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Original Paper

Correlation Between Baseline Osteoprotegerin Serum Levels and Prognosis of Advanced-Stage Colorectal Cancer Patients

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Key Words
Mcrc • OPG • TRAIL

Abstract
Background/Aims: Osteoprotegerin (OPG) is a soluble receptor of the pro-apoptotic cytokine TRAIL which is thought to contribute to tumour development by inhibiting apoptosis or affecting other aspects of tumour biology, including cell proliferation and immune response. Although immunohistochemical studies suggest that OPG correlates with survival in metastatic colorectal cancer (mCRC), only scarce data are available on serum OPG in CRC patients. ***Methods:*** In this pilot study, we assessed the prognostic significance of serum OPG and CEA (Carcinoembryonic antigen) in 81 patients with UICC (Union for International Cancer Control) stage-IV mCRC. OPG was additionally assessed by immunohistochemistry in primary tissue samples from 33 patients of the same cohort. ***Results:*** Baseline serum OPG correlated with CEA (r=0.36, p=0.0011), but independently predicted survival of mCRC patients. Life expectancy was poorer in patients with OPG levels above the median concentration of 51ng/ml (median overall survival [95% confidence interval] 1.8 years [1.3-3.0] vs. 1.0 [0.7-1.2] p=0.013). Patients with high levels of both OPG and CEA had an even poorer life expectancy vs. low-OPG/low-CEA patients (0.9 years [0.6-1.5] vs. 3 years [1.2-4.4], p=0.015), indicating that CEA and OPG have additive prognostic significance. Immunohistochemical analysis of OPG failed to show a correlation between OPG staining and survival (p=0.055) or OPG concentration from matched serum samples. ***Conclusions:*** This pilot study provides evidence of independent prognostic significance of serum OPG in patients with advanced mCRC and warrants its further prospective validation.

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Introduction

Apoptosis mediated by tumour necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) receptors represents a well-established mechanism of immune-mediated tumour surveillance [1]. *In vivo* investigation has shown that the TRAIL-system plays a role in the clearance of metastatic cells [2] and clinical data from human specimens have consistently shown a correlation between TRAIL-Receptor (TRAIL-R) loss and patients' survival across different tumour entities [3-5]. Besides the downregulation of TRAIL-R1 and TRAIL-R2, apoptosis resistance can be caused by overexpression of decoy receptors for TRAIL (such as the membrane receptors TRAIL-R3 and TRAIL-R4 which competitively bind to TRAIL without inducing apoptotic signalling [1]) or by osteoprotegerin (OPG), a third, soluble form of the decoy receptor for TRAIL initially identified as a regulator of bone tissue modelling [1]. According to the proposed role of the TRAIL-system in oncogenesis, OPG is thought to contribute to the development of several tumour entities comprising breast, prostate and gastric cancer [6-8]. More recently, however, it has been proposed that OPG also affects other mechanisms of tumour formation, including enhancement of cell proliferation and paracrine mechanisms influencing tumour microenvironment [9].

We previously provided the first report showing that OPG is a transcriptional target of β-catenin in colorectal cancer, and that its concentration is increased in serum of late-stage mCRC patients [10]. Subsequently, basing on mRNA expression analysis of immunohistochemical samples, other authors independently confirmed that OPG is associated with an aggressive phenotype and metastasis formation in colorectal cancer patients [11]. Very recently, by using a protein screening array, Melzer and colleagues [12] independently observed an increase in OPG serum concentration during neo-adjuvant treatment of rectal tumours. These authors reported a trend towards a poorer survival in CRC patients with high baseline-OPG; on the other hand, an increase of OPG during the neoadjuvant treatment was associated to a better progression-free survival. The concept that OPG favours tumour development has been questioned also by recent data showing that lower immunoreactivity for OPG in tissue samples from CRC is associated to a poorer outcome [13]. These data suggest that OPG plays different roles in different stages of tumour development or in different therapeutic settings. However, in spite of conflicting reports from different immunohistochemical analyses of OPG in colorectal cancer specimens [11, 13], to our knowledge serum OPG has been thus far assessed only in the patients' cohort with rectal carcinoma assessed my Meltzer and colleagues [12]. Following up on these results from the neoadjuvant treatment setting, we contribute to the elucidation of the role of OPG by assessing a cohort of patients with colonic or rectal carcinoma in advanced stage.

