

# Serum Matrix Metalloproteinase-2, -7 and -9 (MMP-2, MMP-7, MMP-9) levels as Prognostic Markers in Patients with Colorectal Cancer

Elena Kostova<sup>1\*</sup>, Maja Slaninka-Miceska<sup>1</sup>, Nikola Labacevski<sup>1</sup>, Krume Jakjovski<sup>1</sup>, Jasmina Trojancanec<sup>1</sup>, Emilija Atanasovska<sup>1</sup>, Vlado Janevski<sup>2</sup>, Redzep Selmani<sup>2</sup>, Gordana Petrushevska<sup>3</sup>, Vesna Janevska<sup>3</sup>

<sup>1</sup> Department of Preclinical and Clinical Pharmacology and Toxicology, Faculty of Medicine, Ss. Cyril and Methodius University, 50 Divizija bb, 1000 Skopje, Republic of Macedonia. <sup>2</sup> Digestive Surgery Clinic, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, R. Macedonia. <sup>3</sup> Department of Pathology, Faculty of Medicine, Ss. Cyril and Methodius University, 1000 Skopje, Republic of Macedonia.

## Abstract

**Introduction:** Matrix metalloproteinases are produced by tumour cells, hence, they may be associated with tumour progression including invasion, migration, angiogenesis and metastasis. Finding prognostic markers to better identify patients with higher risk for poor survival would be valuable in order to customize pre- and postoperative treatment as well as to enable closer follow-up of these patients. Aim of our study was to examine MMP-2, MMP-7 and MMP-9 serum levels and correlated them with pathological data such as stage of the colorectal cancer (CRC) and outcome.

**Methods:** The investigation included 82 patients with operable CRC without distant metastases, who had undergone blood tests in order to determine the MMP-2, MMP-7 and MMP-9 serum levels in the following time periods: preoperatively, 3, 6, 9 and 12 months postoperatively.

**Results:** The values of the investigated MMPs decrease postoperatively and start to increase 6 months later in patients of all stages of the disease, reaching the highest value 12 months postoperatively with statistically important differences of MMP-2, MMP-7 and MMP-9 serum levels in terms of disease staging and defined points of time. Analysis of the results showed that the MMP-2 serum levels obtained 3 and 12 months postoperatively, than MMP-7 serum levels 12 months postoperatively and the MMP-9 serum levels in all analyzed points in time were in significant association with the CRC patients' outcome.

**Conclusion:** The MMP-2, MMP-7 and especially MMP-9 serum values could be important indicators for diagnosis of the patients with CRC and for monitoring of disease progression.

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## Introduction

The degradation of extracellular matrix (ECM) is a crucial step in tumour progression, aggressive growth and metastases. The invasion of cancer cells within the basement membrane depends on matrix metalloproteinases (MMPs) and their inhibition activities (1). MMPs are a family of extracellular structurally related zinc-

dependent endoproteases capable of degrading all the ECM components. At present, 23 members of the human MMP gene family are known. Based on their structure and substrate specificity, MMPs are classified into subgroups of collagenases, stromelysins and stromelysin-like MMPs, matrilysins, gelatinases, membrane-type MMPs (MT-MMPs) and other MMPs (1). MMPs play an important role in the physiologic degradation of ECM, e.g., in tissue morphogenesis, tissue repair and angiogenesis. MMPs have also important functions in pathologic conditions characterized by excessive degradation of ECM, such as rheumatoid arthritis, osteoarthritis, periodon-

\* Corresponding author: Elena Kostova, Department of Preclinical and Clinical Pharmacology and Toxicology, Faculty of Medicine, Ss. Cyril and Methodius University, 50 Divizija bb, 1000 Skopje, Republic of Macedonia  
E-mail: eli\_kos\_pet@yahoo.com, Phone: +389 70 323534

