

## Pretreatment hepatocyte growth factor and thrombospondin-1 levels predict response to high-dose chemotherapy for multiple myeloma

L. POUR<sup>1,2</sup>, H. SVACHOVA<sup>2</sup>, Z. ADAM<sup>1,2</sup>, Z. MIKULKOVA<sup>2</sup>, L. BURESOVA<sup>3</sup>, L. KOVAROVA<sup>1,2</sup>, T. BUCHLER<sup>4</sup>, M. PENKA<sup>1</sup>, J. VORLICEK<sup>1</sup>, R. HAJEK<sup>1</sup>.

<sup>1</sup>Departement of Internal Medicine-Hematooncology, University Hospital Brno, Masaryk University Brno Czech Republic, e-mail: lpour@fnbrno.cz, <sup>2</sup>University research centre – Czech myeloma Group, <sup>3</sup>Institute of Biostatistics and Analyses at the Faculty of Medicine and the Faculty of Science of the Masaryk University, <sup>4</sup>Departement of Oncology, 1st Faculty of Medicine and Thomayer University Hospital, Prague, <sup>5</sup>Departement of Clinical hematology University Hospital Brno.

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Our aim was to establish whether the pretreatment levels of angiogenesis activators and inhibitors can be used to predict clinical responses to treatment that included high-dose chemotherapy with peripheral stem cell support.

We analyzed samples and treatment outcomes of 96 patients with MM enrolled in the CMG 2002 randomized clinical trial and treated with induction chemotherapy and high-dose chemotherapy with stem cell support. Concentrations of vascular endothelial growth factor (VEGF), hepatocytar growth factor (HGF), basic fibroblastic growth factor (bFGF), thrombospondin-1 (TSP-1), endostatin, and angiostatin were measured in the peripheral blood plasma and in the bone marrow plasma at diagnosis.

Pretreatment HGF concentrations in the peripheral blood plasma as well as in the bone marrow plasma of patients who achieved complete or very good partial response were significantly lower than those in patients who had partial or worse response. Patients with complete or very good partial response had higher TSP-1 levels in the bone marrow plasma than the partial or insufficient response subgroups. There were no correlations between the pretreatment levels of VEGF, bFGF, endostatin, or angiostatin and the treatment response.

Pretreatment concentrations of HGF and TSP-1 were predictive factors for treatment response. Patients with low angiogenesis rate as determined by the relative HGF and TSP-1 concentrations were more likely to achieve complete or very good partial response after high-dose chemotherapy.

*Key words: Angiogenesis, cytokines, high-dose chemotherapy, multiple myeloma, therapeutic response*

Multiple myeloma (MM) is a heterogeneous disease with patient survival ranging from a few months to many years. It is clear that the established prognostic factors do not have a universal value, especially for patients treated with novel agents such as thalidomide or bortezomib [1].

Bone marrow angiogenesis is a critical mechanism in the pathogenesis of MM. It has been suggested that the determination of the rate of angiogenesis and its monitoring could provide important independent prognostic information. There is already evidence of the prognostic value of the evaluation of microvascular density of the bone marrow [2, 3].

Malignant plasma cells and their microenvironment produce a number of cytokines including angiogenesis activators and inhibitors. The balance between these counteracting types of cytokines determined whether angiogenesis rate is high or low. There are data suggesting reciprocal interactions between

malignant plasma cells and endothelial cells that result in neovascularization, promote cell adhesion of malignant plasmocytes, and protect them from apoptosis [4, 5].

Our aim was to establish whether the pretreatment levels of angiogenesis activators and inhibitors in the peripheral blood and in the bone marrow can be used to predict clinical responses to treatment that included high-dose chemotherapy with peripheral stem cell support. Our patient cohort received uniform treatment and strict follow-up within the CMG 2002 randomized clinical trial of the Czech Myeloma Group. We evaluated the levels of selected angiogenesis factors including vascular endothelial growth factor (VEGF), hepatocytar growth factor (HGF), basic fibroblastic growth factor (bFGF), thrombospondin-1 (TSP-1), endostatin, and angiostatin in the plasma of peripheral blood (PB) and in the bone marrow plasma (BMP).

## Patients and methods

**Patients.** We have analyzed samples from 96 patients with secretory MM enrolled in the randomized CMG 2002 clinical trial [6]. The study treatment consisted of 4 cycles of induction chemotherapy with vincristin (0,5 mg i.v., day 1-4) doxorubicin (9,0 mg/m<sup>2</sup>, day 1-4) and dexamethasone (40 mg p.o., day 1-4, 10-13, 20-23) /VAD/. Stimulation chemotherapy with cyclophosphamide 2,5g/m<sup>2</sup> followed by G-CSF, and myeloablative chemotherapy with melphalan 200mg/m<sup>2</sup>. Baseline characteristics of the patients are shown in Table 1.

