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ORIGINAL ARTICLE

Biomarkers for the early detection of relapses in metastatic colorectal cancers

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Summary

Purpose: *To assess prognostic/predictive value of carcinoembryonic antigen (CEA), transthyretin (TRT), a-enolase (NNE), β2-microglobulin (β2-micro), B-cell activating factor (BAFF) and circulating tumor cells (CTCs) in metastatic colorectal cancer (mCRC) patients treated with chemotherapy with or without bevacizumab.*

Methods: *72 histologically confirmed mCRC patients treated at Oncology Institute Cluj were included. Biomarker levels were measured through validated methods. A manual method was used for CTCs, involving hemolysis, cytospin centrifugation and immunocytochemical staining for pan-cytokeratin. Statistical endpoints were response, progression-free survival (PFS) and overall survival (OS).*

Results: *Initial chemotherapy was fluoropyrimidine/oxaliplatin-based in 93.1%; bevacizumab was added in 58.3% of the patients. Median PFS and OS were 16.4 and 24.4 months. Two-year OS for CR & PR vs SD vs PD were 90% vs 48% vs 12%, respectively (p<0.01). Two-year OS for chemo/bevacizumab vs chemotherapy: 65% vs 42% (p=0.09). Baseline CEA ≥5 ng/ml had a negative prognostic impact on OS and PFS (p<0.01). High baseline CEA was predictive of improved OS when adding bevacizumab (2-year OS chemo/bevacizumab vs chemo: 60% vs 17%, p<0.01); adding bevacizumab in patients with normal CEA did not improve OS (p=0.29). Higher than cut-off values for TRT had a positive OS prognostic value (p<0.01); higher levels for NNE, β2-microglobulin and BAFF had a negative impact (p<0.01). Two-year OS for baseline <1 CTC/ml vs ≥1 CTC/ml was 74% vs 64% respectively (p=0.15).*

Conclusions: *The evaluated biomarkers could be useful prognostic factors for survival. Baseline CEA also has predictive value, suggesting that patients with low levels do not benefit from bevacizumab. A non-statistically significant correlation was observed between the number of CTCs and outcome.*

Key words: *biomarkers, carcinoembryonic antigen, circulating tumor cells, metastatic colorectal cancer*

Introduction

According to GLOBOCAN 2012, colorectal carcinoma (CRC) is the 3rd most common type of cancer, with 1.36 million new cases each year worldwide. In Romania, CRC age-standardized rate ranked 4th in incidence and 3rd in mortality among all tumors [1]. Half of the patients will develop metastatic disease, with a 5-year estimated survival rate of 12.5% [2]. Availability of new molecular treatments and discovery of biomarkers are both needed to optimize management of patients [3].

Although the treatment definitely improved

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during the past decades, primary or acquired drug resistance still jeopardize the results and recently discovered drugs are very expensive. Therefore, finding new prognostic/predictive biomarkers constitutes a research priority. Immune events around CRCs and their relation with clinical outcomes have led to consider immune microenvironment as one important prognostic factor in this disease [4]. Tumor-associated antigens, such as carcinoembryonic antigen (CEA), have been proposed mainly for early diagnosis of relapse in which CEA has a negative prognostic value [5]. The predictive ability of an individual marker being limited, algorithms were proposed, combining CTCs [6], tumor-associated antigens, and microenvironment biomarkers [7].

The aim of this study was to identify prognostic or predictive biomarkers in mCRC:

a) we explored a locally developed method of counting CTCs, b) revisited the value of CEA and c) evaluated other microenvironment blood biomarkers, measured in patients with mCRC receiving first-line combination chemotherapy with or without bevacizumab. These biomarkers were evaluated in respect with variables such as response rate (RR), PFS or OS.

Methods

This was a prospective study where eligible patients had histologically confirmed mCRC, with ≥1 target lesion by RECIST criteria 1.1, age ≥18 years, ECOG performance status 0-2, life expectancy ≥3 months. All patients provided written informed consent. Adequate hematologic, hepatic and renal function were required. Exclusion criteria included prior chemotherapy or biologic therapy for metastatic disease, major surgery within 28 days before the initiation of study treatment, clinically significant cardiovascular disease, pregnancy, preexisting bleeding diathesis or coagulopathy. The protocol was approved by the Institutional review board and carried out in accordance to the Declaration of Helsinki.

The patients received first-line chemotherapy (FOLFOX, XELOX, FOLFIRI, XELIRI, capecitabine or 5-fluorouracil) with or without bevacizumab, at the investigator's choice. Bevacizumab was added after approval from a National Commission. Treatment continued until disease progression, patient/physician's decision, unacceptable toxicity, or death. Following progression, patients received 2nd, 3rd line chemotherapy at the investigator's choice.