titis, autoimmune blistering disorders of the skin and in tumour invasion and metastasis. MMPs are produced by tumour cells; hence, they may be associated with tumour progression including invasion, migration, angiogenesis and metastasis (2, 3). Among the MMPs, matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9), as members of gelatinases, plays important roles in the migration of malignant cells, because of their ability to degrade type IV collagen (4). The mechanisms of activation of these enzymes are different. MMP-9 modulates permeability of the vascular endothelium, whereas MMP-2 promotes cleavage of extracellular matrix proteins and is intensively expressed by tumour and stromal components of cancer (5). Matrix metalloproteinase 7 (MMP-7) or matrilysin, as a member of stromelysins is able to induce cell apoptotic impairment. Matrilysin can regulate angiogenesis either by inducing a direct proliferative effect on vascular endothelial cells or producing angiogenesis inhibitors (angiostatin, endostatin and neostatin-7) or by enriching the variety of angiogenesis mediators, such as the soluble vascular endothelial growth factor (sVEGF) (6). Increased levels of matrix metalloproteinase in tumour tissues or in blood circulation have been found to correlate with many cancers, including colorectal cancer (CRC). Several previous studies have shown that MMPs may play an important role as an indicator for appearance of CRC and its progression (7, 8, 9). Colorectal cancer (CRC) is a common disease and it is one of the leading causes of cancer related deaths in developed countries (10). Despite improvements in surgical techniques, adjuvant and neo-adjuvant chemotherapy, the 5-year survival rate in patients with CRC ranges from 5-90% with tumour progression (stages: I: 90-95%, II: 75-85%, III: 50-60% and IV: 0-10%). The prognosis in patients without distant metastasis varies from 50-95% depending on the tumour stage (11). The correct staging of each CRC patient is crucial in order to plan an optimal treatment regimen. It is widely recognised that prognostic information based on clinical and histopathological investigation is insufficient, although tumour stage and lymph node involvement are the main prognostic tools in evaluating cancer specific survival. It is

questionable to expose a large number of patients to adjuvant treatment with considerable side effects without indications that they will benefit from such treatment. Finding prognostic markers to better identify patients with higher risk for poor survival (12, 13) would be valuable in order to customise pre- and postoperative treatment as well as to enable closer follow-up of these patients. In our study, we examined MMP-2, MMP-7 and MMP-9 serum levels and correlated them with pathological data such as stage of the disease and the patients' outcome.

## Methods

The study included a total of 82 previously untreated CRC patients, 30 (36.58%) females and 52 (63.41%) males (aged 43 to 75 years, mean age of 67.85; SD±9.67) with operable CRC, without detectable distant metastases, who respected the medical instructions and were available for follow-up. All patients underwent a surgical resection of the primary neoplasm at the University Clinic for Abdominal Surgery in Skopje in the period of 2 years (2007-2009). Blood samples from all patients were drawn before surgical treatment, as well as 3, 6, 9, and 12 months postoperatively in order to examine MMP-2, MMP-7 and MMP-9 serum levels. None of the CRC patients had received chemotherapy before blood sample collection. To standardize clotting conditions, all sera were separated within 1 h after blood collection, aliquoted and stored at -80°C until assayed. Serum levels of MMP-2, MMP-7 and MMP-9 were determined using a quantitative solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) according to the manufacturer's instructions. MMP-2, MMP-7 and MMP-9 technique can detect both pro- and active forms of recombinant human MMP-2, MMP-7 and MMP-9. High concentrations of MMP-2, MMP-7 and MMP-9 were diluted with calibrator, to produce samples with values within the dynamic range of the assay. The surgically removed specimens were histopathologically analyzed at the Institute of Pathology of the Faculty of Medicine, Skopje, where the pathological stage was defined for every patient according to the International Union

against Cancer (UICC-pTNM) and American Joint Committee on Cancer (AJCC) 2010. Forty-three patients with stage II B and III (A,B,C) received adjuvant chemotherapy at the Institute for Radiotherapy and Oncology in Skopje. Correlations were made between the MMPs serum levels and the pathological parameters.

**Statistical analysis**

Descriptive statistics (mean) are given according to normality of the distribution. Normality of the distribution was determined by Kolmogorov-Smirnov’s test. Analysis of variance with Kruskal-Wallis test was first used in the analysis of different sample types. In the case of significant results, the analyses were continued by pairing the variables and analysing them with Mann-Whitney’s U-test. Fisher’s exact probability test and Pearson’s Chi-Square test (r) were used for testing the association (linearity of the correlation of serum concentrations) between MMPs and major prognostic variables in CRC, such as grade and stage. P-values less than 0.05 (p<0.05) were considered as statistically significant.

