

Research Paper

# The Association of Baseline Serum Tumour Markers with Outcome of Patients with Metastatic Colorectal Cancer Treated with Anti-EGFR Monoclonal Antibodies in the First Line

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## Abstract

The measurement of serum tumour markers is a simple and non-invasive method for assessing the response to systemic therapies in metastatic colorectal cancer (mCRC) and estimation of prognosis. The aim of our retrospective study was to evaluate the association of baseline serum levels of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9), thymidine kinase (TK) and tissue polypeptide specific antigen (TPS) with outcome of patients with mCRC treated with combination of chemotherapy and monoclonal antibodies against epidermal growth factor receptor (anti-EGFR mAbs) in the first line. In our study, the cohort included 102 patients treated with therapy based on anti-EGFR mAbs between years 2011 and 2017 at Department of Oncology and Radiotherapy, Medical School and University Hospital in Pilsen, Czech Republic. Serum samples were collected within one month before the initiation of treatment. In multivariate Cox analysis that included serum tumour markers and clinical baseline parameters show that high baseline serum CA 19-9 was significantly associated with worse progression-free survival (HR=1.871, p=0.0330) and also overall survival (HR=3.903, p=0.0006). We have not demonstrated association of baseline levels of CEA, TK and TPS with patients' outcome. CA 19-9 is commonly used serum tumour marker which is simple and readily available and its candidate prognostic importance in the setting of anti-EGFR therapy deserves to be studied in prospective trials.

Key words: colorectal cancer, cetuximab, panitumumab, chemotherapy, tumor markers, serum carcinoembryonic antigen, carbohydrate antigen 19-9, thymidine kinase, tissue polypeptide specific antigen

## Introduction

Colorectal cancer (CRC) is one of the most common causes of morbidity and mortality in developed countries [1]. Cetuximab and panitumumab are monoclonal antibodies (mAbs) targeting

epidermal growth factor receptor (EGFR), widely used in the treatment of metastatic CRC (mCRC). It has been well-established that RAS gene mutations represent predictive biomarker of resistance to the

treatment with anti-EGFR mAbs in patients with mCRC [2-11]. However, there is still a proportion of patients with tumours harbouring wild-type *RAS* gene, who derive no or poor benefit from systemic therapy containing anti-EGFR mAbs. Hence there is a need for surrogate predictive and prognostic biomarkers. The measurement of serum tumour markers is a simple and non-invasive method for assessing the response to systemic therapies in mCRC and estimation of prognosis [12, 13]. The aim of our retrospective study was to evaluate the association of baseline serum levels of carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9), thymidine kinase (TK) and tissue polypeptide specific antigen (TPS) with outcome of patients with mCRC treated with anti-EGFR mAbs in the first line.

## Patients and Methods

### Patients and treatment

We retrospectively analyzed clinical data of 102 adult patients with histologically confirmed mCRC treated with anti-EGFR mAbs containing therapy between years 2011 and 2017 at Department of Oncology and Radiotherapy, Medical School and University Hospital in Pilsen, Czech Republic. Cetuximab (Erbix, Merck & Co., Kenilworth, New Jersey, USA) was administered in combination with chemotherapy or as a single agent in a standard approved dose (initial dose 400 mg/m<sup>2</sup>, further doses 250 mg/m<sup>2</sup> every 7 days). Panitumumab (Vectibix, Amgen Inc., Thousand Oaks, California, USA) was administered in combination with chemotherapy or as a single agent in a standard approved dose (6.0 mg/kg every 14 days). The chemotherapy consisted of the following schedules: fluorouracil and leucovorin in combination with oxaliplatin (FOLFOX) or with irinotecan (FOLFIRI). Cetuximab was combined with FOLFOX in 39 patients and with FOLFIRI in 15 patients, panitumumab was combined with FOLFOX in 47 patients and with FOLFIRI in 1 patient. None of the patients had previously received anti-EGFR therapies. The protocol of our study was approved by the independent ethics committee of the Faculty of Medicine and Teaching Hospital in Pilsen and complied with the International Ethical Guidelines for Biomedical Research Involving Human Subjects, Good Clinical Practice guidelines, the Declaration of Helsinki, and local laws. The patients signed informed consent with the inclusion and subsequent analysis of their data.