Venous blood for biomarkers and CTCs has been collected before chemotherapy on day 1 of cycles 1 (baseline), 2 and 5. Only baseline data are presented here. Response was determined according to the RECIST 1.1 [8], based on CT examinations at baseline and every 6 weeks thereafter.

Molecular biomarkers: 9 ml venous blood samples have been collected in vacutainer tubes, on EDTA. CEA, TRT, NNE, β2-microglobulin, BAFF, belonging to the TNF superfamily, macrophage migration-inhibitory factor (MIF), tumor type-M2 pyruvate-kinase (M2-PK) were assessed. CEA was determined by chemiluminescence immunoassay. Two subgroups of patients were analyzed according to the upper limit of normal for CEA (<5 vs ≥5 ng/mL). The other biomarkers were determined by ELISA technique according to manufacturer's instructions.

CTC analysis: 6 ml venous blood samples have been collected. A manual method was used consisting of a gentle hemolysis with a buffered hypotonic solution of amonium chloride, followed by washing of nucleated cells in Dulbecco's phosphate buffered saline, cytocentrifugation on histologic slides and fixation at 5°C in methanol for 15 min. Then, a standard immunocytochemical staining method was applied, using an antibody against cytokeratin AE1/AE3. Counting was performed by two pathologists under a light microscope. All the positive cells were photo-documented, and a consensual decision was made.

Statistics

The endpoints of statistical analysis were RR, PFS and OS. Kaplan-Meier method was performed to estimate survival and survival differences were assessed by log-rank test. The prognostic score meant to identify patients at risk for relapse and was inferred from the multivariate Cox regression analysis defining a time-related risk. A p value <0.05 was considered statistically significant. Cut-off values were calculated to maximize the value of x^2 from log-rank test. Cox multivariate analysis model was performed with MedCalc version 15. Pearson's r coefficient was used to evaluate the independence between prognostic factors. A coefficient <0.65 in absolute value showed independence [9].

Results

Between 2012-2015, 72 metastatic/relapsed CRC patients treated at the Oncology Institute "Ion Chiricuta", entered the study. Median age was 60 years (range 30-82), and 58.3% were men. The primary tumor was located on the right colon in 19.4% and on the left colon and rectum in 80.6% of the cases; 81.9% had surgery for their primary tumor.

First-line chemotherapy consisted of a fluoropyrimidine/oxaliplatin combination in 91.7%, a fluoropyrimidine/irinotecan combination in 4.2% and fluoropyrimidine monotherapy in 2.8% of the patients. In 58.3% bevacizumab was added to chemotherapy.

The median number of first-line chemothera-

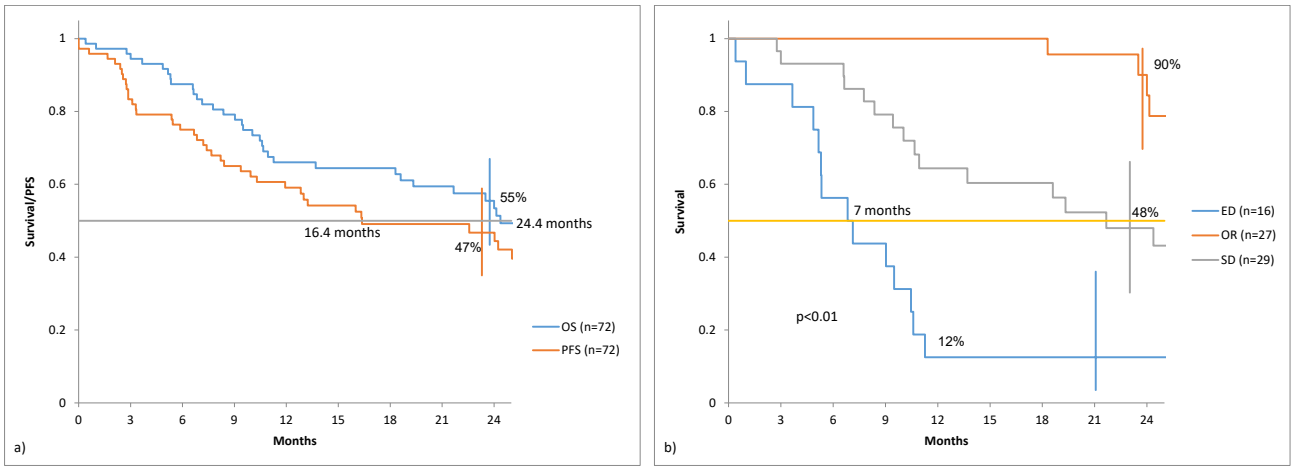

Figure 1. a) Overall survival (OS) and progression-free survival (PFS) at 2 years; **b)** OS by best response to chemotherapy. OR: objective response, SD: stable disease, PD: progressive disease, ED: disease in evolution.

