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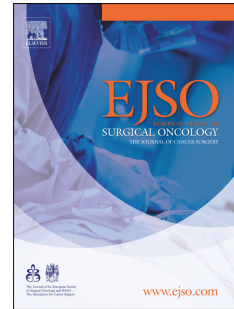
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PERIOPERATIVE TUMOR CELL DISSEMINATION IN PATIENTS WITH  
PRIMARY OR METASTATIC COLORECTAL CANCER

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**ABSTRACT**

**Introduction:** Although there is general correlation between TNM stage of colorectal cancer (CRC) and its prognosis, there is often significant variability of tumor behaviour and individual patient outcome, which is unaccounted for by pathologic factors alone. Our aim was to estimate the perioperative tumor cell dissemination in patients with primary or CRC liver metastases as a possible factor influencing the outcome. **Methods:** Forty patients were prospectively enrolled in the study from year 2007 to 2008. Eighteen patients had histologically proven CRC (50% rectal, 44% colonic, 6% colonic and rectal). Sixteen patients (47%) had CRC liver metastases only. The remaining six patients who underwent colon or liver resection for benign conditions, acted as the control group. All patients with malignant pathologies had R0 resections. Blood samples were taken before the surgical incision (T0), immediately after the tumor resection (T1) and at the end of the surgical intervention (T2). Data acquisition was performed using a dual-laser FACSCalibur flow cytometry. Circulating malignant cells were identified as being CD45-/Cytokeratin+.

**Results:** The analysis of patients overall (CRC resection subgroup and hepatectomy subgroup) revealed that there was no statistically significant difference of the tumoral cell count in the blood per/million of hematopoietic cells at T0, T1 and T2. **Conclusions:** This study demonstrates no differences in the detected circulating numbers of tumor cells at different stages of surgical intervention.

**Key words:** Colorectal cancer, circulating tumor cells, flow cytometry detection

## Introduction

Colorectal cancer (CRC) is a common and often life-limiting disease. Approximately 20-45% of patients with CRC undergoing curative resection subsequently develop local recurrence or metastatic disease in lymph nodes, liver, lung and peritoneum [1, 2].

Although there is general correlation between TNM stage and prognosis, there is often significant variability of tumor behaviour and individual patient outcome, which is unaccounted for by pathologic factors alone. The detection of lymph node (LN) metastases constitutes the most important prognostic factor in CRC and as the primary indicator of disease spread, LN status determines the choice of postoperative adjuvant chemotherapy. However, the limitations of the TNM staging are emphasised by the considerable prognostic heterogeneity of patients within a given tumor stage (not all patients with LN-negative tumors are cured and not all patients with LN-positive tumors die from their disease). This resulted in a number of efforts to develop more accurate staging protocols [3-6].

Tumor progression after curative resection of CRC is caused by tumor cell dissemination, currently undetected by standard clinical staging techniques. The detection of disseminated tumor cells could help to identify a subgroup of patients at risk for disease relapse that could benefit from adjuvant therapy [6,7]. In this context, flow cytometry is one of the methods used to identify subgroups at risk for disease relapse. Flow cytometry allows an accurate quantification of cells and at the same time a multi-parameter characterization of each cell present in blood samples. This technique has become widely used in the detection of rare events, such as minimal residual disease in haematological malignancies. Disseminated CRC cells have immunophenotypic characteristics distinct

from those of haematopoietic cells, which allow their identification and quantification in peripheral blood by flow cytometry.

The aim of the study was to determine whether resection of the primary tumor or CRC liver metastases would lead to an increased dissemination of cancer cells.

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## Patients and Methods

### *Patients*

This prospective study included 40 patients who had undergone surgery in our institution from year 2007 to 2008. There were 24 male patients with a median age and 16 female patients with a mean age of  $64 \pm 10$  (range: 41-90).

Eighteen patients had histologically proven colorectal cancer (50% rectal, 44% colonic, 6% colonic and rectal). Sixteen patients had colorectal liver metastases only. The remaining six patients, which acted as the control group had benign conditions (sigmoid diverticulosis, hepatic adenoma and hemangioma).