**Evaluation of treatment responses.** Very good partial response (VGPR) was defined as reduction of monoclonal immunoglobulin by at least 90% of initial values. In our analysis, this subgroup included patients with complete response, i.e. disappearance of the monoclonal band on electrophoresis and immunofixation. Partial response (PR) was defined as decrease in monoclonal immunoglobulin by 50% to 90%, and non response (NR) as decrease in monoclonal immunoglobulin concentration by less than 50% [7].

**Sample processing** After giving written informed consent to the study, patients had bone marrow aspiration from the sternum or from the iliac crest. Peripheral venous blood sample was taken on the same day. All samples were immediately mixed with EDTA and centrifuged at 3800 rpm for 15 minutes at room temperature. BMP was always prepared from the first milliliter of aspirated bone marrow. Plasma was cryopreserved in 1ml aliquots for later analysis. Samples were not evaluated if hemolysis occurred during sampling or processing.

**Cytokine measurement** Measurements were done using ELISA kits according to the manufacturers' instructions. The following ELISA kit were used: Human VEGF (R&D systems, Minneapolis, USA), Human bFGF (R&D systems, Minneapolis, USA), Human HGF (R&D systems, Minneapolis, USA), Human TSP-1 (Chemicon, Millipore,

**Table 1. Baseline characteristics of patients**

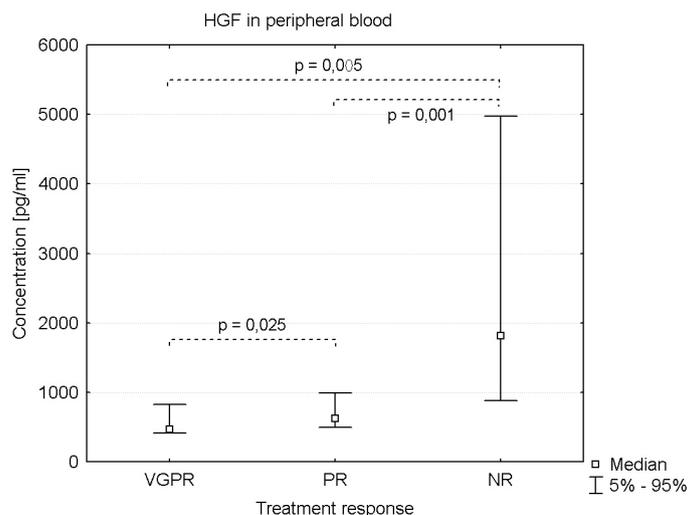
<b>Number</b>	96	
<b>Mean age (range) (years)</b>	55.1 (28 - 72)	
<b>Males/Females</b>	51 (53.1%)/45 (46.9%)	
<b>Durie-Salmon stage</b>	<b>IA</b>	9 (9.4%)
	<b>IB</b>	1 (1.0%)
	<b>IIA</b>	14 (14.6%)
	<b>IIIA</b>	56 (58.3%)
	<b>IIIB</b>	14 (14.6%)
	<b>Not known</b>	2 (2.1%)

Billierica, USA), Human Endostatin (Chemicon, Millipore, Billierica, USA), Human Angiostatin (Ray Biotech, Norcross GA, USA). For each patient, angiogenesis factor levels were measured at diagnosis in the peripheral blood plasma and in the bone marrow plasma.

**Statistical analysis.** The nonparametric Kruskal-Wallis ANOVA test was used to analyze differences in the concentrations of TSP-1, angiostatin, endostatin, HGF, VEGF, and bFGF between subgroups with different treatment responses after high-dose chemotherapy. Statistical significance of ANOVA was confirmed by the nonparametric Mann-Whitney test comparing two subgroups against each other.

## Results

**HGF concentrations and treatment response.** Cytokine concentrations at diagnosis were measured in 88 patients in the peripheral blood plasma and in 95 patients in the bone marrow plasma. HGF concentrations in the peripheral blood plasma of 41/88 patients (47%) who achieved CR or VGPR were significantly lower than those in 33/88 patients (37%)



**Figure 1. HGF Concentrations in peripheral blood in patients with different treatment responses, and statistical differences**