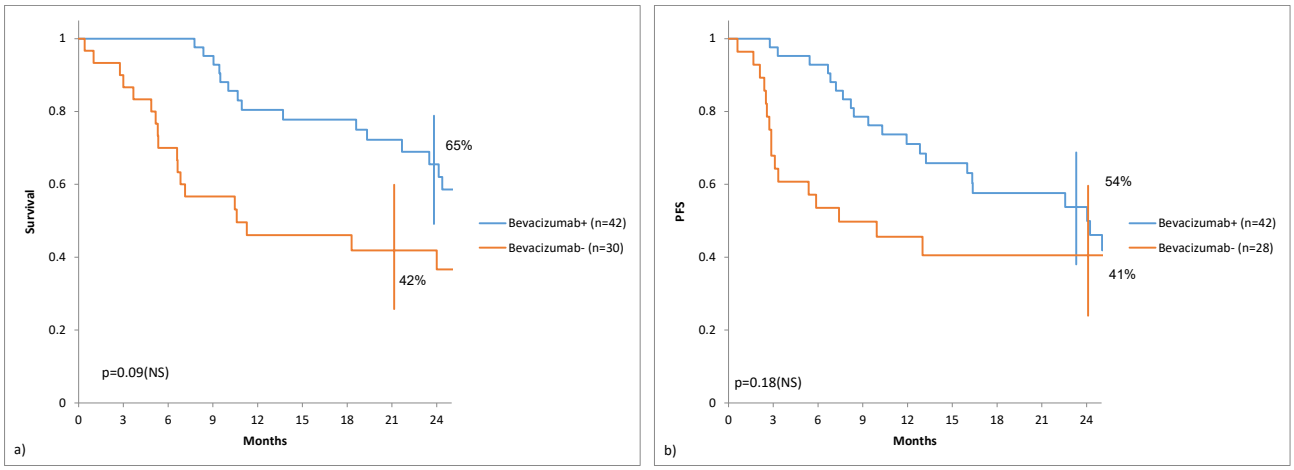

Figure 2. a) Overall survival (OS) according to chemotherapy +/- bevacizumab; **b)** Progression-free survival (PFS) according to chemotherapy +/- bevacizumab.

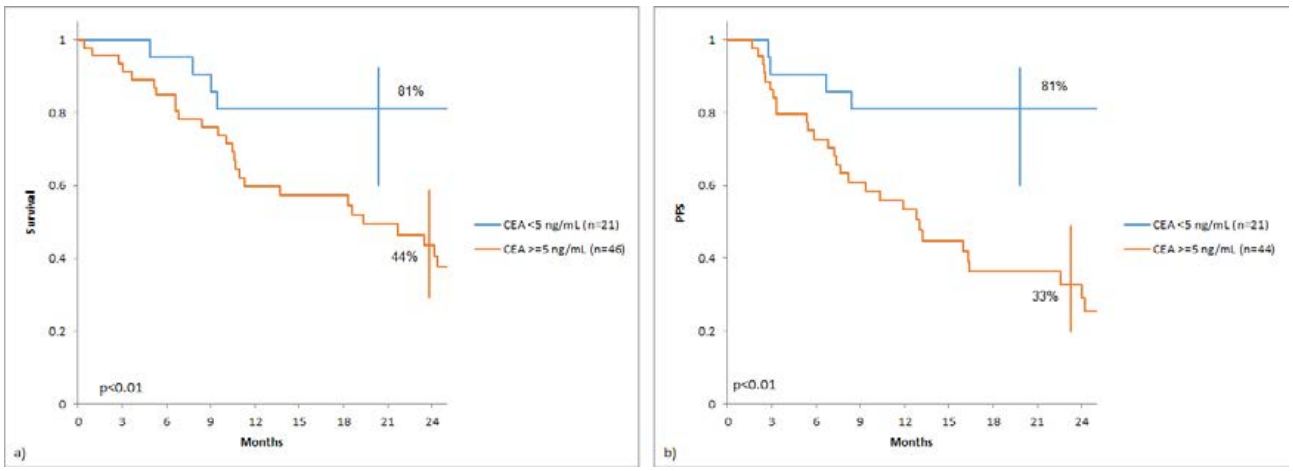

Figure 3. a) Overall survival (OS) by CEA values at baseline; **b)** Progression-free survival (PFS) by CEA values at baseline.

py cycles was 8 (range 1-57).

Responses to first-line chemotherapy were: complete response (CR) in 18%, partial response (PR) in 19.4%, stable disease (SD) in 40.3% and progressive disease (PD) in 22.2%.

Median follow-up was 22.3 months (range 7.8-50.3).