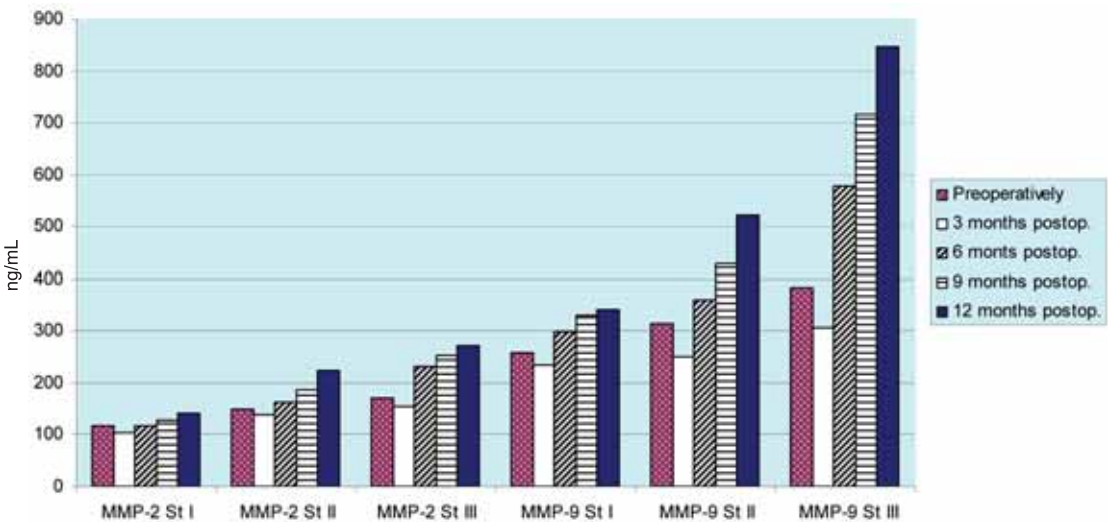
**Results**

There have been 17 (20.73%) patients in stage I of the disease, 40 (48.78%) patients in stage II and 25 (30,48%) patients in stage III. Lymph node

**TABLE 1.** Staging of the disease in CRC patients according to AJCC

Stage	pTNM	Number of patients (n=82)	%
I	pT1 N0 M0	8	20.73
	pT2 N0 M0	9	
II	pT3 N0 M0	22	48.78
	pT4a N0 M0	18	
III	pT3 N1b M0	7	30.48
	pT3 N2a M0	9	
	pT4a N1b M0	4	
	pT4a N2b M0	5	

metastases were substantiated in 25 (30.48%) patients and were not found in 57 (69.51%) patients with different pT category (Table 1). The majority of patients were with pT-3N0M0 (26.82%), i.e. patients in stage II A of the disease, and the smallest number of patients were with pT4aN1M0 (4.87%), i.e. patients in stage III B of the disease. The mean MMP-2, MMP-7 and MMP-9 serum levels in terms of disease staging and defined points of time are shown in Table 2, Figure 1 and Figure 2. The mean MMP-2 serum levels shown in Table 1 and Figure 1, in patients with stage I of the disease, decreased after the operation and started slightly to increase after the 3th month postoperatively. This might be due to the 6 patients with poor outcome in



**FIGURE 1.** Mean MMP-2 and MMP-9 (ng/mL) serum values in terms of disease staging and defined points of time

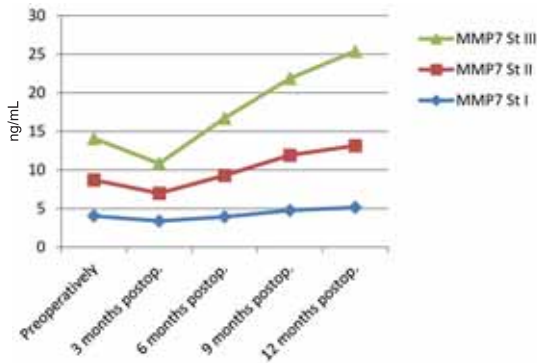


FIGURE 2. Mean MMP-7 (ng/mL) serum levels in terms of disease staging and defined points of time

this group, with mean survival time of 28.5 months. The obtained MMP-2 mean serum levels of the patients in stage II of the disease goes very similarly to the mean serum levels of patients in stage I and reaches the peak in the 12th month postoperatively; that may be due to the 19 out of 40 patients with poor outcome, with mean survival of 26.66 months. The mean levels of MMP-2 in patients in stage III of the disease, decreased after tumour resection, increased abruptly after the 3 months postoperatively and continued to increase slightly after 6 months postoperatively. This might be due to the poor outcome of more than half of the patients, 18 out of 25

patients with mean survival time of 21.8 months. There was a significant difference between the mean MMP-2 serum levels before tumour resection and after the operation, i.e. between the preoperative and postoperative levels during defined control points of time in all encompassed CRC stages. There was a significant difference of the MMP-2 serum levels among the stages ( $p < 0.05$ ). The mean MMP-7 serum levels are separately shown in Figure 2, and they show a similar tendency of postoperative decreasing and permanent increasing after the 3 months, especially in stage III. We did not find a significant difference between the MMP-7 serum levels in stage I and stage II during each control points in time, but there was a significant difference between the MMP-7 serum levels from stage I and II in terms of stage III ( $p < 0.05$ ). There was a significant difference between preoperative and postoperative MMP-7 serum levels ( $p < 0.05$ ). There were significant differences between MMP-9 serum levels in all stages ( $p < 0.01$ ), as well as between preoperative and postoperative serum levels in all defined points of time. The number of patients who received chemotherapy and the outcome of all included patients in the study are shown in Table 3. We found significant differences in terms of

