Abstract. The aim of this study was the detection of circulating tumor cells (CTC) in three tumor types of epithelial origin. Patients and Methods: Four hundred and thirty-eight patients with breast cancer (56.2% localized and 43.8% metastatic), 195 with colorectal tumors (84.1% localized and 15.9% metastatic) and 50 with prostate cancer (52% localized and 48% metastatic) took part in this study. CTC quantification was performed using the CellSpotter Analyzer (Veridex® LLC). Results: 31.5% of patients with cancer had ≥2 CTCs/7.5 mL but none of the healthy volunteers were above this level ($p<0.001$). Among patients with metastatic disease, 62.3% of them had ≥2 CTCs/7.5 mL but only 14.0% of those with localized disease were above this level ($p<0.001$). The presence of CTCs were correlated to stage in the three studied tumor types and no differences in the number of cells were found between them. Conclusion: The presence of more than 2 CTCs/7.5 ml is a frequent event in metastatic cases. In particular, patients with localized disease who have more than 2 CTCs/7.5 ml should be carefully studied to determine the possible prognostic and predictive value of this finding.

Circulating Tumor Cells in Solid Tumor in Metastatic and Localized Stages

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Cancer metastasis is the result of several interacting processes at the end of which cancer cells survive in distant organs. Penetration of primary tumor cells into blood and lymph vessels is central to the whole phenomenon of metastasis and is a common step in all cancer types. The presence of circulating tumor cells (CTCs) in patients with tumors may be a surrogate marker of metastatic disease. Nevertheless, it should be taken into account that the interaction of a cancer cell with host defences and the microenvironment at distant tissues may result in its death and thus the process of metastasis is not successfully completed. The metastatic potential of primary tumors differs among different tumor types and even in a specific tumor not all cells are able to successfully disseminate. The presence of CTCs might be more linked with genotypic characteristics of the tumor cell rather than a sign of tumor burden, as the study of Braun et al. suggested (1).

The detection of these cells in peripheral blood could have clinical usefulness in three aspects: i) as evidence of early dissemination, and then as a risk factor of clinical recurrence in tumors apparently limited to the primary organ; ii) as a relevant risk factor for metastatic progression and worse prognosis; and iii) as a predictive marker of response to treatment (2, 3).

The determination of CTCs can be performed by means of different techniques which, in most cases, include the enrichment of tumor cells with immunomagnetic particles coated with antibodies specific for epithelial antigens. The identification of these isolated cells can be performed by immunohistochemistry or by the analysis of the genetic expression of cytokeratins and tumor antigens (4). The CellSearch detection system represents the first automated and standardized technology that has been approved by the US Food and Drug Administration (FDA) for the detection of CTCs in metastatic breast cancer (5). Subsequently this system was also approved by the FDA for CTC detection in patients with prostate and colon tumors. This technique is reproducible and allows the use of standard protocols for sample preparation and staining and for the interpretation of results. Allard et al. evaluated the accuracy, reproducibility, and linearity of this new detection system and they concluded that this technique has a good reproducibility profile (6). Riethdorf et al. performed a multicentric study to validate the CellSearch technology in patients with metastatic breast cancer and they confirmed its reliability for the assessment of therapeutic effectiveness (7).

A prospective study on the incidence according to the clinical stage and prognostic significance of CTC detection in three different tumor types is being carried out at our
Patients and Methods

Patients. In this study, 789 blood samples were evaluated from 683 patients with breast, colon or prostate cancer, and 106 healthy volunteers. The inclusion criteria for healthy volunteers were: no evidence of disease at the moment of blood extraction, age under 35 years and no personal history of tumor pathology. In cancer patients, blood samples were collected before systemic therapy in those cases with metastatic disease and at least three weeks after the surgical operation and before receiving adjuvant treatment (if indicated) for those with only local disease. In all cases, pathologic diagnoses were made. The project was favourably validated by the Ethics and Clinical Investigation Committee of our hospital. Tumors with M0 classification according to the TNM Staging System (localized primary tumor with or without lymph node metastasis and with no distant metastasis) were considered as localized and M1 (tumor with distant metastasis) as metastatic. A total of 438 patients diagnosed with breast cancer (56.2% in localized stages and 43.8% metastatic), 195 with colorectal tumors (77.4% localized and 22.6% metastatic) and 50 with prostate cancer (52% localized and 48% metastatic) entered into the study. Overall, 36.1% of the selected patients had metastases (247/683).