All patients were preoperatively staged by biological and radiological examinations. In addition, patients with rectal cancer received a mandatory magnetic resonance imaging (MRI). Similarly, MRI was performed also for patients with non-conclusive computed tomography imaging (CT scan) concerning liver metastases originating from either rectal or colonic primary tumors.

Seven patients diagnosed with locally advanced rectal cancer (T3, T4, LN positive) received preoperative radio-chemotherapy.

All patients operated for malignancy had R0 resections, performed according to international standards with at least 10 cm resection margins for colonic tumors or a distal resection margin of at least 2 cm associated with pericolic, perirectal and perivascular truncal lymphadenectomy for rectal cancers. High vascular ligation and no touch technique was used for all resections. Similarly, in the group of patients with liver metastases, the liver resection was performed with at least 1 cm surgical margin.

Liver interventions included: right hepatectomy (n=4), extended left hepatectomy (n=1), left hepatectomy (n=1), central hepatectomy (n=1), left lobectomy (n=4),

bissegmentectomy (n=3), segmentectomy (n=2) and sub-segmentectomy (n=4). Of the four patients who had left lobectomy, two of them had associated right hemicolectomy.

The colorectal procedures included: total coloproctectomy with ileostomy (n=2), total colectomy (n=2), subtotal colectomy (n=1), right hemicolectomy (n=1), left hemicolectomy (n=3), anterior resection (n=6), anterior resection with ileostomy (n=1) and lastly abdominal perineal resection (n=2).

#### *Blood sampling and processing*

Blood was sampled using central venous catheters before placing the surgical incision (T0), immediately after the tumor resection (T1) and at the end of the surgical intervention (T2) and each time the volume of 12 ml was obtained. As negative control, venous blood specimens were collected from the six patients who underwent surgery for benign conditions before (T0), during (T1) and after the surgery (T2).

Peripheral blood samples were centrifuged at 540 x g for 10 minutes, the buffy coat was collected and placed in a 50 ml tube. Fixative-free NH<sub>4</sub>Cl lysing solution was added to each tube (10ml of lysing solution per 1ml of buffy coat) and the samples were incubated for 45 minutes at room temperature (RT) to lyse the remaining red blood cells.

After the incubation period, the samples were centrifuged at 540 x g for 10 minutes. The supernatant was discarded and the cell pellet washed with 10 ml of phosphate-buffered saline (PBS, Gibco; Paisley, Scotland, UK) (540 x g for 10 minutes).

The cell pellet was stained with anti-CD45 (clone 30-F11, BDB, San Jose, CA, USA) conjugated with peridinin chlorophyll protein cyanine 5.5 (PerCP Cy5.5) and anti-Cytokeratin (clone MNF116, DakoCytomation, Glostrup, Denmark) conjugated with fluorescein isothiocyanate and incubated in the dark at RT for 30 minutes. At last, the



samples were washed twice in 10 ml of PBS and the cell pellet resuspended in 1 ml of PBS.

Data acquisition were performed in a dual-laser FACSCalibur flow cytometer (BDB, San Jose, CA, USA) using the CellQuest software (BDB, San Jose, CA, USA). First, a total of 20.000 events, corresponding to all nucleated cells in the sample, was stored. To increase the sensitivity of the technique, in a second step of acquisition, only Cytokeratin+ cells were acquired using an electronic live gate.

Data analysis were performed in the Paint-a-Gate Pro software (BDB, San Jose, CA, USA). Circulating CRC cells were identified as being CD45-/Cytokeratin+. The monoclonal antibody anti-cytokeratin used recognized an epitope present in keratin 5, 6, 8 and 17.

#### *Statistical analysis*

Continuous data was presented as mean and standard deviation (SD). Skewed and non-gaussian continuous data were analyzed using non-parametric tests (Mann-Whitney U test or the Kruskal-Wallis test, whenever there were two, three or more samples to compare); Chi-square test was applied for purpose of comparing proportions. Statistical analysis was performed using Statistica®, version 7. A P value of 0.05 was considered as statistically significant.

#### *Ethics*

Written informed consent was obtained for all enrolled patients. The protocol of the study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and also with the guidelines of our institution.

