the liver. In fact, most liver metastases express EpCAM (26), which supports its relevance in metastatic development. Even in breast cancer, EpCAM has been shown to be overexpressed in metastases (27). However, it has also been observed that EpCAM$^-$ cell fractions from a tumor have tumorigenic activity in immunodeficient mice (28); thus the importance of EpCAM expression for cancer stemness and EMT plasticity is still under debate. Recently, distinct types of CRC-initiating cells have been identified to form human colon cancer metastases, showing the complexity of their detection and targeting (29).

Interestingly, the liver architecture and complex functions may explain the implication of this organ for CRC metastasis (30). Different liver-specific elements constitute a functional microenvironment representing the microenvironment encountered by circulating colon cancer cells entering the liver as described by Vidal-Vanaclocha (30). a) The functional heterogene-
The correlation of our CTC findings with clinicopathologic risk factors revealed an inverse correlation with lymphatic emboli and CTCs, despite the fact that the presence of lymph node metastasis is a strong prognostic marker of the development of subsequent distant metastases in CRC. However, lymphatic emboli do not mean lymph node involvement, and there is no clear correlation between these 2 prognostic factors.

In the peripheral blood, CTCs have been detected in patients with a variety of metastatic cancers, including colorectal cancer, and demonstrated to be a clinically significant risk factor in patients with liver metastases (6, 21, 35–38). In contrast, the prognostic value of CTCs in early-stage CRC patients is still under debate (39). In addition to the different tumor loads in patients with early vs advanced disease, part of this discrepancy might also be because CTCs in stage M0 patients might more frequently undergo EMT than stage M1 patients and are, therefore, missed by current EPCAM-based CTC assays such as CellSearch. CTCs need to undergo the reversal of EMT, called MET, after homing in the liver to form an epithelial overt liver metastasis. This epithelial metastasis might be the source of EpCAM+ CTCs in patients with advanced-stage CRC, and these CTCs can be readily detected by
CellSearch (6). Our present follow-up analysis suggests that only peripheral blood CTCs detected with the Epispot assay tended to have a prognostic impact in stage M0 patients, whereas such an impact was not observed when CellSearch was used. However, this observation needs to be interpreted with great caution. Our preliminary results are based on the analysis of a small cohort of patients with a rather short follow-up and have therefore only an explorative character. Future prospective studies on much larger cohorts respecting the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria and testing various cutoff values need to be performed to validate the prognostic value of CTCs in early-stage CRC.

In conclusion, we have demonstrated that by using different technologies to enrich and detect CTCs, different subpopulations of CTCs could be targeted, confirming the known heterogeneity of CRC cells. In particular, EpCAM+ CTCs seem to be trapped in the liver, and CTC assays should therefore include EpCAM+ and EpCAM− subpopulations of tumor cells. Moreover, the prognostic relevance of these assays including the Epispot applied in this study needs to be examined on large cohorts of CRC patients. A specific microenvironment-related colon cancer gene signature in the liver was identified, as well as genes representing the liver prometastatic reaction, but their specific roles in the pathogenesis of hepatic metastasis is still under investigation (30). Future investigations should focus on defining the best markers of the subpopulation of functional CTCs that are the metastasis-initiating cells, defining the role of EpCAM in liver metastases formation, and identifying factors from colon CTCs able to induce the prometastatic microenvironment of the liver. In view of the controversial role of EpCAM as a potential therapeutic target in CRC (40), these investigations might have also implications for the development of future therapies.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: K. Pantel, the European Commission (DISMAL-project, contract no. LSHC-CT-2005–018911, European Research Council Investigator Grant DISSECT (no. 269081); C. Alix-Panabieres, the European Commission (DISMAL-project, contract no. LSHC-CT-2005–018911.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We are grateful to Delphine Guerout (Epispot assay), Frédérique Lorcy and Emilie Dufourcq (biobank management at the Department of Pathology) at the University Medical Centre of Montpellier, and Cornelia Coith and Oliver Mauermann (CellSearch system) at the University Medical Centre of Hamburg for their excellent assistance.

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