Materials and Methods

Patients and serum samples

Sera from patients diagnosed with metastatic colorectal cancer between 1987 to 2006 were obtained before initiation of therapy and were selected by availability of clinicopathologic and long term follow-up data. A subset of 33 patients, selected according to availability of archival pathological material at the Institute of Pathology of our institution was used for immunohistochemical staining of OPG. Blood samples were delivered to the central laboratory through the internal tube mailing system of our institution within 30 min after blood drawing. All specimens were centrifuged at 2, 000g at 4° C for 10 min. The supernatant was transferred into polypropylene cryotubes and stored frozen at 80° C. The study was approved by the ethical committee of the Medical Faculty of the University of Munich. Analyses of serum samples were performed blinded to patient data.

Determination of CEA and of OPG

CEA was quantified using a microparticle immunoenzymometric assay (AxSYM, Abbott Laboratories, Chicago, IL). OPG concentrations in serum of patients with colorectal cancer were assayed by ELISA (Raybiotech) according to the manufacturer's instructions as previously reported [10].

Immunohistochemistry

Immunohistochemical staining was performed on 5 µm sections of tumor tissue. As primary antibody, osteoprotegerin monoclonal rabbit antibody (Abcam, Cat.No. ab124820, dilution 1:220, Cambridge, United Kingdom) was used. Pre-treatment for antigen retrieval was performed by microwaving for 2 x 15 min at 750 W in Enhancer (Linaris, Cat.No. E7000, Dossenheim, Germany). Detection was performed using ImmPress Reagent Kit Anti-Rabbit Ig (Fa.Vector, Cat.No. MP-7401). AEC+ (Dako, Cat.No. K3468, Hamburg, Germany) was used as a chromogen. Finally, slides were counterstained with Hematoxylin Gill`s Formula (Vector Laboratories, Cat. No. H-3401, Eching, Germany).

Immunohistochemical analysis

Evaluation of immunohistochemical staining was performed by assigning cytoplasmic OPG protein level scores ranging from 0 to 3+ for increasing signal intensities. Samples exhibiting a staining intensity score of 0 (no OPG detectable) or 1+ were referred to as "low staining" samples; "high staining" was defined upon detection of staining scores of 2+ and 3+.

Statistical analysis

All statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC). Spearman Correlation test was used to assess the correlation between OPG and CEA. Wilcoxon-Mann-Whitney test was used to explore the relationship between clinicopathological features and OPG and CEA levels. Overall survival was calculated from the date of diagnosis of the primary tumour to the date of death or end of follow-up. Overall survival curves were calculated with the Kaplan–Meier method. Univariate analysis of overall survival according to clinicopathologic data was performed using the Kaplan–Meier method and log-rank tests. Hazard ratios (HRs) were estimated using Cox's regression model.

Results

Patient characteristics

Altogether, 81 serum samples of patients with colorectal cancer in stage IV treated between 1987 and 2006 at the Hospital of the University of Munich could be retrieved and considered for analysis in this study. By the end of follow-up, 67/81 (82.7%) of all patients had died. Overall median survival was 1.4 years (95% CI 1.1-1.7). The 1-, 3-, and 5-year OS rates were 66.6%, 24.1% and 11.2%, respectively. Altogether, the demographic and clinical-pathological features of this patients collective are in line with the expected characteristics of colorectal cancer patients in Germany. The main characteristics are summarized in Table 1.

OPG-serum concentrations directly correlate with CEA but independently predict the outcome of stage IV mCRC patients

Since CEA is an established tumour marker of colorectal cancer, CEA serum levels were first compared to those of OPG: as assessed by the Spearman correlation coefficient, a positive correlation between the serum concentration of these two se-

Table 1. Patients' characteristics

Characteristic	Frequency	%	Cumulative frequency	Cumulative %
Gender				
Male	46	56.79	46	56.79
Female	35	43.21	81	100.00
Localization				
Sigma	12	14.81	12	14.81
Rectum	23	28.40	35	43.21
Colon	46	56.79	81	100.00
Histology				
Adenocarcinoma	65	89.04	65	89.04
Mucinous Adenocarcinoma	6	8.22	71	97.26
Squamous cell carcinoma	1	1.37	72	98.63
Signet ring cell carcinoma	1	1.37	73	100.00
T-Stage				
2	5	6.25	5	6.25
3	53	66.25	58	72.50
4	22	27.50	80	100.00
N-Stage				
0	16	21.62	16	21.62
1	33	44.59	49	66.22
2	25	33.78	74	100.00
Grading				
2	22	30.56	22	30.56
3	50	69.44	72	100.00