Circulating tumor cell detection. Blood collection was carried out in a 10 ml tube with EDTA and cellular preservative (CellSave tubes; ImmIcon Corporation®, Pennsylvania, USA) and samples were stored for a maximum of 72 hours at room temperature before their processing. Blood (7.5 ml) was then added to a ferrofluid of Immunicon Corporation®, Pennsylvania, USA) for Windows v.11.5 software. The isolated cells were immunomagnetic particles coated with antibodies of an anti-phenylindole (DAPI) for the cellular nucleus, and labelled with fluorescently stained with the nucleic acid dye 4',6-diaminodino-2-epichrome (CK-PE). They were monoclonal antibodies specific for leukocytes (CD45) or epithelial cellular adhesion molecule (EpCAM). The isolated cells were stained nucleus inside of the cytoplasm. The results were expressed as the number of CTCs per 7.5 ml of blood. The samples were processed in a blinded manner and result interpretation was independently accomplished by five specialists specifically trained and with no information about the clinical status of patients. Every sample was reviewed at least by two of the specialists to avoid inter-reader variations.

Statistical analysis. The association between qualitative variables was evaluated using the Chi-square test and, when more than 25% of the expected frequencies fell bellow 5, Fisher’s exact test was applied instead. A receiver operating characteristic (ROC) curve of the CTC value was constructed to determine the cut-off point to discriminate between localized and metastatic cases. The sensitivity and specificity as well as the area under the curve with its 95% confidence interval (CI) are presented. The stratified analysis was performed according to tumor type. In every test the null hypothesis was rejected with a type I error of 5%. The analysis was performed using SPSS (Statistical Product and Service Solutions, Illinois, USA) for Windows v.11.5 software.

Results

In the healthy volunteer group, 100% of the individuals showed fewer than 2 CTCs/7.5 ml, 18 out of 106 had 1 CTC (17%) (Table I). The number and percentage of samples with <2, 2-4, 5-9, 10-49 and ≥50 CTCs/7.5 ml are shown in Table I. According to our results, and considering the CTC values observed in healthy individuals, we established the cut-off point at 2 CTCs/7.5 ml to set the limit of pathological detection. This criterion is in agreement with the cut-off chosen by Allard et al. (6). All the statistical analyses performed here used this cut-off point (Table II). Overall, 31.5% of patients with cancer had more than 2 CTCs/7.5 ml, which is significantly higher compared with healthy volunteers (p<0.001). According to disease stage (localized

Table I. Number of circulating tumor cells per 7.5 ml of blood of patients with breast, colon and prostate tumors, and of healthy volunteers.

<table>
<thead>
<tr>
<th></th>
<th>No. of Mean SD Median P25 P75 Range</th>
<th>&lt;2</th>
<th>2-4</th>
<th>5-9</th>
<th>10-49</th>
<th>≥50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls Healthy</td>
<td>106</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>Patients Tumor</td>
<td>683</td>
<td>28</td>
<td>169</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Breast</td>
<td>438</td>
<td>35</td>
<td>203</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Colon</td>
<td>195</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Prostate</td>
<td>50</td>
<td>58</td>
<td>155</td>
<td>1</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Stage Localized</td>
<td>436</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0-86</td>
</tr>
<tr>
<td>Metastatic</td>
<td>247</td>
<td>74</td>
<td>274</td>
<td>4</td>
<td>0</td>
<td>37</td>
</tr>
</tbody>
</table>

P25, 25th percentile; P75, 75th percentile.
vs. metastatic), CTCs were present in 62.3% of patients with metastatic disease (regardless of tumor type), whereas only in 14% of patients with localized disease were they present ($p<0.001$). With regard to the analysis carried out for each tumor type, 61.5% of patients with metastatic breast tumors had ≥2 CTCs/7.5 ml and 13.0% of the localized cases ($p<0.001$). In colorectal carcinomas, 61.3% of patients with metastasis had ≥2 CTCs/7.5 ml, but only 15.2% in localized stages ($p<0.001$). In prostate tumors, the difference between the patients with metastatic and localized stages was also statistically significant (70.8% vs. 15.4%) ($p<0.001$) (Table II). The analysis of CTCs among the differences tumor types and according to the tumor stage revealed no statistically significant differences between breast, colon and prostate tumors, neither in localized nor in metastatic stages ($p<0.001$). Significant differences between CTCs in patients with breast and those with colon metastatic tumors were observed when considering 50 CTCs as the cut-off point (24.0% vs. 3.2%). The same occurred for metastatic colon and prostate tumors with 10 CTCs (22.6% vs. 58.3%) and 50 CTCs (3.2% vs. 41.7%) (Table II). ROC curves were constructed to determine the cut-off value which better discriminated between localized and metastatic stages. ROC curves confirmed the cut-off point for the three types of tumors to be ≥2 CTCs/7.5ml. The area under the curve for breast cancer cases was 0.76 (95% CI=0.72-0.81), for colorectal cancer was 0.79 (95% CI=0.69-0.89) and for prostate tumors was 0.84 (95% CI=0.72-0.85).