## RESULTS

### *Patients of the control group*

In this group of patients (n = 6), none of the studied tumor markers were detected at any of the mentioned surgical times (T0, T1 and T2).

### *Patients with colorectal cancer or CRC liver metastases*

We analyzed 102 blood samples from 34 patients with malignancy and detected circulating cancer cells in 31 blood samples from 34 patients who underwent curative surgical resection.

The median value of the tumoral cell count in the blood per million of hematopoietic cells was  $7 \pm 7$  (Range 0 - 26) at T0 (before the placement of surgical incision). At T1 (immediately after the removal of the specimen) the tumoral cell count in the blood per/ million of hematopoietic cells was  $4 \pm 4$  (Range 0 – 13) and lastly at T2 (end of the surgical intervention) the tumoral cell count in the blood per million of hematopoietic cells was  $4 \pm 6$  (Range 0 - 26). However, this value was statistically not significant ( $p < 0.501$ ).

### *Patients with colorectal cancer*

In this subgroup of patients the median value of the tumoral cell count in the blood per/million of hematopoietic cells was  $6 \pm 8$  (Range 0 - 26) at T0. Instead, at T1 the tumoral cell count in the blood per million of hematopoietic cells was  $2 \pm 2$  (Range 0 - 6) and lastly at T2 the tumoral cell count in the blood per/ million of hematopoietic cells was  $3 \pm 4$  (Range 0 – 10). Similar to the overall group of patients the value of tumoral cell count at T2 was statistically non significant ( $p < 0.141$ ).

### *Patients with colorectal liver metastases*

The analysis in this subgroup of patients revealed that the median value of the tumoral cell count in the blood per million of hematopoietic cells was  $9 \pm 4$  (Range 0 - 14) at T0. Instead at T1 the tumoral cell count in the blood per million of hematopoietic cells was  $7 \pm 4$  (Range 0 - 13) and lastly at T2 the tumoral cell count in the blood per million of hematopoietic cells was  $6 \pm 9$  (Range 0 - 26), statistically not significant ( $p < 0.088$ ).

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## DISCUSSION

In this study we have investigated the hypothesis that curative resection of the primary or secondary colorectal tumor can lead to shedding of malignant cells into the peripheral circulation. Our results obtained by using the flow cytometry with the pancitokeratin antibody would suggest that such procedures are not associated or do not disseminate tumoral cells in the peripheral blood.

### *Prognostic factors and management of colorectal cancer*

Tumor progression can result from disseminated tumor cells in lymph nodes, blood or bone marrow, sites which are not detected by current staging methods. The objective of adjuvant therapy is to eradicate the viable disseminated tumor cells, thereby decreasing disease relapse and improving patients' survival [8]. Candidates for post-operative adjuvant therapy are usually patients at high risk for disease relapse, as judged by current clinical and pathological staging. In the group of patients without distant metastases, lymph node metastases are the most important prognostic factor [9]. Consequently, adjuvant chemotherapy is recommended for patients with positive lymph nodes. For patients with colon cancer stage I or II, adjuvant chemotherapy cannot achieve a survival benefit, and thus, adjuvant therapy is not recommended for these patients. Although considered at low risk, 10% to 20% of patients with colorectal cancer stage I and II ultimately will develop recurrent disease [10,11]. It is in this population that prognostic markers may identify a subgroup of patients who are at a higher risk for disease relapse and who may also benefit from adjuvant therapy, especially from antitumoral agents with low systemic toxicity such as monoclonal antibodies, which have also proven to be effective against dormant tumor cells [6,7]. In this regard, there are several studies that have demonstrated that tumor cell

detection is clearly related to an early relapse and decreased survival of the respective patients [6,7,12,13]. This new prognostic factor may change the surgical management of patients with colorectal liver metastases and may help to individualize the treatment of these patients with systemic or regional chemotherapy [14].

#### *Flow cytometry in detection of disseminated colorectal cancer cells*

Among the different approaches to screen disseminated colorectal cancer cells in bone marrow aspirates, peripheral and mesenteric venous blood, the immunocytochemistry is the most widely used method. This method has the advantage to allow cell morphology characterization, but presents low sensitivity [6,15,16]. RT-PCR based protocols have further improved the sensitivity and specificity of detection systems for disseminated cancer cells, allowing the identification of approximately of one neoplastic cell in  $10^7$  normal peripheral mononuclear blood cells [17].