Table 2. Multivariate analysis of survival comprising serum OPG and CEA concentration and clinical and pathological variables

	OPG pg/ml			CEA ng/ml		
	Median	Range	p	Median	Range	p
Age						
<65	46.6	19.0 - 112.6	0.132	29.6	1.1 - 3945.0	0.365
≥65	54.7	29.4 - 135.4		19.9	1.0 - 3471.0	
Gender						
M	47.1	20.5 - 135.4	0.257	14.4	1.0 - 3945.0	0.082
F	56.1	19.0 - 112.6		31.5	1.2 - 2298.0	
T-Stage						
T2/T3	48.3	19.0 - 106.3	0.046	26.3	1.0 - 3945.0	0.543
T4	55.6	29.4 - 135.4		13.5	1.1 - 2778.0	
N-Stage						
N0	43.9	20.5 - 106.3	0.208	9.2	2.4 - 2298.0	0.609
N1/2	52.3	19.0 - 135.4		28.2	1.0 - 3945.0	
Grading						
G2	47.0	25.9 - 106.3	0.085	21.4	1.2 - 203.0	0.249
G3	55.2	19.0 - 135.4		29.0	1.0 - 3945.0	

Table 3. Long Rank test of different clinical and pathological variables

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
Age				
<65	34/44	1.7	1.0-2.5	0.134
≥65	33/37	1.2	1.0-1.6	
Gender				
M	38/46	1.6	1.0-2.1	0.541
F	29/35	1.2	0.9-1.9	.
T-Stage				
T2/T3	47/58	1.5	1.1-1.8	0.535
T4	19/22	1.2	0.5-2.5	.
N-Stage				
N0	10/16	2.5	0.5	0.093
N1/2	51/58	1.3	1.1-1.7	.
Grading				
G2	17/22	1.9	1.0-3.6	0.106
G3	43/50	1.2	1.0-1.6	.

rum markers was found (r=0.36, p=0.001).

Subsequently, a survival analysis according to different clinical-pathological variables as well as OPG and CEA serum levels and the respective median concentrations as stratification

factor was conducted. While no clinical and pathological variables significantly correlated with patients' survival (Table 2 and Table 3), outcome was poorer in patients with serum OPG levels above the collective's median concentration of 51 pg/ml (median survival in years and confidence interval: 1.8 [1.3-3.0] vs. 1.0 [0.7-1.2] p=0.013) and in patients with CEA levels above the median concentration of 27ng/ml (2.2 years [1.1-3.3] vs. 1.2 [0.9-1.6] p = 0.014 – Table 4, Fig. 1). A multivariate analysis of survival comprising serum OPG and CEA concentration and clinical and pathological variables confirmed that OPG and CEA have independent prognostic relevance in determining patients' outcome (HR and 95% confidence interval for CEA and OPG were respectively 1.69 [1.03-2.79] and 1.68 [1.03-2.75] - Table 5).