The assessment of *KRAS* and *NRAS* gene status was performed at the time of diagnosis of metastatic disease. As it is a standard practice in Czech Republic, sample analysis was performed using standardized

methods including direct sequencing, real-time PCR (2008-2010) and reverse hybridization method (StripAssay) (since 2010). Although the methods changed over the time, all of them were well-established and certified either by Czech Accreditation Institute (ČIA) or intended for use in clinical laboratories (CE-IVD).

### Clinical monitoring

Clinical data were obtained retrospectively from the hospital information system. Physical examination and routine laboratory tests were performed every two weeks; computed tomography (CT) or positron emission tomography - (PET)-CT was performed every three months of the treatment. The objective tumour response was assessed by the attending physician using Response Evaluation Criteria in Solid Tumors (RECIST) [14].

### Tumour marker measurement

Serum samples were collected and the measurement was performed within one month before the initiation of anti-EGFR treatment. Peripheral venous blood was drawn using the VACUETTE blood collection system (Greiner Bio-one Company, Kremsmünster, Austria). Serum was separated by 10 minutes centrifugation at 1300 g, and immediately frozen to -80°C. Samples were thawed only once, just prior to analyses. Serum levels of CEA and CA 19-9 were measured using chemiluminescent assay on an Unicel DxI 800 analyzer (BeckmanCoulter, Brea, CA, USA). Serum levels of TK were measured using radioenzymatic assay (REA) on an Stratec 300 analyzer (Immunotech, Prague, Czech Republic). Serum levels of TPS were measured using immunoradiometric assay (IRMA) on a Stratec 300 analyzer (IDL Biotech, Bromma, Sweden). The measurements were performed at the Department of Immunochemistry, Medical School and University Hospital in Pilsen, Charles University, Czech Republic, using the following cut-off values: CEA: 3 µg/l; CA 19-9: 28 µg/l; TK: 8 U/l and TPS: 90 µg/l. These are the upper reference values for the tumour markers measured by the used tests.

### Statistical analysis

Standard frequency tables and descriptive statistics were used to characterize the sample data set. Progression free survival (PFS) and overall survival (OS) were estimated using the Kaplan-Meier method and all point estimates were accompanied by two-sided 95% confidence intervals. PFS was determined from the date of anti-EGFR initiation until the date of first documented progression or death. OS was determined from the date of anti-EGFR initiation until the date of death. Statistical significance of the

differences in PFS and OS according to tumour marker levels was assessed using the log-rank test. Multivariable Cox proportional hazards model was used to evaluate the effect of all potential prognostic factors on the survival indicators. Statistical significance of hazard ratios was assessed by means of the Wald test. The association between RECIST response and tumour marker levels was assessed using the Kruskal-Wallis test. All reported p-values are two-tailed and the level of statistical significance was set at  $\alpha = 0.05$ . The statistical analysis was performed using Statistica (version 12 Cz, TIBCO Software Inc., Palo Alto, CA, USA).

## Results

### Patient characteristics

The study included 102 patients. The median age was 64.0 years (range 36.4–77.6 years). Sixty eight (66.7%) patients were male, 65 (63.7%) had a primary tumour localized in the colon, 54 (52.9%) had metastatic disease at diagnosis, 54 (52.9%) received the cetuximab-containing regimen and 48 (47.1%) received panitumumab-containing regimen. Anti-EGFR mAbs were combined with FOLFOX in 86 (84.3%) patients and with FOLFIRI in 16 (15.7%) patients. The baseline patient characteristics are summarized in Table 1. The baseline levels of serum tumour markers are summarized in Table 2. The median PFS was 10.20 months (95% CI 8.44–12.69), and the median OS was 35.13 months (95% CI 27.06–42.17).

