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## Original Research Article

# VEGF-A, sVEGFR-1, and sVEGFR-2 in BCR-ABL negative myeloproliferative neoplasms

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### ABSTRACT

**Background and objective:** Data from the literature indicate the relationship between the bone marrow microvessel density and the blood parameters of angiogenesis. The aim of this study was to evaluate selected parameters of angiogenesis (VEGF-A, sVEGFR-1, and sVEGFR-2) and their correlations with white blood cells, platelets, and red blood cells.

**Materials and methods:** The study included 72 patients (mean age, 61.84 years) with myeloproliferative neoplasms (MPNs): essential thrombocythemia (ET) ( $n = 46$ ), polycythemia vera (PV) ( $n = 19$ ), and primary myelofibrosis (PMF) ( $n = 7$ ). Serum VEGF-A, sVEGFR-1, and sVEGFR-2 were determined using the ELISA assay.

**Results:** We observed a significantly higher level of VEGF-A and reduced concentrations of sVEGFR-1 and sVEGFR-2 in the whole group of patients with MPNs as compared to controls. Detailed analysis confirmed significantly higher level of VEGF-A and lower concentration of sVEGFR-2 in each subgroups of MPNs patients. However, sVEGFR-1 concentrations were significantly lower only in PV and ET patients.

**Conclusions:** The study showed an increased level of VEGF-A, which may indicate the intensity of neoangiogenesis in the bone marrow. Decreased sVEGFR-1 and sVEGFR-2 in the blood of patients with MPNs may reflect consumption of these soluble receptors.

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## 1. Introduction

Myeloproliferative neoplasms (MPNs) are the result of clonal proliferation of stem cells of bone marrow, characterized by the proliferation of one or more myeloid lines (granulocyte, erythrocyte, and megakaryocyte) [1]. Classic myeloproliferative neoplasms include chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).

Since 2008, a new WHO classification distinguishes BCR-ABL-negative myeloproliferative neoplasms including essential thrombocythemia, polycythemia vera and primary myelofibrosis. This group of diseases is identified on the basis of mutations in multipotent stem cells and defined as a lack of Ph chromosome (and the fusion gene BCR-ABL) and potentially allows the presence of the JAK2 mutation.

In recent years, significant progress has been made in understanding angiogenesis process that plays a key role in the pathogenesis of myeloproliferative neoplasms. Regulation of angiogenesis in cancer is complex and depends on angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), transforming growth factor  $\beta$  (TGF $\alpha$ ), tumor necrosis factor (TNF), interleukin-6, interleukin-8, epidermal growth factor (EGF), angiopoietin-1, angiogenin, integrin and anti-angiogenic factors such as thrombospondin-1, angiostatin, endostatin, fibronectin, interferon- $\alpha$ , interferon- $\gamma$ , interleukin-1, interleukin-12, platelet factor 4.

The most specific endothelial cell growth factor is vascular endothelial growth factor (VEGF) [2]. VEGF is a homodimeric glycoprotein of approximately 45 kDa. VEGF family includes seven proteins: VEGF-A, -B, -C, -D, -E, -F, and placental growth factor (PlGF) [3]. The best known factor is VEGF-A, whose gene is located on the short arm of chromosome 6 (6p 21.3) [4]. VEGF is produced by various cells such as macrophages, T lymphocytes, endothelial cells, vascular smooth muscle cells and tumor cells [5]. VEGF exerts its effect by binding to specific receptor VEGFR-2, located on the vascular endothelial cells. Currently there are known two soluble forms of the receptors for VEGF: sVEGFR-1 and sVEGFR-2. The soluble form of VEGFR-1 lacks the seventh immunoglobulin-like domain, transmembrane region, and intracellular signaling tyrosine kinase domain characteristic of VEGFR-1 and VEGFR-2. It is believed that sVEGFR-1, via connecting to each isoform of VEGF, acts as an inhibitor of angiogenesis, reducing VEGF availability to endothelium [6-8]. Currently, very little is known about sVEGFR-2, a product of ectodomain shedding from cell-surface

VEGFR-2 or an alternative mRNA splice variation. According to recent studies, sVEGFR-2 is a natural inhibitor of angiogenesis [9].