TABLE 2. Mean serum levels of MMP-2, MMP-7 and MMP-9 (ng/mL) in terms of disease staging and defined points of time

Stage	MMP-2 (ng/mL)			MMP-7 (ng/mL)			MMP-9 (ng/mL)		
	I	II	III	I	II	III	I	II	III
Preoperatively	117.62	147.96	169.72	4.04	4.66	5.38	259.03	313.35	384.34
3 months postoperat.	104.85	137.5	154.38	3.38	3.6	3.86	234	249.31	307.54
6 months postoperat.	117.33	162.45	231.9	3.92	5.37	7.42	298.63	358.143	576.86
9 months postoperat.	126.99	186	252	4.76	7.15	9.96	329.43	429.83	717.11
12 months postoperat.	140.73	223.34	271.51	5.14	8	12.24	341.11	521.65	846.45

TABLE 3. Patients with different stage of the disease who received chemotherapy and the outcome of the disease

Stage N=82	With chemotherapy		Without chemotherapy		Poor outcome	
		%		%		%
Stage I	/	/	17	20.73	6	7.31
Stage II A	/	/	22	26.82	8	9.75
Stage II B	18	21.95	/	/	11	13.41
Stage III B	20	24.39	/	/	15	18.29
Stage III C	5	6.09	/	/	3	3.65
Total	43	52.43	39	47.56	43	52.43

**TABLE 4.** Statistically significant correlations with poor outcome in CRC patients

Parameter	P	r
Stage	<0.01	0.635
pT	<0.05	0.331
pN	<0.05	0.618
MMP-2 preoperatively	<0.01	0.156
MMP-2 3 months postoperat.	<0.05	0.793
MMP-2 6 m postoperatively	NS	/
MMP-2 9 m postoperatively	NS	/
MMP-2 12 m postoperatively	<0.01	0.548
MMP-7 preoperatively	<0.05	0.391
MMP-7 3 m postoperatively	NS	/
MMP-7 6 m postoperatively	NS	/
MMP-7 9 m postoperatively	NS	/
MMP-7 12 m postoperatively	<0.05	0.728
MMP-9 preoperatively	<0.01	0.619
MMP-9 3 m postoperatively	<0.01	0.351
MMP-9 6 m postoperatively	NS	/
MMP-9 9 m postoperatively	<0.01	0.219
MMP-9 12 m postoperatively	<0.01	0.416

the poor outcome in the CRC patients between stage I and stage II B ( $p < 0.05$ ), between stage I and stage III ( $p < 0.01$ ), as well as between stage II A and stage III ( $p < 0.01$ ). Associations of the examined parameters and poor outcome are shown in Table 4, where it is shown that MMP-2 serum levels preoperatively, at 3 and 12 months postoperatively, are in a significant correlation with the lethal outcome of the CRC patients, than MMP-7 serum levels preoperatively and at 12 months, as well as MMP-9 preoperatively and at 3, 9 and 12 months postoperatively.

## Discussion

MMP-2 is a collagenase discovered for the first time in metastatic murine tumours and in cultured human melanoma cells. It is secreted by fibroblasts, endothelial cells, osteoblasts, keratocytes, macrophages and many malignant cells (14). MMP-2 is expressed in numerous normal tissues as the lungs, heart, kidneys, placenta, and the muscles. MMP-2 is synthesized and secreted as an inactive proenzyme, while as an active enzyme it degrades the type-IV collagen as well as the type I, V, VII and X, the laminin, the elastin, the fibronectin and the proteoglycans (15, 16). MMP-7 (matrilysin) is a proteolytic enzyme,