### Relation between baseline levels of serum tumour markers and treatment efficacy

The median PFS and OS for patients with high ( $>3 \mu\text{g/l}$ ) CEA was 10.24 months (95% CI 8.57–13.06) and 34.32 months (95% CI 26.23–42.92) compared to 10.27 months (95% CI 7.29–17.83) and 36.62 months (95% CI 17.91–52.24) for those with low ( $\leq 3 \mu\text{g/l}$ ) CEA ( $p=0.5239$  and  $p=0.9698$ ). The median PFS and OS for patients with high ( $>28 \mu\text{g/l}$ ) CA 19-9 was 8.97 months (95% CI 5.66–12.67) and 19.19 months (95% CI 14.00–27.24) compared to 12.00 months (95% CI 9.25–17.52) and 41.82 months (95% CI 34.42–51.61) for those with low ( $\leq 28 \mu\text{g/l}$ ) CA 19-9 ( $p=0.0232$  and  $p=0.0012$ ) (Figure 1). The median PFS and OS for patients with high ( $>8 \text{ U/l}$ ) TK was 9.95 months (95% CI 7.35–12.67) and 32.91 months (95% CI 19.58–39.86) compared to 10.56 months (95% CI 7.89–17.50) and 41.76 months (95% CI 28.30–52.51) for those with low ( $\leq 8 \text{ U/l}$ ) TK ( $p=0.1209$  and  $p=0.2161$ ). The median PFS and OS for patients with high ( $>90 \mu\text{g/l}$ ) TPS was 9.44 months (95% CI 7.59–12.15) and 32.92 months (95% CI 24.77–40.62) compared to 12.71 months (95% CI

9.80–17.74) and 42.81 months (95% CI 28.40–54.32) months for those with low ( $\leq 90 \mu\text{g/l}$ ) TPS ( $p=0.3831$  and  $p=0.1654$ ). The PFS and OS data are summarized in Table 3 and survival curves for CA 19-9 are shown in Figure 1. The multivariate Cox proportional hazards model confirmed that high baseline serum CA 19-9 was significantly associated with worse PFS (HR=1.871,  $p=0.0330$ ) and also OS (HR=3.903,  $p=0.0006$ ) (Table 4).

There was not observed any significant association between a RECIST response and baseline serum CEA ( $p=0.6071$ ), CA 19-9 ( $p=0.2777$ ), TPS ( $p=1076$ ) nor TK ( $p=0.7958$ ).

**Table 1.** Baseline patient characteristics

Characteristic	Category	n (%)
Gender	Male	68 (66.7)
	Female	34 (33.3)
Age (range 36.4–77.6, median 64.0)	<70 years	84 (82.4)
	$\geq 70$ years	18 (17.6)
Localization of primary tumour	Rectum	37 (36.3)
	Rectosigma	35 (34.3)
	Ascendens	14 (13.7)
	Descendens	8 (7.8)
	Transversum	4 (3.9)
	Sigma	3 (2.9)
	Caecum	1 (1.0)
Localization L/R	Left	82 (80.4)
	Right	17 (16.7)
	Middle/not defined	3 (2.9)
Type of chemotherapy	FOLFOX	86 (84.3)
	FOLFIRI	16 (15.7)
Response (RECIST)	CR	10 (9.8)
	PR	49 (48.0)
	SD	10 (9.8)
	PD	14 (13.7)
	Not Assessed	19 (18.6)
	Grading	G1
	G2	68 (66.7)
	G3	15 (14.7)
Synchronous/metachronous	Unknown	5 (4.9)
	Synchronous	54 (52.9)
	Metachronous	47 (46.1)
RAS/KRAS wild-type	Unknown	1 (1.0)
	RAS	71 (69.6)
	KRAS	31 (30.4)

**Table 2.** Baseline levels of serum tumour markers

	Valid n	Mean	Median	25%-75%	Min-Max
CEA	88	54.3	5.6	2.3–28	0.6–900
CA 19-9	87	112.2	15.0	9–55	1–1900
TPS	75	271.5	144.0	79–275	11.6–2400
TK	85	11.6	8.3	5.2–11.9	2.5–103