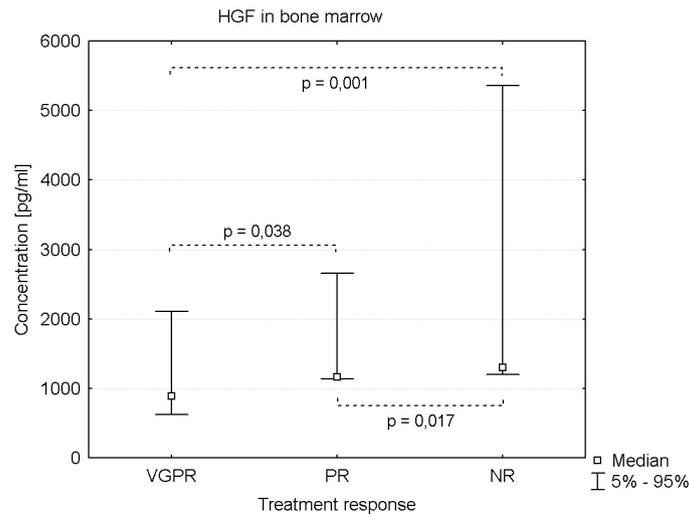


Figure 2. HGF Concentrations in bone marrow in patients with different treatment responses, and statistical differences

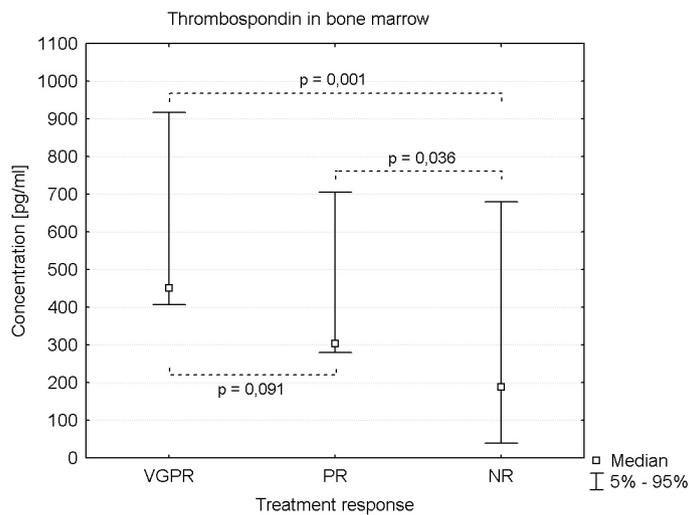


Figure 3. Thrombospondin concentrations in bone marrow in patients with different treatment responses, and statistical differences

who had PR ( $p = 0.025$ ). HGF concentrations in the 14 patients with NR (16%) were significantly higher in comparison with those in both CR+VGPR and PR subgroups ( $p=0.005$  and  $p=0.001$ , respectively). Similar results were obtained for the bone marrow plasma measurements in 95 patients. HGF concentrations were significantly lower in the 45 CR+VGPR patients (47%) compared with the subgroup of 36 patients (38%) who had PR ( $p= 0.038$ ) as well as with the subgroup of 14 patients (15%) with NR ( $p = 0.001$ ). The concentrations in the PR patients were significantly lower than those in the NR patients ( $p = 0,017$ ) as shown in figure 1 and figure 2.

*TSP-1 concentrations and treatment response.* Total 14 patient (15%) with NR had significantly lower TSP-1 con-

centrations in the bone marrow plasma than 36 (38%) PR patients ( $p = 0.036$ ). Forty five (45%) CR+VGPR patients had higher TSP-1 levels in the bone marrow plasma than NR but not PR patient subgroups ( $p = 0.001$  and  $p =0.091$ , respectively) as shown in figure 3. There were no statistically significant differences between the TSP-1 concentrations in the peripheral blood in the different treatment response subgroups.

*Concentrations of VEGF, bFGF, endostatin, and angiostatin.* There were no statistically significant differences between the pretreatment levels of VEGF, bFGF, endostatin, or angiostatin in the peripheral blood plasma in the bone marrow plasma between the subgroups with CR+VGPR, PR, or NR. Patients

**Table 2. Levels of bFGF, VEGF, Endostatin and angiostatin in peripheral blood plasma (PB) and bone marrow plasma (BMP) in patients with different response.**

Response	VGPR		PR		NR	
	PB	BMP	PB	BMP	PB	BMP
bFGF median (95%IS) pg/ml	11,0 (7,3-23,4)	27,9 (10,7-210,9)	12,3 (1,1-37,5)	9,5 (4,0 -124,8)	8,6 (0,4-22,3)	21,1 (12,3-391,0)
VEGFmedian (95%IS) pg/ml	71,1 (60,9-188,2)	58,0 (38,5-178,1)	76,9 (63,8 - 168,7)	39,8 (21,4-114,3)	162,3 (78,2 - 437,4)	129,0 (41,4-284,2)
Endostatin median (95%IS) pg/ml	472,3 (405,5-697,8)	241,0 (127,3-410,2)	558,0 (396,7- 702,8)	210,5 (164,1 -432,1)	635,3 (326,8-777,3)	83,7 (5,1-293,7)
Angiostatin median (95%IS) pg/ml	373,5 (275,7-547,4)	138,0 (104,6-621,4)	635,0 (373,2-700,1)	165 (126,3-686,3)	551,0 (78,6-1102,2)	119,2 (104,0-603,4)

