SUMMARY

JAK2V617F mutation is a well-recognized feature in most Ph-negative myeloproliferative neoplasms (MPNs). An activated bone marrow (BM) angiogenesis has been established in these disorders as well. Consequently, the rational question is to ascertain a possible relation among JAK2 mutation, morphological features and angiogenesis in MPNs bone marrow.

**Aim:** To assess bone marrow microvessel density (MVD), bone marrow cellularity and fibrosis in newly diagnosed patients with BCR-ABL-negative MPNs, and define a correlation between the degree of angiogenesis in the bone marrow and JAK2V617F mutant allele burden.

**Methods:** JAK2 mutational burden was determined by RT-PCR, BM angiogenesis was defined by MVD assessment using anti-CD34 for BM endothelium staining. The BM fibrosis was evaluated according to the Hanover system. The statistical analysis was performed with SPSS 17.0 software. 52 patients with newly diagnosed MPN were included in the study.

**Results:** The distribution of the mutational burden was as follows: 26 patients with polycythemia vera (PV), 16 patients with primary myelofibrosis (PMF) and 10 patients with essential thrombocythemia (ET). In patients with PV the homozygosity was found prevalent in frequency whereas in PMF and ET the heterozygous variants were dominant. In all patients a significant positive correlation between JAK2V617F and BM MVD (r=0.306, p<0.002) and between MVD and fibrosis, (r=0.523, p<0.0001), was found. JAK2 correlated positively but borderline with fibrosis. The MVD and JAK2 burden were found in significant negative correlation with the BM cellularity (r=-0.405; p<0.002 and r=-0.431, p<0.0001, resp.).

**Conclusions:** The significant correlation between JAK2V617F, BM angiogenic activity and the fibrosis marks out the JAK2 allele burden as a feasible parameter with prognostic significance for evolution and progression of MPN.

**Key words:** JAK2V617F mutation, angiogenesis, myeloproliferative neoplasm, polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF),
nosed patients with BCR-ABL-negative MPNs and to define a correlation between the degree of angiogenesis in the bone marrow and JAK2V617F mutant allele burden.

PATIENTS AND METHODS

A prospective study was performed according to the regulations of the local ethics committee. We included 52 patients with newly diagnosed and previously untreated MPNs. Bone marrow biopsies were analyzed for the extent of angiogenesis. Bone marrow samples were fixed in buffered neutral formalin for 12-48 hours. Slides were stained with Hematoxylin and Eosin and immunohistochemically for the evaluation of endothelial cells with FLEX Monoclonal Mouse Anti-Human CD34 Class II, Clone Q5Bend 10 (DAKO). Visualization system was Envision High pH (Link) (Code K8000). Sections were examined for bone marrow MVD at x400 magnification. Five hot spot areas (areas of highest neovascularization) in CD34 stained sections were selected and the mean number of microvessels was measured. The degree of bone marrow fibrosis was established according to the Hannover system.

Analysis of JAK2 V617F mutation was performed by Polymerase Chain Reaction (PCR). Peripheral blood mononuclear cells were separated after red blood cells destruction with a lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) (Silva et al., 2002) [10]. Genomic DNA (gDNA) and/or total cellular RNA was isolated using Trizol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol. JAK2 V617F mutation status was determined by two different approaches using allele-specific (AS)-PCR [1] and PCR restriction fragment length polymorphism (RFLP) analysis [2] using either the genomic DNA or complementary DNA (cDNA), synthesized via reverse transcription of RNA with 200 U MMLV reverse transcriptase (USB Products, Affimetrix, Cleveland, Ohio, USA) according to the previously reported method (van Dongen et al., 1999) [11].

gDNA AS-PCR: Reaction was performed using 100 ng gDNA as a template in a total volume of 25 µl containing 1x PCR buffer, 1.5 mM MgCl2, 40 pmol of each primer (control forward primer: 5’-ttccctgctgctgatgta-3’; common reverse primer: 5’-ctgaatagtcctacagtgtttcagtttca-3’; mutation specific forward primer: 5’-agcatttggttttaaattatggagtatatt-3’, 200 µM each of dNTPs, and 1 U of Taq polymerase (Promega). Thirty eight cycles of amplification were performed with an annealing temperature of 57°C on instructions on a Thermocycler Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia). The amplified fragment was digested overnight at 37°C with 2 µl BsaXI endonuclease (New England BioLabs, Ipswich, MA).

