Circulating endothelial cells and endothelial progenitors as predictive markers of clinical response to bevacizumab-based first-line treatment in advanced colorectal cancer patients

M. Ronzoni1*, M. Manzoni2, S. Mariucci2, F. Loupakis4, S. Brugnatelli2, K. Bencardino1, B. Rovati2, C. Tinelli3, A. Falcone4, E. Villa1 & M. Danova2

1Medical Oncology, Scientific Institute S. Raffaele, Milano; 2Medical Oncology; 3Biometry and Clinical Epidemiology Unit, Foundation IRCCS Polcínico S. Matteo, Pavia; 4Medical Oncology 2, Department of Oncology, University Hospital of Pisa, Pisa, Italy

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Background: Despite the consistent clinical results demonstrated by studies on anti-angiogenic drugs targeted against the vascular endothelial growth factor in metastatic colorectal cancer (mCRC) patients, no specific direct/indirect biomarker of their efficacy has been validated. In this field, circulating endothelial cells (CECs) and endothelial progenitor cells (CEPs) have recently been proposed as noninvasive biomarkers.

Patients and methods: The absolute numbers of CEPs, total CECs (tCECs) and their resting (rCECs) and activated subsets were evaluated by multiparameter flow cytometry in 40 mCRC patients at baseline and before the administration of the third and sixth course of a bevacizumab-based first-line treatment. Fifty healthy subjects were utilized as control.

Results: The overall response rate was 80%, overall clinical benefit was 90% and median progression-free survival (PFS) was 13.8 months. In our patients, tCECs and rCECs were significantly increased compared with healthy subjects. The patients who achieved a radiological response showed, at baseline, a significant decrease of rCECs and a trend in decrease of tCECs in comparison with patients not achieving response. Finally, a baseline absolute number of tCEC and rCEC <40 cells/ml was evidenced in patients with a longer PFS. No correlation was found regarding CEP.

Conclusions: Our study suggests significant correlations between both tCEC and rCEC baseline levels and the antitumor efficacy of a bevacizumab-based combination therapy in mCRC patients, thus confirming that these biomarkers could be used in the clinical setting as an early predictor of tumor response.

Key words: anti-angiogenic therapy, biomarkers, circulating endothelial cells, clinical outcome, colorectal cancer

introduction

The process of neoangiogenesis [1] is one of the key mechanisms involved in neoplastic transformation and tumor growth. The crucial regulator of this process is the vascular endothelial growth factor (VEGF), overexpressed in many tumors and in particular in 40%–60% of colorectal cancer, where its level is correlated with intratumoral vascular density and progression of disease [2, 3]. Although the introduction into clinical practice of anti-angiogenic drugs targeted against VEGF, such as the monoclonal antibody (mAb) bevacizumab (Avastin®, Basel, Switzerland), has shown consistent clinical results in metastatic colorectal cancer (mCRC) patients [4], to date no specific direct/indirect biomarker of their efficacy has been validated. In fact, the predominantly cytostatic action of these compounds makes it an inadequate assessment of their activities as it is based only on reduction of the tumor and may not reflect the true biological efficacy. Many potential biomarkers, both tumor and systemic, are under evaluation in clinical trials, but no one has been able to carry out a close monitoring of the vascular structure of a particular cancer during the various clinical stages and in relation to treatment undertaken [5, 6].

In this field, the multiparameter flow cytometric evaluation of absolute number and viability of circulating endothelial cells (CECs) and their progenitors (CEPs) could represent a novel and easily applicable predictive and prognostic approach for the selection of responder patients and the monitoring of benefit over time [7–11].

Rare in healthy individuals, CECs are shed from vessel walls and enter the circulation reflecting endothelial damage or dysfunction. Increased numbers of different types of CECs have
been documented in cancer patients without specific treatment, and their number appears to correlate with the progression of the tumor [12]. CEPs, which derive from bone marrow rather than from vessel walls, are mobilized following tissue ischemia and may be recruited to complement local angiogenesis supplied by existing endothelium [13, 14].