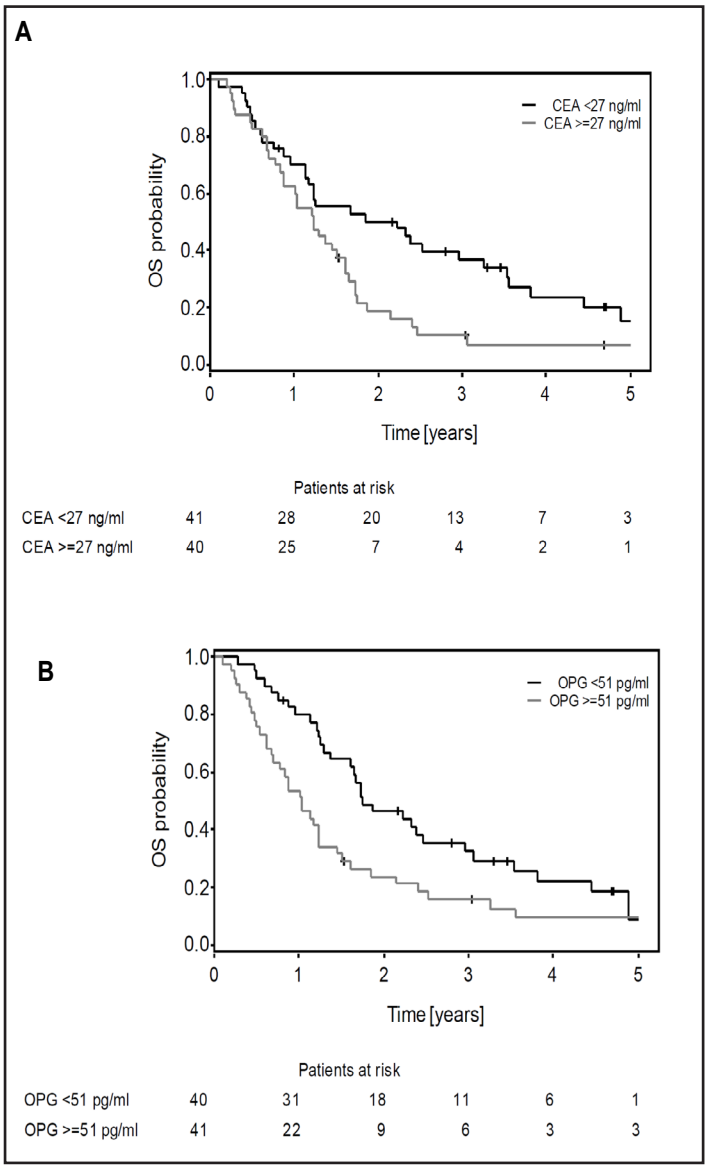

Fig. 1. OPG correlates with patients' survival. Survival curves showing overall survival according to median values of CEA (A) and OPG (B) concentrations. In graphs, censored cases are indicated by a cross.

Combined assessment of CEA and OPG defines a patients' population with poor outcome

Due to the independent prognostic effect of CEA and OPG, a further analysis was conducted to assess patients' outcome according to the combined assessment of these both biomarkers. Patients with both CEA and OPG concentrations above the cut-off levels defined by the respective median values showed, as expected, a poorer prognosis in comparison to patients with both low CEA and OPG concentrations. Survival rates at 1, 3, and 5 years in these two groups were 78.4 vs. 50%, 46.5 vs. 10% and 13 vs. 10% respectively (p=0.015 – Fig. 2, Table 4). In line with the results of the multivariate analysis showing an independent prognostic value of OPG and CEA, these data show that the combined assessment of CEA and OPG enhances the prognostic significance of each biomarker considered individually.

Immunohistochemical staining shows a trend toward increased survival in tumour specimens with high OPG-immunoreactivity

To assess whether the effect of OPG serum concentrations on survival reflects a tumour-derived increased synthesis of OPG, OPG immunoreactivity was assessed in a subgroup of

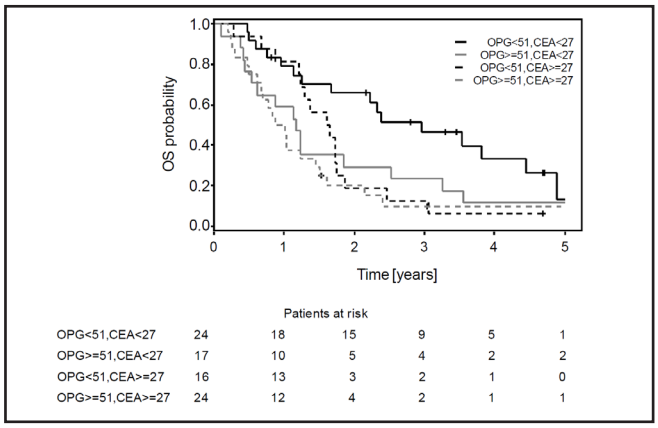

Fig. 2. Patients' stratification by combined assessment of CEA and OPG defines a patients' population with poor outcome. Survival of patients according to serum levels of both OPG and CEA. Kaplan-Meier curves represent overall survival according to: both OPG and CEA "high" serum levels, low-OPG and high-CEA, low-CEA and high-OPG and both OPG and CEA "low" serum levels. In graphs, censored cases are indicated by a cross.