Many studies have shown that angiogenesis plays an important role in the pathogenesis of myeloproliferative neoplasms. Due to the fact that bone marrow is the site of tumor cell formation, early studies focused on the bone marrow microvascular density assessment. An increased density of vessels has been demonstrated mostly in PMF and PV patients. Significantly higher concentration of VEGF-A in patients with myeloproliferative neoplasms correlated with microvascular density, as well as with the clinical severity of the disease progression. For this reason, VEGF-A may be used as a diagnostic and prognostic marker of MPNs [7].

The aim of this study was to measure VEGF-A, sVEGFR-1 and sVEGFR-2 concentrations in patients with myeloproliferative neoplasms and to examine their correlations with other parameters (number of leukocytes, erythrocytes and platelets).

## 2. Materials and methods

The study involved 72 newly diagnosed patients (mean age, 61.84 years) with myeloproliferative neoplasms, hospitalized and diagnosed at the Hematology Clinic Dr. J. Biziel University Hospital No. 2 in Bydgoszcz, Poland. The study group included 46 patients with ET, 19 with PV, and 7 with PMF. Patients at the time of inclusion in the study were not taking drugs affecting angiogenesis. The diagnosis of ET was based on the diagnostic criteria of ET according to the WHO (2008) [10] and the exclusion of other malignant and non-malignant diseases in the course of which there can be observed essential thrombocythemia. The diagnosis of PV was based on the diagnostic criteria for PV according to the WHO (2008) [10] including a genetic test for the gene JAK2 V617F mutation. In all patients with PV there were initially excluded secondary causes of PV. Myelofibrosis diagnosis was based on the diagnostic criteria for spontaneous bone marrow fibrosis, according to the WHO (2008) [10], including the study on the presence of cytogenetic V617F mutation in gene JAK2.

Mutational status of MPNs patients and JAK2 V617F-negative patients is presented in Tables 1 and 2.

The control group consisted of 39 healthy volunteers (mean age, 59.22 years) who were age and sex matched.

The material for the test was venous blood collected from the elbow vein into 2 tubes containing different anticoagulants: 3.2% sodium citrate and K2EDTA. Peripheral blood counts were performed on Advia 120 hematology analyzer. Concentration of fibrinogen was marked using the Siemens

**Table 1 – Mutational status of MPNs patients.**

	Groups		
	Total N (%)	JAK2 V617F positive N (%)	JAK2 V617F negative N (%)
Essential thrombocythemia	46 (100)	27 (58.70)	19 (41.30)
Polycythemia vera	19 (100)	16 (84.21)	3 (15.79)
Primary myelofibrosis	7 (100)	5 (71.43)	2 (28.57)

**Table 2 – Mutational status of JAK2 V617F negative patients.**

	JAK2 V617 F negative subgroups		
	MPL W515K/L positive N (%)	CARL positive N (%)	Triple negative <sup>a</sup> N (%)
Essential thrombocythemia	1 (5.26)	6 (31.58)	12 (63.16)
Primary myelofibrosis	0 (0.00)	0 (0.00)	2 (100.00)

<sup>a</sup> Triple negative: JAK2 (-), MPL (-), CARL (-).

analyzer. ELISA technique was used to measure following parameters: concentrations of VEGF-A, sVEGFR-1 and sVEGFR-2 (R&D Systems), USA. Assays were performed according to the manufacturers' instructions. All samples were analyzed in duplicate.

The statistical analysis was performed with the use of Statistica 9.1 software (StatSoft®). The Shapiro–Wilk test was used to assess the normality of the distribution. For the parameters with a normal distribution, arithmetic mean and standard deviations were determined, and the parameters with the abnormal distribution were presented as a median and quartiles: lower (Q1) and upper (Q3). The Student t test or the Mann Whitney U test was used to compare the difference between groups. Spearman correlation coefficients were calculated to determine if there were any correlations between the parameters. The P values of <0.05 were considered significant.

The study was approved by the Bioethics Committee of Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Toruń, Poland. Informed written consent was obtained after the purpose and nature were explained to the subjects.