The 1- and 2-year OS was 66% (95%CI 54-76) and 55% (95%CI 43-67) respectively, and the median OS was 24.4 months; PFS at 1 and 2 years was 59% and 47%, respectively, and the median PFS was 16.4 months (Figure 1a).

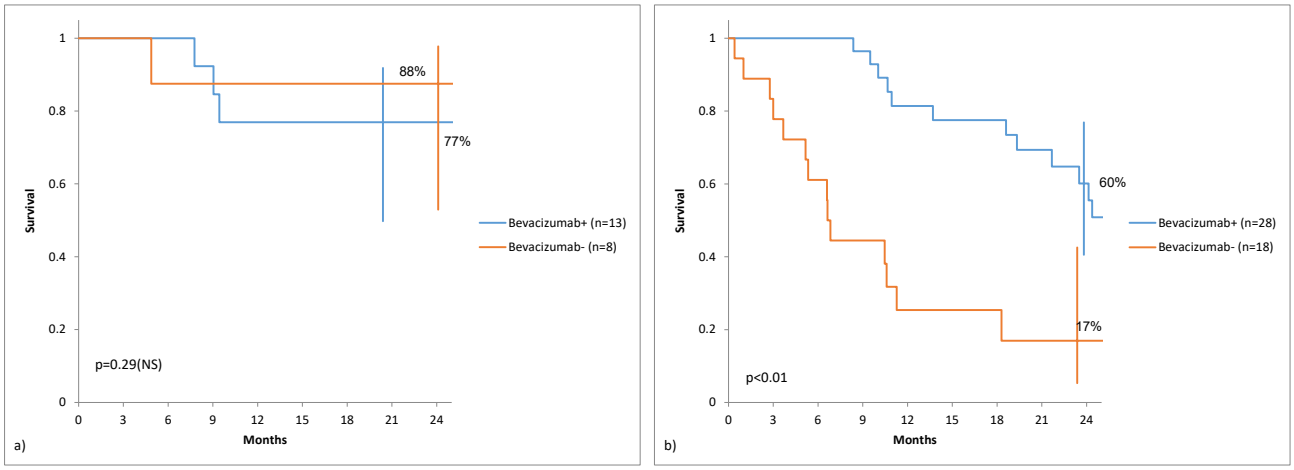

Figure 4. a) Patients with normal baseline CEA: adding bevacizumab to chemotherapy did not improve overall survival; **b)** Patients with elevated baseline CEA ≥5ng/ml: adding bevacizumab improved overall survival.

Table 1. Statistical data for biomarkers evaluated by ELISA technique: transthyretin (TRT), α-enolase (NNE), β2-microglobulin (β2-micro), B cell activating factor (BAFF), macrophage migration-inhibitory factor (MIF), tumor type M2 piruvate kinase (M2-PK)

	BAFF (pg/ml)	*MIF (ng/ml)*	*β2-micro (mg/l)*	*M2-PK (ng/ml)*	*NNE (ng/ml)*	*TRT (mg/ml)*
Min	352.58	30.66	2.40	23.24	6.40	238.97
Max	3142.93	143.27	5.80	103.07	62.18	808.70
Mean	1480.70	57.32	3.49	50.29	31.60	522.45
Median	1344.14	54.85	3.42	48.18	34.33	515.09
Standard deviation	633.46	18.90	0.73	16.10	12.78	142.56
Coefficient of variation (%) (Standard deviation/ Median)	42.78	32.96	20.82	32.02	40.44	27.29

Table 2. Pearson's coefficient correlation between evaluated parameters: BAFF, MIF, β2-micro, TRT, NNE, M2-PK

	BAFF (pg/ml)	*MIF (ng/ml)*	*β2-micro (mg/L)*	*M2-PK (ng/ml)*	*NNE (ng/ml)*	*TRT (mg/L)*
BAFF		0.068	0.380	0.252	-0.142	-0.402
MIF			0.141	0.188	-0.197	-0.175
B2-micro				0.256	0.237	-0.382
M2-PK					-0.197	-0.444
NNE						0.064
TRT						

For abbreviations see text

Response to chemotherapy significantly influenced survival (Figure 1b). For those patients who achieved an objective response the 2-year actuarial survival was 90%, compared to 48% for those with SD and only 12% for PD (p<0.01). Median survival for objective responders was not reached, it was 21.7 months for SD and only 7 months for PD patients.

Addition of bevacizumab gave a trend towards longer survival, with 2-year actuarial survival of 65% for bevacizumab combinations vs 42% for chemotherapy alone (p=0.09; Figure 2a, 2b).