33 tissue specimens from primary tumours of the same patients' collective. Immunohistochemical evaluation was performed by assigning OPG staining scores ranging from 0 to 3+ (Fig. 3A-D). A trend towards a poorer outcome was observed in patients with high OPG-staining (2+ and 3+) in comparison to patients with low staining intensity (0 and 1+, p=0.055) (Fig. 3E, Table 6). However, no correlation was found between serum OPG and OPG-immunoreactivity in matched histological specimens (p=0.47).

Table 4. Survival of stage IV patients after stratification acc. to OPG and CEA median concentration

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
OPG (ng/ml)				
<51	31/40	1.8	1.3-3.0	0.013
≥51	36/41	1.0	0.7-1.2	
CEA (ng/ml)				
<27	31/41	2.2	1.1-3.3	0.014
≥27	36/40	1.2	0.9-1.6	
OPG/CEA				
<51/<27	16/24	3.0	1.2-4.4	0.015
<51/≥27	15/17	1.2	0.4-2.5	
≥51/<27	15/16	1.6	1.2-1.8	
≥51/≥27	21/24	0.9	0.6-1.5	

Table 5. Multivariate analysis of survival according to CEA and OPG serum levels

		HR	95% CI	P
OPG	≥51 vs <51	1.68	1.03-2.75	0.0385
CEA	≥27 vs <27	1.69	1.03-2.79	0.0397

Discussion

Our assessment of a cohort of patients with metastatic colorectal cancer shows for the first time that high serum OPG has a prognostic significance in mCRC patients which is independent of the well-established prognostic value of CEA. Our data are in agreement with previous immunohistochemical findings provided by Tsukamoto and colleagues [11], who found that OPG staining was increased in tumours of patients with metastasic disease and was associated with poorer prognosis. Our results are also in keep with the Tromsø study, a large Norwegian study which prospectively investigated a large population cohort showing that serum OPG is associated with increased risk of developing cancers of gastrointestinal origin and that OPG predicts cancer-related mortality [14].

This data also confirm the very recent findings by Meltzer et al. showing that high baseline OPG tends to correlate with poor survival in the neoadjuvant treatment setting of rectal cancer [12].

Our data are instead inconsistent with the observations reported by Kim and colleagues [13] who found that low immunohistochemical staining intensity for OPG correlated with hepatic metastasis formation and poor outcome. Such results were corroborated by the high degree of methylation found in the promoter region of OPG in cancer cells and by *in vitro* experiments showing decreased MMP-2 and VEGF-A in response to incubation with recombinant OPG. These data show that beyond the postulated role of OPG in apoptosis resistance, OPG might play different roles yet to be defined e.g. in cell proliferation and angiogenesis. In addition, these data suggest that hypermethylation is a mechanism contributing to OPG regulation in addition to the beta-catenin-driven transcription previously reported by us [10].

Independently of possible additional roles of OPG in tumour biology, however, the discrepancies between the observations by Kim et al [13]. and ours on the effect of OPG on patients' survival may be attributable to differences in size and characteristics of the investigated collective and to the different methods used, and in particular to the utilization of immunohistochemistry to assess OPG in tissue specimens vs. ELISA-based assessment of OPG in serum. OPG has been shown to be expressed not only by cancer cells