**Discussion**

The detection of CTCs has been pointed out as a potential useful prognostic factor and of predictive value for tumor recurrence and response to treatment. For this reason, several attempts have been made to find a reliable method to measure them. The latest methods for CTC detection have included cytometry with immunocytochemical labelling and immunomagnetic enrichment coupled to DNA and RNA technologies. The only automated and highly reproducible system available so far is the one used in this study and combines immunomagnetic enrichment and immunofluorescent labelling. According to the criteria applied in our

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Tumor stage</th>
<th>&lt;2 (%)</th>
<th>≥2 (%)</th>
<th>$p$-Value</th>
<th>&lt;5 (%)</th>
<th>≥5 (%)</th>
<th>$p$-Value</th>
<th>&lt;10 (%)</th>
<th>≥10 (%)</th>
<th>$p$-Value</th>
<th>&lt;50 (%)</th>
<th>≥50 (%)</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Localized</td>
<td>214 (87.0)</td>
<td>32 (13.0)</td>
<td>$&lt;0.001$</td>
<td>234 (95.1)</td>
<td>12 (4.9)</td>
<td>0.81</td>
<td>241 (98.0)</td>
<td>5 (2.0)</td>
<td>$&lt;0.001$</td>
<td>245 (99.6)</td>
<td>1 (0.4)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Metastatic</td>
<td>Localized</td>
<td>74 (38.5)</td>
<td>118 (61.5)</td>
<td>98 (51.0)</td>
<td>94 (49.0)</td>
<td>114 (59.4)</td>
<td>78 (40.6)</td>
<td>146 (76.0)</td>
<td>46 (24.0)</td>
<td>$&lt;0.001$</td>
<td>164 (100)</td>
<td>0 (0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Colon</td>
<td>Localized</td>
<td>139 (84.8)</td>
<td>25 (15.2)</td>
<td>$&lt;0.001$</td>
<td>157 (95.7)</td>
<td>7 (4.3)</td>
<td>0.56</td>
<td>114 (59.4)</td>
<td>78 (40.6)</td>
<td>0.07</td>
<td>146 (76.0)</td>
<td>46 (24.0)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Metastatic</td>
<td>Localized</td>
<td>12 (38.7)</td>
<td>19 (61.3)</td>
<td>18 (58.1)</td>
<td>13 (41.9)</td>
<td>24 (77.4)</td>
<td>7 (22.6)</td>
<td>30 (96.8)</td>
<td>1 (0.5)</td>
<td>$&lt;0.001$</td>
<td>164 (100)</td>
<td>0 (0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Prostate</td>
<td>Localized</td>
<td>22 (84.6)</td>
<td>4 (15.4)</td>
<td>$&lt;0.001$</td>
<td>26 (100)</td>
<td>0 (0)</td>
<td>$&lt;0.001$</td>
<td>26 (100)</td>
<td>0 (0)</td>
<td>$&lt;0.001$</td>
<td>26 (100)</td>
<td>0 (0)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Metastatic</td>
<td>Localized</td>
<td>7 (29.2)</td>
<td>17 (70.8)</td>
<td>10 (41.7)</td>
<td>14 (58.3)</td>
<td>10 (41.7)</td>
<td>14 (58.3)</td>
<td>14 (58.3)</td>
<td>10 (41.7)</td>
<td>$&lt;0.001$</td>
<td>26 (100)</td>
<td>0 (0)</td>
<td>0.16</td>
</tr>
</tbody>
</table>
technology, a CTC is a round to oval cell, immunomagnetically selected with antibody anti-EpCAM with nucleus (DAPI stained) and a cytoplasm positive for antibody anti-CK 8, 18 and 19 and negative for anti-CD45. These markers have proven to be valid for the detection of epithelial cells arising from different tumor types in peripheral blood (6, 7-10).

Our results of CTCs detection in healthy volunteers confirm those reported by Allard et al. (6). A threshold of ≥2 CTCs/7.5 ml discriminates between cancer patients and healthy people. Nevertheless, it does not mean that this is the appropriate cutoff when used as prognostic factor or for monitoring response to treatment. In fact, a threshold of 6 CTCs and 3 CTCs have been established in metastatic breast and colon cancer respectively when used as a predictive factor for response to chemotherapy (9, 11). The presence of CTCs correlated with stage: with 62.3% of patients with metastatic tumors having ≥2 CTCs/7.5 mL in contrast to those with the localized tumors at 14.0%. At the moment, we do not have enough surveillance of our patients to determine if the presence of CTCs in non-metastatic stages is a surrogate marker of metastatic disease, but in the near future, CTC determination may be of help to select people at high risk of recurrence. Adjuvant treatment with hormonal therapy or chemotherapy is sometimes not indicated in early stages of these tumors because of the low expected rate of recurrence. CTC determination could be a simple test to select patient candidates for adjuvant treatment if the prognostic value for recurrence can be demonstrated.