## Discussion

Randomized clinical trials have provided evidence for the efficacy and safety of cetuximab as well as panitumumab in the treatment of patients with mCRC [15–27]. The efficacy of anti-EGFR mAbs was reported to be restricted to patients with tumours

harbouring wild-type *KRAS* gene. Thus, *KRAS* gene mutations, occurring in 35-45% of cases, became the most important predictive biomarker in patients with mCRC [2-6]. Extended *RAS* analyses have demonstrated lack of response to anti-EGFR mAbs also in patients with tumours harbouring *NRAS* gene mutations, occurring in 1-6% of CRC cases [2, 7-11]. However, there is still a large proportion of patients with tumours harbouring wild-type *RAS* gene, who derive poor benefit from systemic treatment based on anti-EGFR mAbs.

Molecules used as serum tumour markers play a role in several cancer-related processes including cell

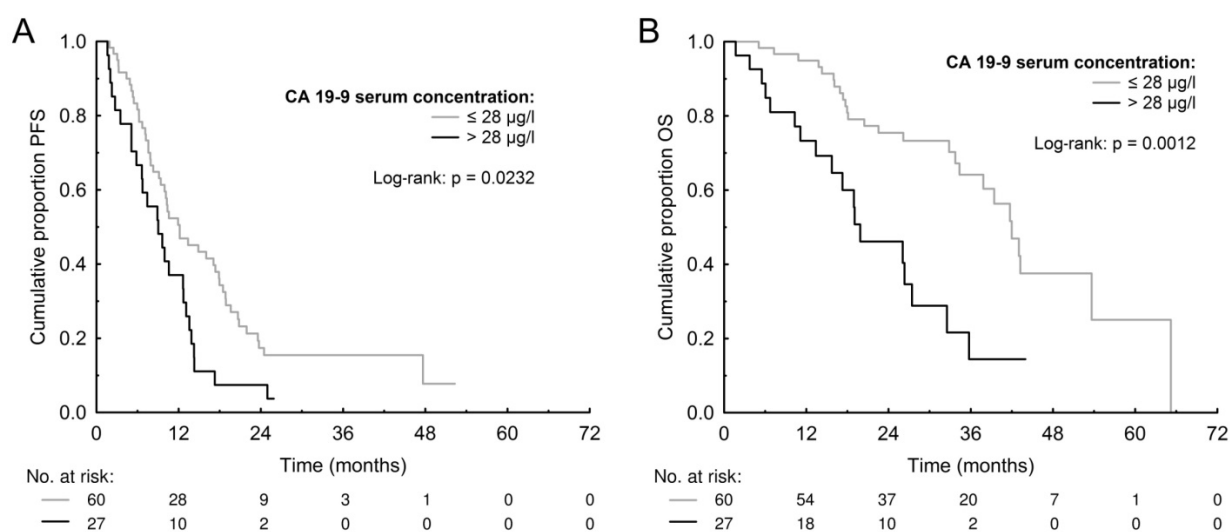
adhesion, proliferation, and tumour angiogenesis and could be useful as a surrogate marker for molecular characteristics of a tumour. CEA, CA 19-9, TK, and TPS are serum tumour markers widely used for primary diagnostics and also follow-up monitoring of patients with CRC. In the present study we focused on their association with the efficacy of anti-EGFR mAbs containing therapy in patients with mCRC treated in the first line. In order to facilitate the clinical interpretation of the results, we used the upper normal values for all the studied tumour markers as a cut-off.