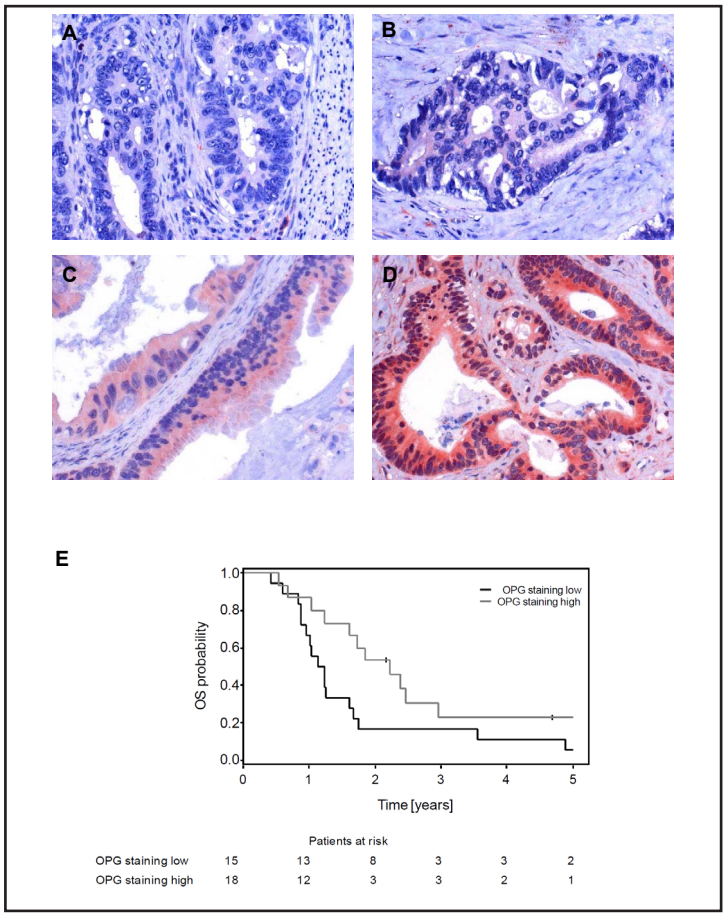

Fig. 3. Immunohistochemical staining of OPG shows a trend toward an increased survival in OPG-high tumors. Representative negative staining of OPG in tumor tissue (A) and (B-D) of increasing staining intensity of OPG (1 to 3+). Original magnification ×400. (E) Survival of patients according OPG staining as defined by high vs. low staining intensities.

Table 6. Survival according to immunohistochemical staining of OPG

	Events / Cases	Overall Survival (years)		P
		Median	95% CI	
OPG staining intensity				
low	11/15	2.2	1.0-3.0	0.055
high	17/18	1.2	0.9-1.6	

but also by cells of the tumor microenvironment, ([15, 16] and reviewed by Goswami and Sharma-Walia [9]); assessment of serum OPG has therefore the advantage of accounting for OPG deriving also from other sources than the tumour cells (e.g. blood vessels and immune cells [9]). Furthermore, measurement of OPG in serum is less influenced by the investigator-related variability of immunohistochemical investigation, and is likely more representative than immunohistochemical assessment of OPG in biopsies from single tumour lesions, which can be influenced by clonal effects and the tumour heterogeneity typical of late-stage tumours. The lack of correlation between serum OPG and immunohistochemical staining of OPG from the subset of matched tissues samples in our cohort might reflect these factors.

Our data therefore reinforce the notion of OPG as marker of poor survival in late-stage colorectal cancer patients. Our report is consistent with the proposed role of the TRAIL-system in carcinogenesis [3-5], with previous observations from different tumour entities [6-8], with the recent report on pre-therapeutic baseline levels of OPG in rectal carcinoma patients [12], and with data from a large prospective epidemiological Norwegian study showing that OPG in serum correlates with cancer-related mortality [14].

The additional recent finding by Meltzer et al. that increasing OPG levels during treatment correlate with a favourable prognosis [12] suggests that OPG may have properties which deserve to be further investigated. In particular, additional studies should assess whether changes in OPG concentration during therapy play a functional role in determining response to treatment or rather reflect increased release of OPG from tumours responding to chemotherapy or radiation-treatment.

Confirming a biological significance of OPG in the development of colorectal cancer could open potential therapeutic perspectives: the discovery of a different role of OPG within the OPG–RANKL–RANK system led to the development of denosumab, which is employed to prevent the consequences of bone fragility in patients with bone metastases [17]. In a similar way, antibodies targeting OPG might be used as cancer treatment in tumours overexpressing OPG.

Conclusion

In summary, our paper has some limitations due to the fact that immunohistochemical and genetic characterization and treatment data could not be retrieved for all individuals of this cohort. However, our pilot study is to our knowledge the first report on the prognostic effect of OPG in pre-therapeutic sera of metastatic colorectal cancer patients and warrant prospective investigation of OPG in serum of patients in different tumour stages and therapeutic settings.

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Disclosure Statement

The authors have no conflicts of interest to declare

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