### 3. Results

Clinical characteristics of patients with myeloproliferative neoplasms are shown in Table 3. In patients with PV there were an increased number of red blood cells, increased HCT and HGB concentration. In PMF patients, a low degree of anemia was diagnosed. Moreover, the WBC count was elevated in patients with PV, ET as well as in PMF. The platelet count was elevated in patients with ET and PV, but reduced in PMF patients.

Table 4 presents selected angiogenic factors (VEGF-A, sVEGFR-1, sVEGFR-2) measured in the plasma of patients and controls. VEGF-A concentration was significantly increased in patients with MPNs in relation to the controls ( $P < 0.001$ ). Moreover sVEGFR-1 and sVEGFR-2 concentrations were decreased in patients with MPNs in relation to the control group, and the differences were statistically significant ( $P < 0.01$  and  $P < 0.001$ , respectively).

Table 5 shows that the concentration of VEGF-A was significantly higher in patients with ET, PV, and PMF compared to the control group. It was also found that the concentration

**Table 3 – Clinical data of patients with myeloproliferative neoplasms.**

Parameter	ET N = 46	PV N = 19	PMF N = 7	Control group N = 39
Age, mean (SD), years	61.69 (16.72)	61.21 (16.60)	64.57 (6.83)	59.22 (12.23)
Sex, n (F/M)	28/18	10/9	2/5	20/19
RBC, $\times 10^{12}/L$	4.85 (4.66; 5.31)	7.13 (6.34; 7.55)	3.28 (3.01; 4.19)	4.21 (3.68; 4.42)
HGB, mean (SD), g/dL	14.01 (2.54)	17.94 (2.07)	10.13 (2.43)	13.86 (1.24)
WBC, $\times 10^9/L$	10.00 (8.11; 12.46)	9.83 (6.26; 15.95)	10.46 (4.66; 18.24)	6.10 (4.90; 7.30)
PLT, $\times 10^9/L$	857.00 (724.00; 1163.50)	357.00 (209.00; 524.00)	101.50 (40.00; 188.00)	250.00 (222.00; 287.00)
Fibrinogen, g/dL	3.10 (2.65; 3.90)	2.90 (2.10; 3.20)	4.60 (4.20; 5.80)	2.55 (2.10; 3.00)

Values are expressed as median (Q1; Q3) unless otherwise stated.

RBC, red blood cells; HGB, hemoglobin; WBC, white blood cells; PLT, platelets.

**Table 4 – Concentrations of VEGF-A, sVEGFR-1 and sVEGFR-2 in the study group compared to the control group.**

Parameter	Study group N = 72	Control group N = 39	P
VEGF-A, median (Q1; Q3) [range], pg/mL	87.35 (49.52; 188.33) [11.84–732.92]	18.82 (12.13; 26.87) [6.32–82.85]	<0.001
sVEGFR-1, median (Q1; Q3) [range], pg/mL	87.03 (70.635; 111.21) [40.54–447.10]	133.70 (75.57; 213.09) [8.90–323.10]	<0.01
sVEGFR-2, mean (SD) [range], pg/mL	7475.57 (2426.95) [317.77–12 650.00]	12 121.87 (2530.04) [7902.07–9686.70]	<0.001

**Table 5 – Comparison of VEGF-A, sVEGFR-1, sVEGFR-2 concentration in various subgroups of patients with MPNs and in relation to the control group.**

Parameter	ET	PV	PMF	Control group	P
	n = 46 (I)	n = 19 (II)	n = 7 (III)	n = 39 (C)	
VEGF-A, median (Q1; Q3), pg/mL	105.74 (51.40; 208.95)	71.72 (47.63; 143.89)	64.80 (38.46; 156.90)	18.82 (12.13; 26.87)	I vs C, <0.001 II vs C, <0.001 III vs C, <0.01
sVEGFR-1, median (Q1; Q3), pg/mL	87.44 (69.66; 111.62)	87.66 (68.04; 111.62)	72.92 (72.11; 98.37)	133.70 (75.57; 213.09)	I vs C, <0.01 II vs C, <0.05
sVEGFR-2, mean (SD), pg/mL	7783.44 (1989.81)	6490.86 (2941.62)	8125.21 (3123.31)	12121.87 (2530.04)	I vs C, <0.001 II vs C, <0.001 III vs C, <0.01

**Table 6 – Correlation between white blood cell count and VEGF-A in patients with MPNs and PV.**

Parameter	WBC	
	R	P
VEGF-A all patients with MPNs	0.34	<0.01
VEGF-A patients with PV	0.52	<0.05

of sVEGFR-1 was lower in all patients with MPNs, as compared to the control group, but only in patients with PV and ET differences were statistically significant. sVEGFR-2 concentration was significantly lower in all subgroups of patients with MPNs, as compared to the control group.