**Table 3.** Progression free survival (PFS) and overall survival (OS) according to baseline levels of serum tumour markers

	CEA		<i>p</i> -value (Log rank test)
	≤ 3 µg/l (n = 26)	> 3 µg/l (n = 62)	
Median PFS (95% CI)	10.27 months (7.29–17.83)	10.24 months (8.57–13.06)	0.5239
3-month PFS (95% CI)	84.44% (70.52–98.37)	92.21% (85.54–98.87)	
6-month PFS (95% CI)	76.47% (60.17–92.76)	75.66% (64.98–86.34)	
12-month PFS (95% CI)	45.26% (25.86–64.65)	44.50% (31.97–57.02)	
18-month PFS (95% CI)	31.24% (13.03–49.45)	22.07% (11.51–32.63)	
Median OS (95% CI)	36.62 months (17.91–52.24)	34.32 months (26.23–42.92)	0.9698
12-month OS (95% CI)	82.25% (67.47–97.03)	89.51% (81.77–97.24)	
24-month OS (95% CI)	63.32% (44.24–82.41)	66.98% (54.60–79.36)	
36-month OS (95% CI)	51.18% (27.83–74.54)	46.26% (30.93–61.59)	
	CA 19-9		<i>p</i> -value (Log rank test)
	≤ 28 µg/l (n = 60)	> 28 µg/l (n = 27)	
Median PFS (95% CI)	12.00 months (9.25–17.52)	8.97 months (5.66–12.67)	0.0232
3-month PFS (95% CI)	95.17% (89.75–100.58)	80.20% (65.19–95.21)	
6-month PFS (95% CI)	81.52% (71.70–91.34)	65.95% (48.09–83.82)	
12-month PFS (95% CI)	49.96% (37.07–62.85)	34.49% (16.58–52.41)	
18-month PFS (95% CI)	34.20% (21.84–46.55)	7.06% (n.a.)	
Median OS (95% CI)	41.82 months (34.42–51.61)	19.19 months (14.00–27.24)	0.0012
12-month OS (95% CI)	94.22% (88.27–100.18)	71.75% (54.48–89.03)	
24-month OS (95% CI)	74.58% (63.21–85.95)	42.32% (21.75–62.90)	
36-month OS (95% CI)	62.36% (47.84–76.89)	< 14.42% (n.a.)	
	TPS		<i>p</i> -value (Log rank test)
	≤ 90 µg/l (n = 22)	> 90 µg/l (n = 53)	
Median PFS (95% CI)	12.71 months (9.80–17.74)	9.44 months (7.59–12.15)	0.3831
3-month PFS (95% CI)	87.53% (73.78–101.27)	90.75% (82.96–98.55)	
6-month PFS (95% CI)	81.74% (65.59–97.88)	75.99% (64.50–87.49)	
12-month PFS (95% CI)	62.18% (41.93–82.42)	36.91% (23.66–50.15)	
18-month PFS (95% CI)	29.95% (10.84–49.07)	19.83% (8.80–30.86)	
Median OS (95% CI)	42.81 months (28.40–54.32)	32.92 months (24.77–40.62)	0.1654
12-month OS (95% CI)	89.38% (76.59–102.18)	87.94% (79.14–96.75)	
24-month OS (95% CI)	73.50% (54.87–92.13)	64.27% (50.69–77.86)	
36-month OS (95% CI)	63.45% (39.55–87.35)	39.89% (23.75–56.04)	
	TK		<i>p</i> -value (Log rank test)
	≤ 8 U/l (n = 39)	> 8 U/l (n = 46)	
Median PFS (95% CI)	10.56 months (7.89–17.50)	9.95 months (7.35–12.67)	0.1209
3-month PFS (95% CI)	87.08% (76.55–97.60)	91.42% (83.33–99.51)	
6-month PFS (95% CI)	76.84% (63.60–90.08)	75.17% (62.69–87.65)	
12-month PFS (95% CI)	47.06% (30.90–63.21)	40.61% (26.42–54.79)	
18-month PFS (95% CI)	31.69% (16.37–47.00)	17.35% (6.41–28.30)	
Median OS (95% CI)	41.76 months (28.30–52.51)	32.91 months (19.58–39.86)	0.2161
12-month OS (95% CI)	86.00% (74.85–97.15)	88.28% (78.99–97.57)	
24-month OS (95% CI)	73.62% (59.06–88.18)	59.28% (44.49–74.07)	
36-month OS (95% CI)	56.62% (37.60–75.64)	42.41% (25.35–59.48)	