In previous experiences in breast cancer patients treated with metronomic therapy, an approach that in preclinical studies appears to have an important anti-angiogenic effect, baseline number and the kinetic modifications in number and viability of CEPs, CECs and their subsets (as activated and resting) have predictive value for the response to therapy [15, 16]. Similar results have been found when bevacizumab was combined with chemotherapy (CT) and hormonotherapy [17].

Currently, no data are available regarding the modification in number of these cells in patients with mCRC treated with bevacizumab or their possible role as biomarker.

The aim of our study was to determine the baseline values and the modification of absolute number of total CECs (tCECs), their subsets (resting—rCECs and activated—aCECs) and CEPs in patients with mCRC treated with first-line bevacizumab-based antineoplastic therapy and to

Figure 1. Flow cytometric dot plot panels show representative pictures relative to the gating strategy utilized for the identification of mature circulating endothelial cells (CECs), both resting and activated (rCECs and aCECs) and circulating progenitor endothelial cells (CEPs). (A) The analysis gate used to exclude platelets and debris (panel a1) and the gate used to exclude hematopoietic cells expressing the CD45 antigen versus side scatter (SSC) (panel a2). (B) The dot plots to determine CECs (with rCECs and aCECs subsets) and CEPs. In detail, panel b1 shows the gate restricted to a CD34+/CD133— population, used to evaluate mature CECs (panel b2). Panel b3 shows the expression of antigens used to evaluate rCEC and aCEC subsets. Panel b4 shows the gate restricted to a CD34+ population employed to detect CEP population (panel b5).
evaluate their prognostic or predictive role regarding clinical response and progression-free survival (PFS).

**patients and methods**

**study population**

Adult patients with histologically documented mCRC with measurable disease and suitable for first-line bevacizumab-based antineoplastic treatment as currently used in clinical practice were eligible. Three centers were involved: Foundation IRCCS Policlinico S. Matteo (Pavia, Italy), Scientific Institute S. Raffaele (Milano, Italy) and University Hospital of Pisa (Pisa, Italy). The biological analysis was centralized and carried out at Flow Cytometry and Cellular Therapy Unit, University and Foundation IRCCS Policlinico S. Matteo, Pavia, Italy.

Each patient gave written informed consent before entry into the study and the experimental trial was conducted after the approval of the Ethical Committee and Study Protocols Review Boards of the above-mentioned institutions. All the procedures utilized in this study are in agreement with the 1975 Helsinki Declaration [18].

**clinical study plan**

The CT regimen combined with bevacizumab was chosen according to daily clinical practice by each center among the following: FOLFIRI regimen [(CPT11 180 mg/m² on day 1, folinic acid 100 mg/m² on days 1 and 2, 5-fluorouracil (5-FU) bolus 400 mg/m² on days 1 and 2, 5-FU 600 mg/m² 22-h continuous infusion on days 1 and 2, every 14 days)], FOLFOX regimen (oxaliplatin 85 mg/m² on day 1, folinic acid 100 mg/m² on days 1 and 2, 5-FU bolus 400 mg/m² on days 1 and 2, 5-FU 600 mg/m² 22-h continuous infusion on days 1 and 2, every 2 weeks), XELOX regimen (oxaliplatin 130 mg/m² on day 1, capetitabine 1000 mg/m² from days 1 to 14, every 2 weeks) and FOLFOXIRI regimen [Irinotecan 165 mg/m² on day 1, oxaliplatin 85 mg/m² on day 1, leuokovorin 200 mg/m² on day 1, 5-FU 3200 mg/m² 48-h continuous infusion starting on day 1, every 2 weeks). Bevacizumab was administered on the first day of each cycle at the dose of 5 mg/kg in combination with 2-week regimens and at the dose of 7.5 mg/kg in combination with 3-week regimens.

CEPs, CECs and their subsets were evaluated in all patients at baseline and before the administration of the third and the sixth courses. According to RECIST criteria, the patients were evaluated for response and PFS at the end of treatment.

For comparison with patient baseline data, the absolute numbers of CEPs, CECs and their subsets in a group of healthy subjects, selected as previous report [19], were used as reference.