CEA at baseline was available in 93% of the patients. Mean CEA was 413.6 ng/mL and median 27.6 (range 0.7–9176). Increased baseline values of CEA (≥5ng/ml) proved to be a negative prognostic factor for OS and PFS. Two-year OS was 81% for patients with normal baseline CEA vs 44% for patients with higher CEA values (p<0.01; Figure 3a). Two-year PFS was 81 vs 33% in patients with normal vs high CEA (p<0.01; Figure 3b).

Adding bevacizumab to chemotherapy in patients with normal baseline CEA did not improve OS in comparison with patients that had received only chemotherapy (2-year OS 88 vs 77%, p=0.29; Figure 4a). On the other hand, in the subset of pa-

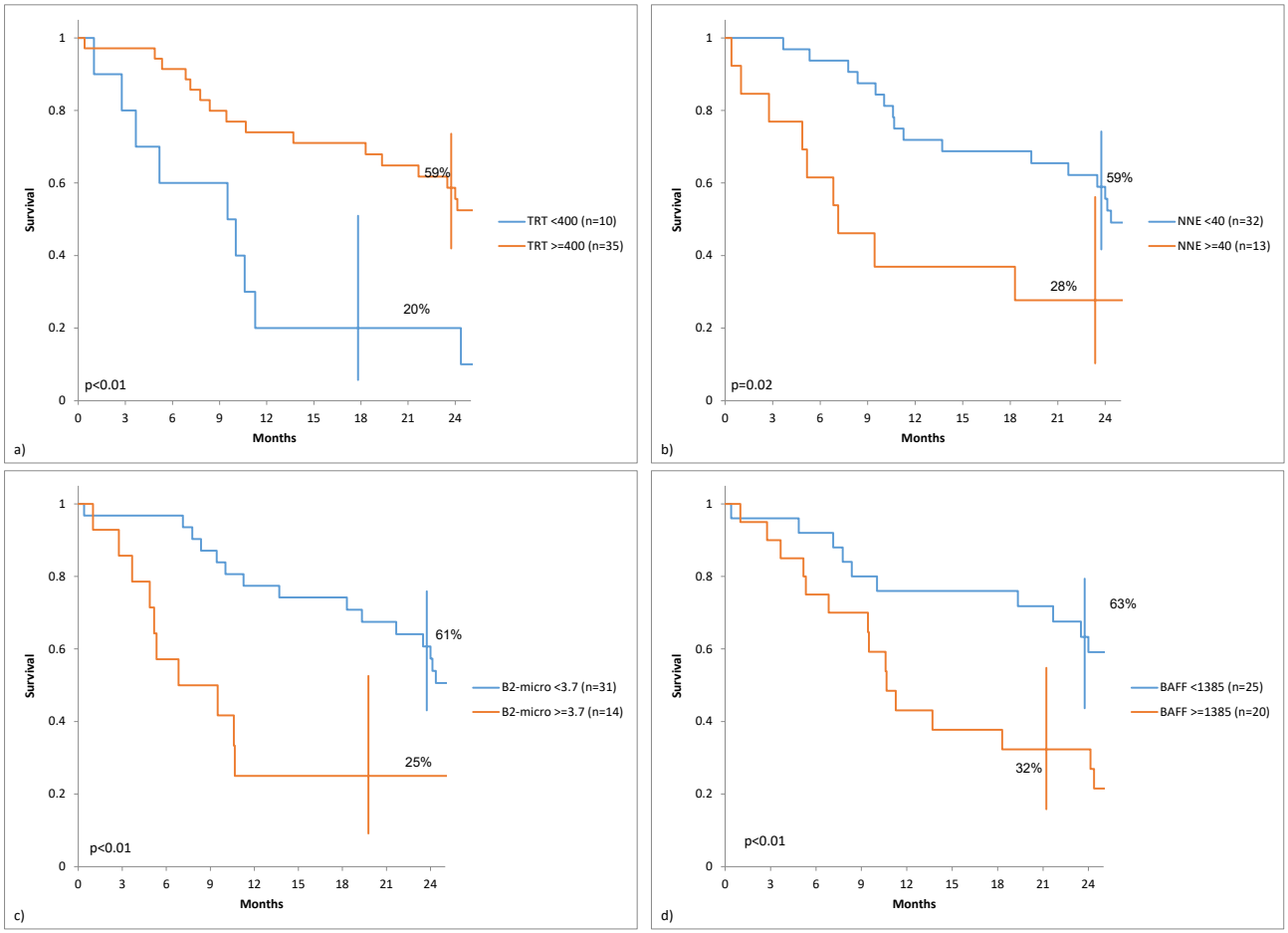

Figure 5. Overall survival by: **a)** TRT, **b)** NNE, **c)** β2-micro, **d)** BAFF levels.

tients with high CEA levels, adding bevacizumab improved OS (2-year OS 60 vs 17%, p<0.01; Figure 4b). The same correlations were found between CEA and PFS. In normal baseline CEA, adding bevacizumab to chemotherapy did not influence PFS (2-year PFS 88 vs 77%; p=0.27). For patients with elevated CEA, 2-year PFS for chemotherapy/bevacizumab vs chemotherapy alone was 42 vs 13% (p<0.01).