There was a positive correlation between VEGF-A concentration and WBC count in all patients with BCR-ABL-negative MPNs and in patients with PV (Table 6). A positive correlation between VEGF-A and sVEGFR-1 in the whole group of patients with MPNs and in patients with PV and ET was found (Table 7).

#### 4. Discussion

In the recent years, many studies emphasized the important role of angiogenesis in solid tumors. The newly formed blood vessels supply oxygen and nutrients to the growing tumor, which determines its growth. Blood vessels allow the movement of cancer cells to other organs, where they form a metastasis. VEGF – a vascular endothelial growth factor – plays a key role in vascular neoplasia.

The available literature shows elevated levels of VEGF and its receptors in a number of cancers such as breast cancer, colorectal cancer, esophageal cancer, pancreatic cancer, laryngeal cancer, non-small lung cancer, brain tumors [11-13].

**Table 7 – Correlation between sVEGFR-1 and VEGF-A in patients with MPNs, ET, and PV.**

Parameter	sVEGFR-1	
	R	P
VEGF-A all patients with MPNs	0.44	<0.001
VEGF-A patients with ET	0.32	<0.05
VEGF-A patients with PV	0.72	<0.001

Activation of proangiogenic factors in solid tumors correlates with clinical stage of the disease, prognosis and incidence of metastases.

In the literature, there are only few reports describing angiogenesis in the pathogenesis of hematopoietic malignancies. Many researchers indicate the prognostic and clinical importance of neovascularization in the pathogenesis of acute myeloid leukemia, acute lymphocytic leukemia, myelodysplastic syndromes, multiple myeloma [14,15]. Verstovsek et al. demonstrated that in patients with myelodysplastic syndromes, VEGF expression in bone marrow cells is an unfavorable prognostic factor and correlates with shorter disease-free survival [16]. Interesting are the results of Bellamy et al. who demonstrated that angiogenic factors in patients with acute myeloid leukemias may not only be responsible for angiogenesis, but also exert autocrine and paracrine effect on blast cells [17].

Data concerning the potential role of angiogenesis in pathophysiology of myeloproliferative neoplasms are scarce. This study involved 72 patients diagnosed with BCR-ABL negative myeloproliferative neoplasms. We demonstrated significantly elevated levels of VEGF (about 5-fold) in the blood of all patients with myeloproliferative neoplasms in comparison to the control group. Detailed analysis of the results obtained in the different subgroups confirmed the general observations. The concentration of VEGF-A in patients with polycythemia vera, essential thrombocythemia and primary myelofibrosis was significantly higher than in healthy subjects.

Our findings are consistent with reports available in the literature [18-20]. A study performed by Krasowska-Kwiecień et al. showed that VEGF expression is dependent on the clinical course of CML. VEGF expression was increased at the time of the diagnosis, while decreased expression occurred during remission of the disease [21]. Research by Musolino et al., Maktouf et al., Alonci et al., Panteli et al. and Cacciola et al. also showed significantly elevated levels of VEGF in patients with myeloproliferative neoplasms. According to the authors, a high concentration of VEGF as well as positive correlation between VEGF and the density of capillaries in the bone marrow may indicate increased angiogenesis and predispose to the development of thromboembolic events [22-25].

Current views on the contribution of VEGF-A in angiogenesis in MPNs relate to its role in maintaining the survival of blast cells as well as stimulating and supporting proliferation and migration of endothelial cell. There are also data on excitatory effect of VEGF-A on endothelial cells to secrete G-CSF, GM-CSF

and IL-6, which stimulate proliferative activity of bone marrow hematopoietic cells.