**flow cytometric analysis**

Peripheral blood samples were collected in a 3-ml Vacutainer tube containing liquid tri-potassium EDTA as an anticoagulant. Blood samples will not be retained for further assessment and were immediately disposed of. CECs and CEPs were evaluated by the four-color flow cytometry method according to the previously described procedure [19], using a panel of mAbs: fluorescein isothiocyanate-labeled anti-CD34 (8G12) (BD Biosciences, San Jose, CA); and anti-CD106 (51-10C9) (BD Pharmingen, San Jose, CA); allophycocyanin-labeled anti-CD34 (8G12) (BD Biosciences) and anti-CD133 (AC133) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany); R-phycocerythrin-labeled anti-CD146 (P1H12; BD Pharmingen™), CA and peridinin chlorophyll protein cyanine 5-labeled anti-CD45 (2D1) (BD Biosciences). Nonspecific antibody binding was blocked using 20 μl FcR blocking reagent and 200 μl of mouse serum (Sigma-Aldrich, St Louis, MI), and dead cells were excluded with 7-aminoactinomycin D (7-AAD Viability Dye; Beckman Coulter, Miami, FL) before conjugated antibodies staining. Stained whole-blood samples were subjected to red blood cell lysis with 2 ml of lysing solution (Auto Lyse BD Biosource, Europe SA, Nivelles, Belgium).

Evaluation of nucleated cells from whole-blood specimens was carried out using a FACSComp flow cytometer (BD Biosciences) and data were analyzed using BD FACSDiva software. A range of internal quality assurance procedures was employed, including daily calibration of flow cytometry optical alignment and fluidic stability using seven-color SetUp Beads (BD Biosciences). The exactitude and stability of cell count were tested using international quality controls purchased from United Kingdom National External Quality Assessment Scheme (UK NEQAS LI, Sheffield, UK) and daily monitoring of whole-blood preparation procedures and mAbs reactivity using Immuno-Trol (Beckman Coulter, Fullerton, CA) control cells.

Total CECs were defined as CD45+ , CD146+, CD34+ and CD133--; rCECs as CD45−, CD146+, CD34+, and CD106−; and aCECs as CD45−, CD146+, CD34+, and CD106+. CEPs were identified as CD45−, CD34+, CD146+ and CD133+.

The gating strategy for sample analysis is shown in Figure 1A, panel a2. The dot plot windows utilized for identification of CECs and CEPs are shown in Figure 1B, panels b1, b2 and b3 for CECs with their subsets and panels b4 and b5 for CEPs.

The absolute CEC and CEP number was derived from the absolute number of white blood cell (WBC) provided by the hematological analyzer (Coulter, Miami, FL) and the percentage of CECs and CEPs as determined by FCM, using the following formula: percentage of cells × WBC count/100.

**Table 1. Patient clinical features**

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Number of patients</th>
</tr>
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<tbody>
<tr>
<td>Assessable patients</td>
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<tr>
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<tr>
<td>Median</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Female</td>
<td>19</td>
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</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<tr>
<td>Primary tumor, localization</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Rectum</td>
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<td>38</td>
</tr>
<tr>
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<tr>
<td>Metastatic tumor at diagnosis</td>
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</tr>
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<td>29</td>
</tr>
<tr>
<td>No</td>
<td>11</td>
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<tr>
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<tr>
<td>3 sites</td>
<td>4</td>
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<tr>
<td>Metastatic sites, localization†</td>
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<td>Nodes</td>
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</table>

†Some patients have several metastatic sites.

ECOG, Eastern Cooperative Oncology Group.
Statistical analysis
Means and standard deviations were used to summarize quantitative variables or median and interquartile range if data were not normally distributed; 95% confidence intervals (CIs) are also shown. Count and percentage were used for qualitative variables. The Mann-Whitney U test was used for comparisons between healthy subjects and patients. The Kaplan-Meier methodology was used to describe PFS and log-rank test for univariate comparisons. Cox regression analysis was used to identify independent predictors. Data analysis was carried out with STATA statistical package (Stata Corporation, College Station, TX). All tests were two-sided.

Results
Clinical results
From October 2007 to March 2009, 40 mCRC patients were enrolled.
Clinical features of the patient population are reported in Table 1.