which is expressed in glandular and ductal epithelium of many tissues. It degrades type IV and X collagen, the elastin, the fibronectin, the laminin, the osteopontin, the proteoglycans, as well as numerous other substrates. MMP-7 is also synthesized and secreted by cancer cells as an inactive proenzyme. After the activation, the MMP-7 is found in soluble active form or bound to the membrane of the tumour epithelium cells, which has also a proteolytic capability (17). MMP-9 was discovered for the first time as an elastin binding protein, which is synthesized by the macrophages and the polymorphonuclears. In normal conditions, MMP-9 is expressed only in several cell types as trophoblasts, osteoclasts, leukocytes, and dendritic cells. It is also being expressed by several types of tumours, in the tumour cells and in the stromal cells. MMP-9 also degrades the components of the ECM, especially type IV, V, VII, X, XI, XIV collagen, fibronectin, elastin, osteonectin and entactin (18). Diverse results have been obtained from numerous examinations which have been made in order to determine the significance of the MMPs in the diagnosing of malignancies and to determine their influence on the disease outcome (19). In 1998, in order to determine the active and inactive MMP-2 and MMP-9 expression, Pearsons, Warson, Collins, et al. examined 53 colorectal carcinomas, 15 colorectal adenomas and 15 gastric carcinomas. They found out overexpression of the two enzymes in both the colorectal and gastric carcinomas (20). The aim of the examination conducted by Tuton, George, Eccles, et al. was to determine the MMP-2 and MMP-9 distribution in CRC patients and to compare the levels of the two enzymes in patients' plasma and the changes that occur in the plasma after the resection. They wanted to discover whether plasma levels are a reflection of the clinical staging and the development of the disease. Their results showed that MMP-2 plasma levels were considerably elevated in patients with CRC; they considerably decreased after surgical resection of the tumour, and MMP-9 serum levels were considerably elevated in all stages of the disease in CRC patients, while they decreased after the surgical resection of the neoplasm (21). On the contrary, the Ruokolainen's investigation for the prognostic role of the MMP-2 and

MMP-9 and their tissue inhibitors (TIMP-1 and TIMP-2) in squamous head and neck cancer, showed that serum MMP-2 immunoreactive protein levels in healthy patients were higher than those in patients with cancer, while the MMP-9 and TIMP-1 levels were considerably higher in patients with squamous carcinoma. The authors determined an important correlation between the serum levels of MMP-9 and TIMP-1 with immunohistochemical expression of MMP-9 and TIMP-1 from tumour tissue (22). Dragutinovic, Radonjic, Petronijevic, et al. in their study of 32 CRC patients and another 11 controls using immunohistochemistry and serum values of CEA, CA 19-9 and MMP-2 and 9 reported an important correlation of the MMP levels with the staging, but not with the CEA and CA 19-9 serum levels. They concluded that the serum MMP-2 and MMP-9 detection can be a useful tool for identification of the CRC patients (23). Maurel, Nadal, Garcia-Albeniz et al. examined the MMP-7 serum levels in 87 healthy patients and in 120 CRC patients in order to determine the serum level prognostic significance of this enzyme. They found out that patients with advanced cancer had considerably higher mean MMP-7 levels in comparison with those without metastases and in comparison with healthy subjects. They discovered a significant correlation between the MMP-7 levels and shorter survival time, which led them to the conclusion that the elevated MMP-7 serum levels are an independent prognostic factor for survival in patients with advanced CRC (24). We have observed (unpublished data) that in CRC patients with low MMP-7 but high LDH levels, MMP-7 values can increase during chemotherapy treatment, and would be therefore implicated in early acquired resistance, after initial response.

Therefore, we speculate that MMP-7 would be implicated in primary chemoresistance in the subgroup of patients with well-known poor prognosis, to an even more aggressive phenotype, or both. Leelawat, Sakchinabut, Narog, and Wannaprasert analyzing the CEA, CA 19.9, MMP-7 and MMP-9 serum levels in patients with cholangiocarcinoma detected that only MMP-7 level was considerably higher in patients with cancer (25). Levels of total MMP-7 can be measured in human serum and it is feasible using a simple ELISA technique, as this has been recently shown in few other studies. Serum measurements of total MMP-2, MMP-7 and MMP-9 can be considered as an indirect estimation of tumour MMP-2, MMP-7 and MMP-9 expression. Other techniques, such as zymography, are useful to distinguish between activated MMP-2, MMP-7, MMP-9 and pro-forms and might be implemented in the near future for further analysis.

## Conclusion

In our examination, we have determined that the MMP-2, MMP-7 and MMP-9 serum levels decrease considerably after the resection of the primary neoplasm, as well as the MMP-2 serum levels at 3th and 12th month postoperatively, than MMP-7 serum levels preoperatively and at 12th month and the MMP-9 serum levels at 3th, 9th and 12th month postoperatively are in correlation with the poor outcome of the CRC patients. Subsequently, detection of serum MMP-2, MMP-7 and MMP-9 is feasible and done through a non-invasive technique. They could be potential serum markers which may be useful in the CRC detection and in monitoring of the disease progression.

## Competing interests

Authors declare no competing interests.

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