**Table 4.** Hazard ratios from multivariable Cox proportional hazard model for progression-free survival (PFS) and overall survival (OS)

Parameter	Category	Progression-free survival (PFS)		Overall survival (OS)	
		HR (95 % CI)	p-value	HR (95 % CI)	p-value
Gender	Female	1	0.2167	1	0.4608
	Male	0.747 (0.470–1.187)		0.789 (0.421–1.481)	
Age	<70 years	1	0.4231	1	0.0677
	≥70 years	1.274 (0.704–2.305)		2.046 (0.949–4.410)	
Localization of primary tumour	Left	1	0.1215	1	0.0941
	Right	1.549 (0.890–2.695)		1.841 (0.901–3.761)	
Synchronous/ metachronous metastases	Synchronous	1	0.1298	1	0.7900
	Metachronous	1.418 (0.902–2.230)		1.092 (0.571–2.087)	
Type of chemotherapy	FOLFOX	1	0.2233	1	0.2146
	FOLFIRI	0.681 (0.367–1.264)		0.588 (0.254–1.360)	
CEA	≤ 3 µg/l	1	0.8426	1	0.2610
	> 3 µg/l	0.940 (0.513–1.723)		0.638 (0.291–1.397)	
CA 19-9	≤ 28 µg/l	1	0.0330	1	0.0006
	> 28 µg/l	1.871 (1.052–3.327)		3.903 (1.789–8.514)	
TPS	≤ 90 µg/l	1	0.7177	1	0.8633
	> 90 µg/l	0.888 (0.465–1.694)		1.078 (0.458–2.540)	
TK	≤ 8 U/l	1	0.2877	1	0.9580
	> 8 U/l	1.315 (0.794–2.178)		0.982 (0.503–1.916)	

**Figure 1.** Kaplan-Meier estimates of progression-free survival (PFS) (A) and overall survival (OS) (B) according to baseline levels of carbohydrate antigen 19-9 (CA 19-9).

CEA plays a role in cell-to-cell adhesion and has a dominant effect in blocking cell differentiation and it cooperates with Myc and Bcl-2 in cellular transformation [28, 29]. The prognostic value of CEA in patients with locoregional CRC or those after metastasectomy has been previously clearly reported [30–34]. On the other hand, it seems to be controversial whether it is an independent prognostic parameter in patients with mCRC [35–37]. In the present study, we did not demonstrate any significant difference in PFS or OS between patients with high compared to those with low baseline levels of CEA. Interestingly, in our previous retrospective study, we observed that high baseline levels of CEA were independently associated with shorter PFS in patients with mCRC treated with systemic therapy based on a monoclonal antibody against vascular endothelial growth factor (VEGF),

bevacizumab [38]. Similar results have been previously published by Prager et al., who reported the lack of association between baseline CEA levels and the treatment efficacy of cetuximab-based therapy and significant association of baseline CEA levels with shorter PFS for patients treated with bevacizumab-based therapies [39]. The study by Prager et al. showed significant association of baseline CEA levels with OS for patients treated with cetuximab-based treatment and also for those treated with bevacizumab-based treatment [39]. The results of our present and also the previous study did not confirm the association of serum CEA with OS [38]. In comparison with our studies, Prager et al. used different cut-off value (26.8 µg/l), which was obtained as a median value of pretreatment CEA levels. The association of baseline CEA levels with PFS in mCRC

patients treated with bevacizumab contrasting with no association in patients treated with anti-EGFR mAbs suggests use of CEA as a surrogate predictive biomarker for targeted treatment in mCRC. This could be explained by several proangiogenic effects of CEA recently described by Bramswig et al., who reported that soluble CEA activates endothelial cells and tumour angiogenesis *via* paracrine manner and the CEA-induced endothelial cell activation was independent of the VEGF-VEGFR 1/2 system [40].