cDNA AS-PCR: Reaction was performed using 2 µl of cDNA as a template in a total volume of 25 µl containing 1x PCR buffer, 1.5 mM MgCl2, 25 pmol of each primer specific forward primer: 5’-agcatttggttttaaattatggagtatatt-3’; 25 pmol control forward primer: 5’-agcatttggttttaaattatggagtatatt-3’; 200 µM each of dNTPs, and 1 U of Taq polymerase (Promega). Thirty eight cycles of amplification were performed with an annealing temperature of 58°C on instructions on a Thermocycler Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia).

cDNA PCR-RFLP: PCR amplification was carried out using 4 µl of cDNA as a template in a total volume of 32 µl containing 1x PCR buffer, 1.5 mM MgCl2, 25 pmol of each primer 5’-taaggctg-3’ and 5’-ggcctgtccc-3’ (Jamieson et al., 2006) [14], 200 µM dNTPs, and 1 U of Taq polymerase (Promega). Thirty eight cycles of amplification were performed with an annealing temperature of 58°C on a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Following amplification, JAK2 amplicons were digested overnight at 37°C with 2 µl BsaXI endonuclease (New England BioLabs, Ipswich, MA, USA).

All amplification and digestion products were run in a conventional 2% (allele-specific PCR) or 3% (PCR RFLP) agarose gel stained with Sybr Safe (Invitrogen, Karlsruhe, Germany) and visualized after UV irradiation. Appropriate controls comprising wild-type, heterozygous and homozygous JAK2 V617F mutants were included in all experiments.

The statistical analysis was performed with SPSS 17.0 software.

RESULTS

Demographic data:

The patients with Polycythemia vera (PV) were 26 with average age of 55.5 years, between 23 and 75 years. A small prevalence of the female sex was established (women: men 54%: 46%). Patients with primary myelofibrosis (PMF) were 16 with an average age of 64 years (44-74), female: male ratio 40%: 60%. 10 patients with essential thrombocytopenia (ET) had an average age of 61 years (37-72), with prevalence of the female sex (women: men –60%: 40%).

JAK2 mutation burden

In the group of patients with PV (26 patients) JAK2V617F mutation was registered in 81% of cases, 62% of them were homozygotes and 38% heterozygotes. In the group of patients with PMF (16 patients) 69% were carriers of the mutation (27% homozygotes and 73% heterozygotes). Among the patients with ET (10 patients) mutation carriers were 60%, as only one patient was homozygote. The distribution according to MPN type is presented on Fig.1.
Fig. 1. Myeloproliferative neoplasms (MPNs) and JAK2V617F mutational burden distribution of the tumor load in three entities.

Microvessel density (MVD)

The most significant grade of MVD was found in the group of patients with PMF (average MVD 85), followed by PV (average MVD 70) and ET (average MVD 52). Kruskal-Wallis Test showed a statistically significant difference between the studied groups (p < 0.05), the most distinct between PMF and PV and PMF and ET (p < 0.01) (fig. 2).

Fig. 2. Bone marrow microvessel density, determined in three groups of malignancies (PV, PLF and ET)

Significant positive correlation was found between MVD and the degree of fibrosis in the bone marrow (r = 0.503, p < 0.0001) as well as between the degree of MVD and the presence of JAK2 mutation (r = < 0.306, p< 0.002) in all patient groups (table 1). There was a pronounced significant inverse correlation between MVD and bone marrow cellularity (r =-0.44; p < 0.002). A negative correlation was found between the presence of JAK2 mutation and the cellularity in the bone marrow (r =-0.508; p < 0.0001) (table 1).
### Table 1. Correlations

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**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

### DISCUSSION

The constitutive activation of the JAK2/STAT3 signaling pathway has been implicated in events involved in tumor-host interactions, such as angiogenesis [15]. JAK2/STAT3 signaling pathway activation mediates tumor angiogenesis by up-regulation of VEGF and basic fibroblast growth factor (bFGF) in different carcinoma types [16, 17]. There are only few publications on the relationship between the existence and the extent of a carrier of JAK2 mutation and the magnitude of the angiogenesis in the bone marrow in MPNs. In a study of Tretinski J et al. [18] a significant increase of angiogenesis in patients with PV and ET was established, but there was no correlation with the carrier of JAK2V617F mutation. In a previous study we have found that increased angiogenesis is present in all Ph negative MPNs, in highest degree in PMF [19]. Recently, new genetic mutations were detected in JAK2V617F negative patients with MPNs (ASXL1, TET2, DNMT3A, SUZ12, etc.) [20, 21]. However, the role of these mutations in the pathogenesis of Ph negative MPNs and especially in the initiation and permanent stimulation of angiogenesis still remains to be established.

### CONCLUSIONS

The significant correlation between JAK2V617F, BM angiogenic activity and the fibrosis marks out the JAK2 allele burden as a feasible parameter with prognostic significance for evolution and progression of MPN.

### REFERENCES:

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