There has been found a positive correlation between VEGF-A concentration and white blood cells count in all patients with MPNs, as well as in PV patients. Aref et al. found a positive correlation between VEGF-A and WBC in the blood of patients with acute myeloid leukemia (AML). According to the authors, leukemic blasts are the source of VEGF-A [26]. Another source of VEGF-A may be platelets, particularly in patients with ET and PV, where they are present in large numbers. Möhle et al. demonstrated the presence of VEGF-A mRNA in platelets [27]. Misso et al. found a positive correlation between the number of platelets and VEGF-A concentration in patients with chronic myeloid leukemia (CML) [28].

Our findings demonstrated a significantly lower concentration of sVEGFR-1 in patients with ET and PV compared to the healthy subjects. Higher sVEGFR-1 concentration showed Aref et al. and Hu et al. in the blood of patients with AML and myelodysplastic syndromes (MDs) [26,29]. Ratajczak et al. found VEGFR-1 mRNA expression in all samples collected from bone marrow from patients with CML [30]. Wierzbowska et al. observed higher concentration of sVEGFR-1 in patients with AML and acute lymphoblastic leukemia (ALL) than in the blood of healthy individuals [8]. They also reported that sVEGFR-1 level was the highest in patients with a number of blasts in bone marrow >50% and in patients with WBC count exceeding 20 g/L in the peripheral blood [8].

The assessment of sVEGFR-1 concentration was the subject of few studies in MPNs patients. Treliński et al. showed elevated level of sVEGFR-1 in the blood of patients with ET and PV [31].

Our study showed a significant positive correlation between concentrations of VEGF-A and sVEGFR-1 in the blood of patients with MPNs, particularly expressed in patients with PV.

Analysis of the available literature indicates that there is a relationship between VEGF-A and sVEGFR-1, but its nature is still unclear. The ratio of sVEGFR-1 to VEGF was found to be an important prognostic indicator for cancers such as astrocytic glioma, primary breast cancer, pancreatic cancer and acute myeloid leukemia [32]. Unclear is the role of sVEGFR-1 in MPNs. In vitro studies and reports of animal models indicate that sVEGFR-1 can act as an inhibitor of angiogenesis. It is believed that sVEGFR-1 is responsible for sequestering VEGF ligand and reducing the availability of VEGF to VEGFR-2 located on endothelial cells [32]. Significantly lower sVEGFR-1 concentration and multiple times higher VEGF-A level observed in our study in Ph-negative MPNs patients may indicate the consumption of sVEGFR-1 in process of inactivation of VEGF-A.

A recent immunohistochemistry study conducted by Boiocchi et al. indicated the presence of VEGF and VEGFR-1 in megakaryocytes, macrophages and in immature myeloid precursors [33]. Moreover, VEGFR-1 immunoreactivity in endothelial cells was not observed. According to the authors between VEGF-A and VEGFR-1 exists an internal autocrine loop mechanism.

In the present study we have found significantly reduced sVEGFR-2 level in MPNs patients. There are only two clinical studies assessing sVEGFR-2 in MPNs patients. Treliński et al. found no difference in concentration of sVEGFR-2 in patients with PV and ET in relation to healthy subjects. Moreover Hu

et al. found no correlation between serum level of sVEGFR-2 and WBC count in patients with MPNs [29,31]. Recombinant forms of sVEGFR-2 had an inhibitory effect on angiogenesis induced by VEGF-A (both in vivo and in vitro models) [8]. It can therefore be assumed that reduced level of sVEGFR-2 in blood of patients with Ph-negative MPNs is a result of its consumption in the process of inactivation of VEGF-A.

In the recent years a great progress has been made in the field of antiangiogenic therapy. Several angiogenesis inhibitors have been developed to target vascular endothelial cells and block tumor angiogenesis. Anti-angiogenic agents like monoclonal antibodies, receptor tyrosine kinase inhibitors, immunomodulatory drugs, and proteasome inhibitors have been already approved for solid tumors and some hematological malignancies. Further studies should be undertaken to evaluate the potential role of antiangiogenic therapy in myeloproliferative neoplasms.

## 5. Conclusions

Summarizing, the study showed increased level of VEGF-A, which may indicate the intensity of neoangiogenesis in the bone marrow. Decreased sVEGFR-1 and sVEGFR-2 in the blood of patients with MPNs may be a result of consumption of these factors.

## Conflict of interest

The authors stated that there are no conflicts of interest regarding the publication of this article.

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