CA 19-9 is an antigen expressed by the glycosylated extracellular MUC1 protein. It plays an important role in cancer invasion by enhancing cell adhesion and promoting angiogenesis indirectly [41]. In the present study, we observed significantly shorter PFS and OS for patients with high CA 19-9 compared to those with low CA 19-9, and the multivariate Cox proportional hazards model confirmed that high CA 19-9 was independently associated with shorter PFS and OS. In CRC patients, the prognostic role of CA 19-9 remains controversial since it has a lower sensitivity as compared to CEA [42]. Elevated CA 19-9 levels have been reported to be strongly associated with poor prognosis, especially in nodal-positive locoregional CRC treated with adjuvant chemotherapy [43, 44]. A large meta-analysis including seventeen studies has been conducted by Yu et al., recently [45]. The meta-analysis included 6,434 patients and its results show that baseline serum CA 19-9 can be used as a prognostic indicator for both metastatic and locoregional stage of CRC [45]. This is in agreement with our results. Interesting data suggesting possible predictive role of CA 19-9 have been recently provided by Formica et al. and Narita et al. [46, 47]. In observational studies, they found that only patients with high baseline levels of CA 19-9 benefited significantly from the administration of bevacizumab in comparison with chemotherapy alone [46, 47]. To our best knowledge, there are currently no data published on the possible predictive role of baseline CA 19-9 level in patients with mCRC treated with anti-EGFR mAbs. The present study did not include a control group not treated with anti-EGFR mAbs, and, therefore, it cannot be concluded whether serum CA 19-9 could serve as a potential predictive biomarker also for anti-EGFR mAbs containing therapy, or it is a prognostic factor associated with mCRC in general.

TK is an enzyme present in most cells, correlating with their proliferative characteristics. It has a key function in the synthesis of DNA and therefore in cell division, it is expressed during cell division in the G<sub>1</sub> and S phase while it is absent in resting cells [48]. In the present study, we did not observe any significant association between PFS or OS

and the baseline level of TK, although several authors have reported on the putative role of TK as a prognostic factor [49, 50].

The TPS assay detects the M3 epitope of cytokeratin 18 or of tissue polypeptide antigen. Cytokeratin 18 is an acid-type cytosolic protein expressed in simple epithelial cells and also by tumor cells [51, 52]. TPS has been shown to indicate tumor cell proliferative activity. TPS has been mostly studied as a biomarker for monitoring of treatment response to palliative chemotherapy in patients with gastrointestinal tumors including mCRC. [53-55]. No impact of baseline level of TPS and survival has been detected in our study.

The principal limitations of the present study are its retrospective design and relatively small number of patients. We should also mention the fact, that 31 (30.4%) patients were tested only for *KRAS* mutation, not for *NRAS* mutation. However, with respect of low incidence of *NRAS* mutations in CRC, this could hardly influence the results [2, 11]. Nevertheless, it is the first study published so far to use a comprehensive serum tumour marker panel in patients with mCRC treated with anti-EGFR mAbs containing therapy.

In conclusion, the results of the conducted retrospective study suggest that the baseline level of CA 19-9 was independently associated with PFS and OS in patients with mCRC treated with anti-EGFR mAbs containing therapy in the first line. CA 19-9 is commonly used serum tumour marker which is simple and easy to detect and thus it is feasible for the use in the routine clinical practice. We have not demonstrated association of baseline levels of CEA, TK and TPS with patients' outcome. Prospective studies on the prognostic and predictive role of serum tumour markers should be performed to confirm these results.

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## Conflict of Interest

OF received honoraria from Roche, GSK and Pfizer for consultations and lectures unrelated to this project. TB and AP received lecture honoraria from Novartis, Pfizer, Bayer-Schering and Roche. TB also received research support from Roche and Novartis.

JF has received honoraria from Astra Zeneca, Roche and Novartis for consultations and lectures unrelated to this project. PH, OS, VL, RK, OT and MS declare that they have no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence this work.

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