Several studies have proved that the sensitivity of flow cytometry is similar to PCR's, ranging from  $10^{-4}$  to  $10^{-5}$  [16,19,20]. In a study based on serial dilutions of breast cancer cells in normal peripheral blood, showed that, using the appropriated markers, flow cytometry presented a sensitivity ranging between  $10^{-6}$  and  $10^{-7}$  [21]. Despite this, flow cytometry is not widely used in the detection of disseminated tumor cells in peripheral blood or bone marrow samples, probably because of the limited information in the literature regarding the phenotype of these cells. Because of the absence of tumor-specific target antigens, the disseminating tumor cells are identified based on the expression of epithelium-specific antigens such as cytoskeleton-associated cytokeratins, surface adhesion molecules, or growth factor receptors, whose quantitative expression obtained by flow cytometry are not well documented. Moreover, cytokeratin expression might vary along

malignant transformation and different tumor stages [21,22]. Other reasons that have limited the use of flow cytometry in this field are the absence of consensus for the reagents and methods applied, the need for technical expertise and the long period required for sample acquisition, in order to obtain a large number of cells to achieve an acceptable sensitivity.

However, flow cytometry allows an accurate quantification of cells and enable the immunophenotypic characterization of each cell in the sample. Besides the improvement of flow cytometer technology, the development of new high-speed flow cytometers allowing the acquisition of 20 000 events per second, has reduced dramatically the acquisition period. Altogether these factors make the flow cytometry an attractive method for the quantification of rare events. This technique has become a method of choice for the detection of minimal residual disease in hematological malignant neoplasms [17,19,23,24] and, more recently, an important tool in quantifying other rare events such as circulating endothelial cells, circulating progenitor cells [19,25], mesenchymal stem cells [25] and disseminated tumor cells from CRC [26], small and non-small lung cancer [26], prostate cancer [27] and rhabdomyosarcoma [28].

In this study, we used an anti-pancytokeratin monoclonal antibody able to recognize an epitope present in cytokeratin 5, 6, 8 and 17; and anti-CD45 monoclonal antibody. The analysis of the expression of both pancytokeratin and CD45 together with light scatter properties, enabled us to distinguish between non-hematopoietic cells (pancytokeratin+/CD45-) and peripheral blood cells (pancytokeratin-/CD45+). However, before we proceeded to the quantification of disseminated CRC cells in peripheral blood, we evaluated pancytokeratin expression in tumor cells from tumor biopsies and verified the presence of two distinct populations: the first with a low pancytokeratin expression and the

second with a higher expression. Based on previous proteomic studies in CCR showing a heightened cytokeratin 8 expression in tumor tissue compared to normal mucosa from the same individual, we assume that only those cells with higher pancytokeratin expression were CRC cells [29;30]. Therefore, when peripheral blood samples were analysed, we only considered as circulating CRC cells those with characteristic light scatter properties, CD45- and with high pancytokeratin expression.

In our results, none of the samples from patients with benign conditions who had surgical resection demonstrated the evaluated tumor markers. However, these tumor markers are not limited only to the gastrointestinal epithelium, as they have been found in a variety of cell types including urothelial, Merkel cells and leucocytes 1982 [31]. On the other hand, several studies have also reported that in terms of molecular screening of circulating blood, the expression of several tumor markers is limited to patients with colorectal cancer with no expression seen in controls [32,33].

### *Conclusion*

This prospective study by using the flow cytometry (a very specific and sensitive technique) to detect circulating tumor cells, demonstrated no differences in the circulating numbers of tumor cells were detected at different times of the surgical intervention. These results would lead to the logical questions: what is the impact of the “no touch” technique in the oncological outcome of the patients with primary colorectal cancer or liver metastases? Do we need to use perioperative adjuvant therapy or to change the surgical strategies to prevent intraoperative tumor cell shedding?

Our study does not suggest so, however, further studies should be performed to answer the raised questions.

**Conflict of interest**

Concerning the present manuscript, the authors declare that there are no personal, financial or non-financial competing interests or conflicts.

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