Table 1 presents the statistical data for the other 6 possible prognostic biomarkers tested. Correlations between each pair of biomarkers analyzed by Pearson's correlation coefficient are shown in Table 2. Because none of the Pearson's coefficient, in absolute value, was over 0.65 we concluded that these biomarkers were statistically independent. In this study, a significant link was found between TRT, NNE, β2-microglobulin and BAFF and survival (detailed below), but not for MIF and M2-PK.

For TRT the identified cut-off value was 400 mg/L. Patients with baseline TRT levels >400 mg/L had a better 2-year OS (59 vs 20%, p<0.01; Figure 5a). For PFS the difference was not significant (p=0.06; Figure 6a).

For NNE the identified cut-off value was 40 ng/ml. Patients with baseline NNE levels >40 ng/ml had a worse 2-year OS (28 vs 59%, p=0.02; Figure 5b). For PFS the difference was not significant (p=0.10; Figure 6b).

β2-microglobulin levels higher than the cut-off value of 3.5 mg /L were a negative prognostic factor: 2-year OS 25 vs 61% (p<0.01; Figure 5c) and 2-year PFS 19 vs 50% (p<0.01; Figure 6c).

BAFF levels higher than the cut-off value of 1385 pg/ml were a negative prognostic factor: 2-year OS 32 vs 63% (p<0.01; Figure 5d) and 2-year PFS 29 vs 50% (p=0.03; Figure 6d).

CTCs were detected before chemotherapy in 96% of the 51 tested patients (Figure 7). The mean value was 1.64 CTC/ml blood (corresponding to ~12 CTC/7.5 ml) with a standard deviation ± 1.29 and a median of 1.16 CTCs/ml (corresponding to ~9 CTC/7.5 ml). A cut-off value of 1 CTC/ml of whole blood was chosen for the correlation with OS and PFS. Two-year OS was 74 % in patients under the cut-off and 60% in those over this value (p=0.15; Figure 8a). PFS was 66 and 50% respectively, also without statistical significance (p=0.19; Figure 8b).

Only items with statistically significant prognostic relevance in univariate analysis were used