Regarding CT regimens combined with bevacizumab, 30 patients received FOLFIRI, 3 patients received FOLFOX4, 2 patients received XELOX and 5 patients received FOLFOXIRI. All patients received 12 cycles of CT plus bevacizumab; only eight among the patients with clinical benefit (CB) continued bevacizumab alone until progression.
The healthy subject population was composed of 50 healthy subjects (males/females: 25/25, median age: 43 years; range 21–65 years).
At the end of the treatment, complete response (CR) was obtained in 11 patients (27.5%) and partial response was observed in 21 patients (52.5%); 4 patients (10%) had stable disease (SD) and 4 patients (10%) had progressive disease (PD). The overall response rate was 80% (95% CI: 68% to 92%) and the overall CB (CR + PR + SD) was 90% (95% CI: 80% to 99%). With a median follow-up of 13 months (range 7–27 months), the median PFS was 13.8 months. PFS after 6 months was 92% (95% CI: 78% to 97%) and after 24 months was 33% (95% CI: 13% to 55%) (Figure 2A). At univariate
test, only the log-rank test between PFS of one and three metastatic sites was significant ($P = 0.006$).
analysis, among the clinical features, only the number of metastatic sites was related to PFS \( (P = 0.006); \) Figure 2B.

**biological results**

At baseline, tCEC and rCEC counts were significantly increased in our patients \( (P = 0.009 \text{ and } P = 0.01, \) respectively) relative to healthy subjects (Figure 3).

Among the mCRC population, the patients with one metastatic site had a significant decrease of tCECs and rCECs \( (P = 0.006 \text{ and } P = 0.001) \) at baseline compared with those with three sites of metastasis. No other correlation was found between clinical characteristics and baseline values of circulating cells.

Regarding the correlation with clinical response, the patients who obtained a radiological response \( (CR + PR) \) showed a significant decrease of rCECs \( (P = 0.02) \) at baseline and a slight trend in decrease of tCECs \( (P = 0.07) \) in comparison with patients who obtained SD or PD (Figure 4A).

Finally, patients at baseline with tCEC and rCEC absolute number <40 cells/ml \( (i.e. \) the 75th percentile distribution value) showed a longer PFS \( (P = 0.01 \text{ and } P = 0.007); \) Figure 4B and C).

No substantial modification at the third and sixth cycles, relative to baseline, was found in tCECs or in activate and resting subsets (Figure 5).

No correlation was discovered regarding CEP baseline values and their kinetic modifications with the response to treatment or PFS.

**discussion**

Inhibition of tumor angiogenesis suppresses tumor growth and metastatic spread in many experimental models, suggesting that anti-angiogenic drugs may be successfully used to treat human cancer. Recent results stemming from phase III trials with anti-VEGF antibodies, used both alone and in combination with CT, have indicated that this systemic approach has a noticeable impact on cancer progression and patient survival.

Nevertheless, it has also become clear that the classical end points used in anticancer clinical trials, based on changes in tumor size by radiological assessments, lack sufficient discriminative power to monitor the effects of anti-angiogenic drugs that have cytostatic rather than cytotoxic effect [20]. Conventional imaging evaluation needs to be integrated with other novel sensitive indicators of anti-angiogenic response, which must be validated. In this field, promising candidate markers include the absolute number and the kinetic modifications of CECs and CEPs, detected by multiparameter flow cytometry, as recently suggested both in preclinical models [21, 22] and in cancer patients [23–25].

Despite the widespread utilization of antibody directed against VEGF such as neutralizing antibody bevacizumab in mCRC, only an evaluation of CECs in the neoadjuvant treatment of rectal cancer is to date available, with promising results [26]. The present study provides, for the first time, data regarding the baseline quantification, the kinetic modification and the correlation with clinical outcomes of CECs and CEPs after a combined treatment containing bevacizumab in mCRC patients treated in first line.