Allard et al. found in their study of metastatic tumors that the presence and the number of CTC varied depending on the tumor type. They suggested that these differences can be explained by differences in tumor vascularization, sites of metastasis and tumor aggressiveness (6). However, we did not observe any significant differences in the CTC quantification among breast, colon and prostate tumors, when stratifying by stage and using a threshold of 2 CTCs. The percentage of patients with CTC in localized and metastatic stages was similar in the three studied tumor types (localized stages of breast 13%, colon 15.2% and prostate 15.4% and metastatic stages of breast 61.5%, colon 61.3% and prostate 70.8%). It should be taken into account that all three tumor types tend to develop hematogenous metastasis frequently, even in apparently early stages. It would be interesting to evaluate the presence of CTCs in patients with tumors with low risk of distant dissemination, such as of the head and neck, or endometrial carcinoma. The differences between localized and metastatic stages were still significant when thresholds of 5, 10 or 50 CTCs/7.5 ml was considered. There is one exception, in that for patients with colon tumors, the 50 CTCs limit could not be evaluated because only one patient had more than 50 CTCs/7.5 ml. We found that the cut-off point which better discriminates between localized and metastatic stages was 2 CTCs/7.5ml and this threshold was applied in the analyses for the three types of tumors. The mean number of CTCs (±SD) observed in localized tumors was 1 (±5) /7.5 ml and the maximum was 86 CTCs. In some patients at metastatic stages, no CTCs were detected (93/247). We excluded patients under systemic therapy to avoid the effect of therapy on CTCs.

Another possibility to take into account is that cancer cells might not continuously pass into blood vessels. To test this hypothesis, we carried out an assay to examine the CTC release from the metastatic sites in patients with metastatic breast cancer, and we observed no circadian pattern but a constant flow throughout the day (11). In some cases, a preferential pattern of dissemination throughout the lymphatic vessels may explain the absence of CTCs from the bloodstream. Several studies have recently been reported which used the CellSearch system in patients with metastatic breast cancer. Overall, the percentage of patients with >5CTCs/7.5 ml ranged from 24 to 45% (6, 7, 12, 13). Different studies have shown that CTCs in patients with metastatic breast cancer are related to disease-free survival (DFS) and with overall survival (OS), as well as with prediction of therapeutic response (14). No studies in patients with localized tumors have been carried out to date. Regarding our population of patients with colorectal carcinoma, 61.3% of those with metastatic tumors had ≥2 CTCs/7.5 ml. Allard et al. studied 196 patients with metastatic colorectal carcinoma and 30% had ≥2 CTCs/7.5 ml. This percentage is significantly lower than that observed in our population (6). In a recent study of 456 metastatic colorectal cancer patients, Meropol et al. demonstrated that the number of CTCs evaluated using the CellSearch system is an independent prognostic factor for OS and DFS (9). We are the only group with published results of CTC in localized stages of colorectal cancer using the CellSearch system. In a previous study, we found a correlation between the number of CTCs and the tumor stage in this tumor type (8). In our experience, 70.8% of patients with metastatic prostate tumors had ≥2 CTCs/7.5 ml and 41.7% ≥50 CTCs/7.5 ml. Shaffer et al. quantified CTCs using the CellSearch system in 59 patients with metastatic prostate cancer: 84.7% presented ≥2 CTCs/7.5 ml and 30.5% ≥50 CTCs/7.5 ml (10). Allard et al. studied 123 patients with metastatic prostate tumors: 57% had ≥2 CTCs/7.5 ml and 14% ≥50 CTCs/7.5 ml (6).

In conclusion, CTC detection by the CellSearch system is an easy and highly reproducible method that deserves more evaluation in epithelial tumors. A cut-off of ≥2 CTCs can be considered as pathological, although the threshold for prognostic significance may differ among the different tumor types. The number of CTCs detected was significantly higher in the metastatic stage compared with earlier stages, and no differences were found among epithelial tumors with high affinity for hematogenous dissemination such as breast, colon and prostate adenocarcinomas (15). A longer follow-up is necessary in our series to assess the prognostic value of the presence of CTCs and the predictive value for response to systemic therapy.
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References