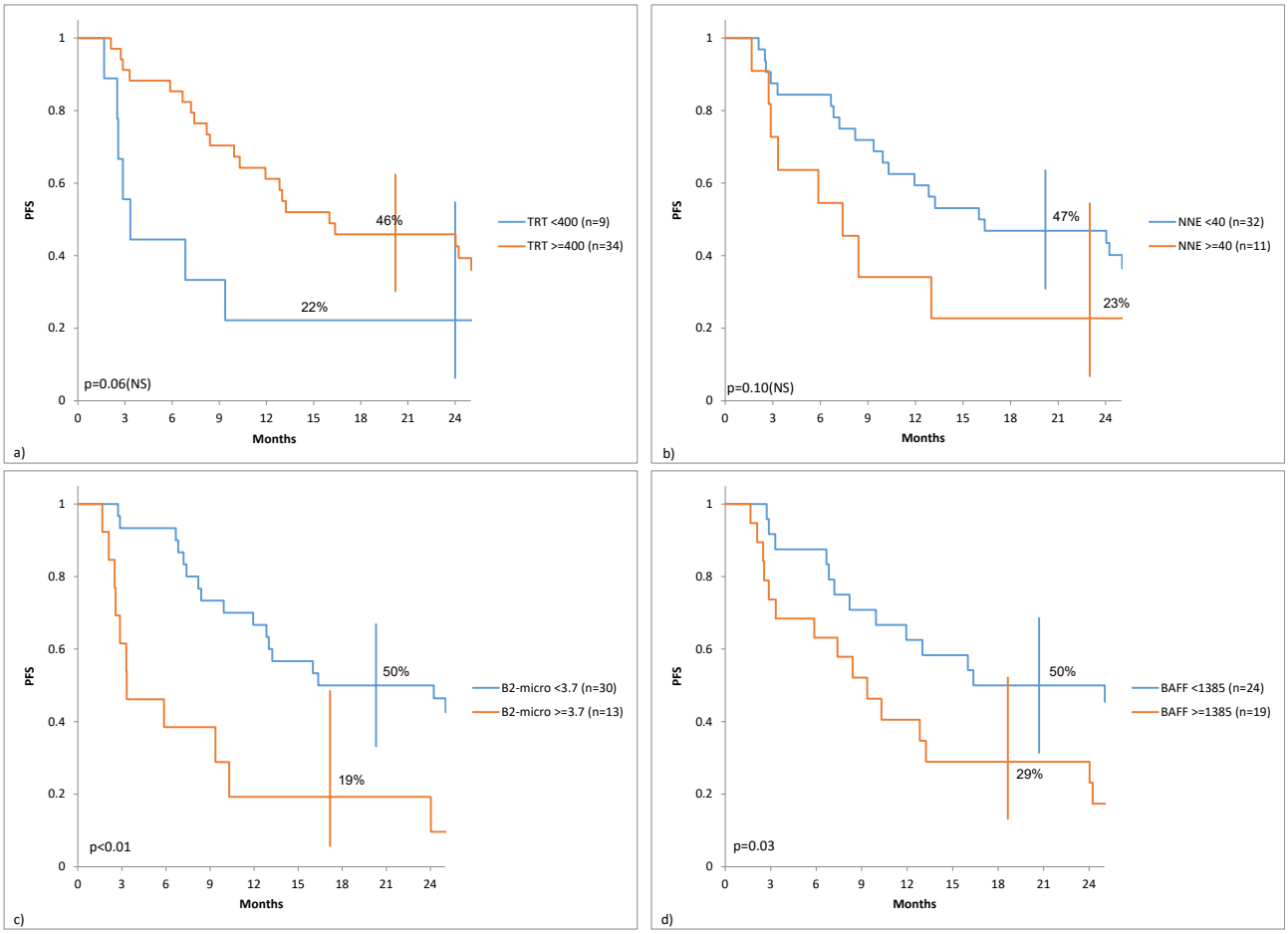

Figure 6. Progression free survival by: **a)** TRT, **b)** NNE, **c)** β2-micro, **d)** BAFF levels.

for multivariate analysis (CEA, TRT, NNE, β2-microglobulin, BAFF, bevacizumab). In multivariate analysis only β2-microglobulin remained as independent prognostic factor.

Discussion

Tumors depend on neoangiogenesis to grow and metastasize. Bevacizumab, added to standard chemotherapy, improves PFS and OS in mCRC [10]. Only a trend towards improved outcome was found in our non-randomized study (p=0.09) where the addition of bevacizumab was delayed until an approval was obtained from a centralized commission.

CEA induces proangiogenic behaviors such as *in vitro* endothelial cell adhesion, spreading, proliferation and migration and *in vivo* tumor vascularization [11]. When the VEGF pathway is blocked, CEA pathway may substitute neo-angiogenic effect [12].

According to our data and consistent with the literature [13-15], higher baseline CEA carries a negative prognostic value. Addition of bevacizumab improved OS and PFS only in the subset of patients with higher baseline CEA. This finding differs from other reports [12,16], where CEA level was inversely correlated with the OS and PFS benefit among bevacizumab-treated patients.

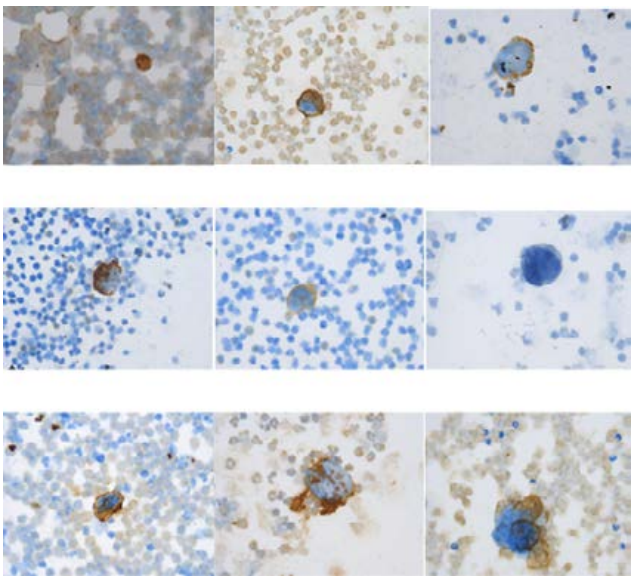

Figure 7. Photomicrographs 400x magnification, AE1/AE3 staining: Various types of CTCs, ranging from small to large (first row), from intense positive in cytokeratin staining to negative (second row), and from unique to cluster of multiple tumor cells (third row), often, present in the same blood sample.