At baseline, our advanced cancer patients had higher tCEC and rCEC counts than healthy controls, as noted in the previous reports [25, 27]. The median age and range of the control patients were much younger than the age of the patients. We do not think that the age of the healthy subjects could impact on their CEC median baseline value; this point is supported in our hands by the fact that in a preliminary work [19], we have carefully evaluated the CEC profile on a cohort of healthy donors and we did not observe statistically significant difference of median value of tCECs and of their subsets and progenitors between selected age groups.

Only number of metastatic sites was correlated with CEC value, suggesting a correlation between spread of disease and endothelial cell count.

The baseline values of these cells were also correlated with clinical response: the patients who achieved a radiological...
response showed, at baseline, a significant decrease of rCECs and a slight trend in decrease of tCECs in comparison with patients achieving SD and PD; these data appear strong if we consider the high clinical efficacy of bevacizumab-based treatment in our population. Using a cut-off of 40 cells/ml, baseline values of tCECs and rCECs were also predictive for PFS. In our experience, lower tCEC and rCEC counts seem to be positive predictive factors for clinical outcomes. In breast cancer experiences, the only data available to date are conflicting; in metronomic approaches, CEC baseline value was not predictive for response and PFS [15]; on the other hand, where bevacizumab was combined with metronomic CT, patients with higher baseline CEC values and apoptotic CECs have better clinical response and PFS [16]. These data suggest that CEC predictive baseline value could be different in different cancer and patient subsets, reflecting a specific cancer endothelium activation.

In our population, no significant modification of the CEC and subpopulation kinetic was discovered during treatment, despite the baseline value (it must be noted that baseline median count of aCECs was zero). No clear modifications during other treatments in global analyzed population without a correlation with clinical response were described in previous reports [15, 16, 27]. Only one experience in renal cell cancer showed a strict relation between CEC kinetic and sunitinib administration, but the identification markers of cells in flow cytometric analysis were in part different from the present experience. Probably, CECs, and in particular their subsets, are extremely rare events and only evaluation in a large population could evidence significant modification during treatment.

Finally, as already noted in Mancuso et al. [25], no predictive value was discovered for CEPs. In our experience, median baseline value was zero, and this finding was confirmed in subsequent checkpoint of detection; as suggested by Shaked et al. [28], CEP mobilization was influenced not only by anti-angiogenetic treatment but also by other factors, such as granulocyte colony-stimulating factor, with a limitation of their predictive and prognostic value.

In conclusion, our data demonstrate, for the first time, the value of baseline mature and resting endothelial cells as good predictive biomarkers in mCRC patients treated with CT plus bevacizumab. We can hypothesize that the positive treatment effects in patients with metastatic disease and low baseline tCECs and rCECs are achieved by the addition of anti-VEGF treatment to CT on the basis of our previous experience showing that no significant modifications of these parameters occurred in patients with the same disease and treated only with CT [29].

The early identification of patients who might benefit from targeted anti-angiogenetic therapies is crucial for the optimization of the treatment strategy as well as the reduction of drug costs and the onset of serious adverse events in no responder patients. The evaluation of these cells in a large, well-designed ad hoc trial, requiring close collaboration between clinical investigators and laboratory-based researchers, could confirm these data and provide new insight regarding their kinetic modification and their role as surrogate biomarkers for anti-angiogenetic therapy.

Figure 4. Correlation between baseline values and clinical response. (A) The patients who obtained a radiological response (CR + PR) showed significant decrease of resting circulation endothelial cells (rCECs; P = 0.02) at baseline and a slight trend to decrease of tCECs (P = 0.07) in comparison with patients with SD and PD (tCECs, total CECs; rCECs, resting CECs). (B) The median progression-free survival (PFS) was greater in patients whose total CEC count was <40 cells/ml (i.e. the 75th percentile distribution value) than in patients whose total CEC count was higher. (C) The median PFS was greater in patients whose rCEC count was <40 cells/ml (i.e. the 75th percentile distribution value) relative to patients whose resting CEC count exceeded 40 cells/ml.
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disclosure
The authors do not have any conflict of interest to declare or founding sources that may generate conflict of interest.

references

Figure 5. Early modification of circulating endothelial cell (CEC). No statistically significant modification at the third and sixth relative to baseline were found either in total CECs (tCECs) or in activate (aCECs) and resting (rCECs) subsets.