**Table 3. p values if compared Levels of bFGF, VEGF, Endostatin and angiostatin in peripheral blood plasma (PB) and bone marrow plasma (BMP) in patients with different response**

	Peripheral blood plasma			Bone marrow plasma		
	VGPR vs PR	VGPR vs NR	PR vs NR	VGPR vs PR	VGPR vs NR	PR vs NR
<b>bFGF p value</b>	0,356	0,268	0,148	0,125	0,251	0,152
<b>VEGF p value</b>	0,211	0,112	0,144	0,258	0,096	0,079
<b>Endostatin p value</b>	0,187	0,254	0,241	0,145	0,067	0,084
<b>Angiostatin p value</b>	0,225	0,107	0,124	0,201	0,235	0,194

number was the same as mentioned in TSP-1 and HGF. Results are shown in Table 2 and Table 3

There was an only trend to increased concentrations of VEGF in the bone marrow plasma in the NR patients as compared to the PR patients ( $p = 0.079$ ). There were also differences bordering on statistical significance in endostatin concentrations, where patients with NR had lower endostatin levels in the bone marrow plasma PR and CR+VGPR ( $p = 0.084$  and  $p = 0.067$  respectively).

## Discussion

Increased rate of angiogenesis is one of the key factors in the pathogenesis of MM [3, 8]. Angiogenesis is a complex process controlled by a number of cytokines with activator or inhibitor activities. One of the way to monitor angiogenesis in vivo is the measurement of concentrations of the most important angiogenesis factors in relevant tissues [9, 10, 11, 12].

The published studies on the levels of angiogenesis factors in hematological diseases have brought rather discordant results. One of the reasons behind this heterogeneity may be the technique of bone marrow sampling. We have shown previously that angiogenesis markers must be measured from the first milliliter of the aspirated bone marrow; otherwise the

admixture of peripheral blood will skew the results [13]. Comparison between patients is only possible if there is stringent adherence to the proper sampling technique.

The main objective of our retrospective study was to determine the predictive value of selected angiogenesis markers in MM treated with high-dose chemotherapy. We have chosen three angiogenesis activators including VEGF, HGF, and bFGF, and three inhibitors including TSP-1, endostatin, and angiostatin which have been shown to of importance in MM or in other malignancies [9, 11, 12, 14, 15, 16, 17, 18]. There is substantial literature on the roles of VEGF, HGF, and bFGF in multiple myeloma. Especially HGF seems to be a key molecule in the myeloma-associated angiogenesis and a factor stimulating survival and adherence of malignant plasma cells [19]. Much less has been published about angiogenesis inhibitors in MM. There is some evidence that the concentrations of endostatin in the peripheral blood are higher in MM patients compared to healthy controls [16]. In addition, angiostatin was reported to inhibit neovascularization, delay tumor growth, and decrease HGF levels in a murine model of plasmocytoma [20].

Our most important novel finding is that TSP-1 levels in the bone marrow plasma at diagnosis predict the success or failure of high-dose chemotherapy in MM. It is likely that patients

with partial or worse response to high-dose chemotherapy also have poorer survival than those with complete or very good partial response [21]. The differences in peripheral blood TSP-1 concentrations between the subgroups with different treatment responses were non-significant probably due to its release from activated platelets and endothelial cells in direct contact with peripheral blood [14]. It has been proposed that the type of endothelium which is present in the tumor microenvironment produces less TSP-1 than the endothelium of normal capillaries, thus leading to disinhibition of tumor-associated angiogenesis [22].

The presence of high concentrations of angiogenesis activators in aggressive disease has already been described [11, 12, 15, 18]. Our results are in agreement with previously published reports which show that HGF is the critical angiogenesis activator in MM [2, 18, 23]. It also seems to be the most useful marker for monitoring of angiogenesis activity because in contrast to all other measured cytokines even its peripheral blood levels were predictive of treatment response. On the other hand, in our homogeneous cohort of patients and using a standardized technique we have been unable to confirm the correlation of VEGF and bFGF levels with treatment response [9, 24, 25].

Overall, the changes in angiogenesis activators and inhibitors suggest that MM patients with lower rate of angiogenesis at diagnosis respond better to high-dose chemotherapy while the patients with high angiogenesis rate respond poorly. Our results indicate that TSP-1 and HGF pretreatment concentrations are predictive factors for MM patients treated with high-dose chemotherapy. Their relative importance and independence of established predictive and prognostic factors will need to be confirmed in prospective studies.

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