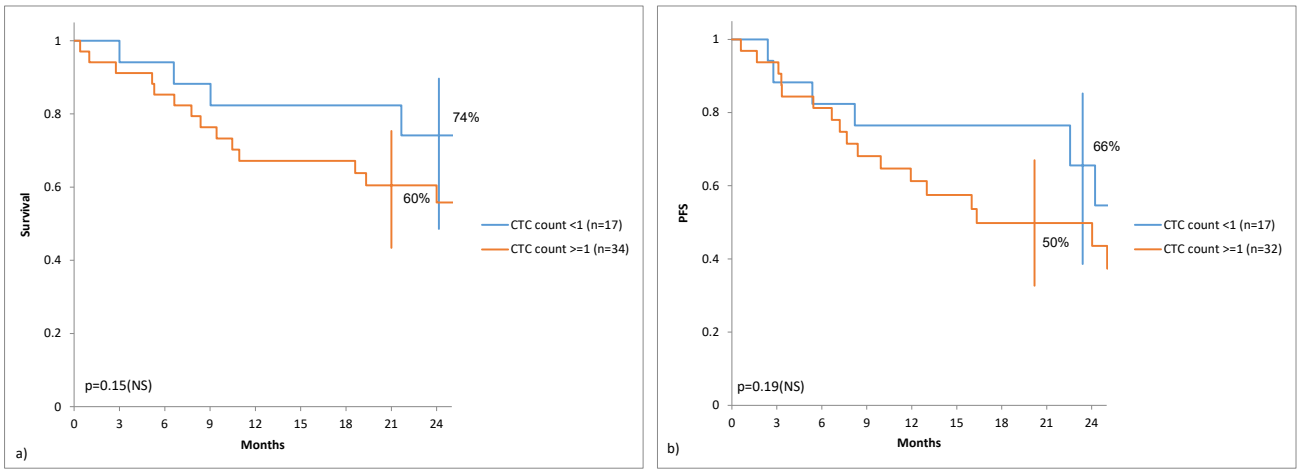

Figure 8. a) Overall survival according to CTCs baseline level; **b)** Progression-free survival according to CTCs baseline level.

Ramucirumab, a new anti-VEGFR monoclonal antibody, was found active in 2nd line patients irrespective of CEA level, but the activity was more important for CEA ≤10 ng/mL [17]. Additional prospective investigation is necessary to clarify the predictive role of CEA related to antiangiogenic therapy.

TRT, a transporter of tiroxine and retinol, is an acute inflammatory phase protein and a measure of tumor burden and metabolic status. We found a significant correlation between TRT and OS in univariate analysis. In a similar series of 106 patients, low baseline levels of TRT were also correlated with poor OS [7].

NNE sustains energetic metabolism of tumor cells under anaerobic conditions and mediates activation of plasmin and extracellular matrix degradation [18]. Our results showed a negative impact of high NNE for OS in univariate analysis.

β2-microglobulin forms complexes with the MHC class I molecules, contributing to regulation of immune recognition of antigens presented to cytotoxic T-cell [19]. β2-micro-globulin is involved in the functional regulation of growth, survival, apoptosis and metastasis of cancer cells [20]. In our study high β2-microglobulin was a negative prognostic factor for OS and PFS in both univariate and multivariate analyses.

BAFF is a cytokine belonging to the TNF family involved in the humoral immune response, acting as a costimulator for B-cell maturation, function and survival [21]. In our study high BAFF values were a negative prognostic factor for OS and PFS in univariate analysis.

CTCs proved their prognostic and predictive value in various types of cancers [6]. We found a tendency towards a negative prognostic value of high CTCs baseline count, but the limited number of patients enrolled precluded it to reach statistical significance [22,23]. The number of CTCs detected in our study is comparable to those found in other studies, using Cell Search method [24]. The high sensitivity of our method of detection doesn't rely on the expression of a surface antigen (e.g. EpCAM) like for Cell Search method [24] nor is dependent on the size of CTCs like filtration based methods (e.g. Screen Cell, ISET) [25]. Our method is based on the expression of cytokeratins by CTCs, and this is complemented by the malignant morphology spotted by the human eye. Thus, an experienced cytologist is able to identify a tumor cell, despite its negativity to cytokeratin, seen for example in tumor stem cells or after epithelial-to-mesenchymal transition.

Conclusions

OS and PFS results of mCRC patients treated with chemotherapy with or without bevacizumab in our study are similar with those reported in the literature and correlated with best response to first-line chemotherapy.

CEA baseline increased levels are a negative prognostic factor for OS and PFS.

A positive predictive value of CEA related to the addition of bevacizumab in first-line treatment was found, that needs to be confirmed in further studies.

Among the biomarkers evaluated, high TRT had a positive prognostic value, while high β2-microglobulin, NNE and BAFF carried a negative prognostic value for survival. Only β2- microglobulin retained its significance in multivariate analysis.

A negative correlation was observed between

the number of CTCs and the therapeutic outcome, but did not reach statistical significance.

Conflict of interests

Tudor Ciuleanu: Consultant, advisory board, accommodation or expenses: Merck Serono, Roche.

The other authors report no potential conflict of interest.

Acknowledgement

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