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Original Research Article

Bone marrow osteoprotegerin (OPG) and interleukin-8 (IL-8) immunostaining in patients with Ph-negative myeloproliferative neoplasms (MPN): Clinical correlations

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Abstract

We evaluated the expression and intensity of IL-8 and OPG immunostaining in BM biopsies of MPN patients at diagnosis and correlated the results with clinical parameters. Seventy-six patients were studied, diagnosed with MPN from 1984 to 2012 with a median follow up period of 55 months. IL-8 and OPG immunostaining was performed on paraffin-embedded sections of formalin fixed BM biopsies, carried out at the time of diagnosis, using the two-step peroxidase conjugated polymer technique. Grade of positivity and intensity of IL-8 and OPG expression was scored according to a 0 to 3 scale. Statistical analysis was performed conventionally, using the SPSS version 22.0 package, and p values <0.05 were considered significant. High intensity of IL-8 expression in megakaryocytes correlated, in the whole cohort, with neutrophilia (p=0.005) and high haemoglobin (p=0.001). High intensity of OPG expression in megakaryocytes correlated high haemoglobin (p=0.017) and a favorable karyotype (p=0.01). Most importantly, low OPG intensity in megakaryocytes correlated with poor survival (p=0.026) in the whole patient series. In conclusion, our most important finding is that high intensity of OPG expression in megakaryocytes correlated with increased survival and a favorable karyotype in patients with MPN, a finding that has not been reported yet.

Keywords: Myeloproliferative Neoplasms (MPN), Polycythemia Vera (PV), Essential Thrombocytosis (ET), Primary Myelofibrosis (MF), Osteoprotegerin (OPG), Interleukin-8 (IL-8)

INTRODUCTION

Polycythemia Vera (PV), Essential Thrombocytosis (ET) and Myelofibrosis (MF), are BCR-ABL negative myeloproliferative neoplasms, characterized by dysregulated signaling pathways with production of inflammatory markers and subsequent changes in the bone marrow stroma. Alterations of the bone marrow microenvironment are found in all MPN types. In addition, elevated levels of proinflammatory and microenvironment regulating cytokines are also found. The concept of cytokine mediated bone marrow stromal reaction is not

new, and was discussed by Groopman J E in 1980.Tefferi et al investigated the prognostic significance of cytokines in MF by determining serum levels of a comprehensive cytokine panel. All MPN, arise from genetic defects within the pluripotent stem cell populations that accumulate during the disease course and the burdensome symptom profile seems to arise from the dysregulated synthesis of cytokines, particularly TNFa, IL-8, IL-1, IL-6 and OPG (Tefferi A et al, 2013). There are many other studies in the literature that support this concept which is well established (Vaidya R et al., 2013; Le Bousse-Kerdiles M.C et al., 1996; Le Bousse-Kerdiles M.C et al., 2001; Martyre M.C et al., 1997). It seems that in MPN, disease burden is not only mediated by the neoplastic clone, but also by a secondary inflammation process with significant cytokine production referred as cytokine storm, and changes of the bone marrow (BM) microenvironment (Hoermann G et al., 2015). Interleukin-8 (IL-8), a CXC motif-related chemokine family member, has been shown to play an important role in tumor growth, angiogenesis and metastasis (Li.A et al., 2003). IL-8 was found expressed in various haematopoietic cell types, including granulocytes, monocytes, megakaryocytes, platelets and CD34+ cells and was additionally shown involved in MPN pathogenesis as already mentioned. Osteoprotegerin (OPG), was first identified in 1997 by 2 independent groups; Amgen investigators in the USA and investigators in Japan, that considered it a potent negative inhibitor of osteoclastogenesis . It is also known as Osteoclastogenesis Inhibitory Factor (OCIF), or tumor necrosis factor receptor superfamily member 11B and is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL) (Simonet WS et al., 1997; Tsuda E et al., 1997). By binding RANKL, OPG prevents RANK mediated nuclear kappa B (NF-Kb) activation, which is a rapid acting transcription factor for immune related genes and a key regulator of inflammation, innate immunity, cell survival and differentiation. In the context of bone biology, RANKL describes the osteoclastogenic cytokine, OPG describes its inhibitor and RANK describes the receptor for RANKL (Weitzmann MN, 2013).

Distinct clinical manifestations are associated with the aforementioned Ph negative MPNs. The somatic mutation JAK2V617F is present in nearly all patients with PV (Baxter EJ et al., 2005). Symptoms of PV are usually related to blood hyperviscosity, secondary to a marked increase in haematocrit (Ht) and red cell mass. The consequences of cell accumulation can manifest as organ enlargement, hyperuricemia, cytokine-mediated events such as pruritus, or/and microvascular occlusive or haemorrhagic phenomena causing headache, dizziness, vertigo, tinnitus and visual disturbances (Spivak J et al., 2002). Venous thrombosis is more common in PV such as Budd Chiari syndrome (BCS), mesenteric, splenic (SVT) or portal vein thrombosis (PVT). PVT is the most common type of splachnic vein thrombosis (40%), while BCS is the least common (5%). (De Stefano V et al., 2008; Smalberg JA et al., 2012).

ET is characterized by an unexplained and persistent thrombocytosis (plts>450x10^9/lt). The JAK2V617F mutation identifies 55% of patients with isolated thrombocytosis, as possibly having ET (Tefferi A et al., 2018). Both thrombosis and bleeding are common complications and a platelet count of >1500x10^9/L is an indication to initiate cytoreductive treatment. Patients can develop neurological symptoms, such as paresthesias of the lower extremities and cerebrovascular accidents (CVAs) due to microvascular circulation clotting disturbances. The identification of somatic mutations of JAK2, CALR or MPL is found in 90% of patients and they have improved the diagnostic and clinical approach to this disorder.

MF is a clonal proliferation of the pluripotent haematopoietic stem cell, characterized by the production of cytokines and growth factors, that affect the bone marrow stroma leading to fibrosis. Neoplastic cells infiltrate extramedullary organs such as the liver and the spleen, leading to hepatomegaly and splenomegaly respectively. Anaemia, teardrop poikylocytes and a leukoerythroblastic blood film characterize the disease. JAK 2 is present in 60% of patients with MF or post ET MF, and in 95% with post PV MF (James C et al., 2005; Kralovics R et al., 2005; Baxter EJ et al., 2005). Mutations in the thrombopoietin receptor gene (MPL), were subsequently found in 3% to 8% of patients with MF and post ET MF, whereas mutations in the calreticulin gene (CALR) have been observed in half of patients with MF and post ET MF lacking JAK2 and MPL mutations.

However, there are overlapping clinical features such as fever, night sweats, splenomegaly and fatigue, which are thought to be cytokine driven. Fatigue is the most common symptom regardless of MPN subtype. It is important to emphasize that an accurate histologic diagnosis is essential to precisely distinguish each condition.

The primary aim of the present study was to assess the intensity and degree of expression of IL-8 and OPG by immunostaining in BM biopsies of MPN patients at diagnosis and investigate if they are related to disease clinical characteristics. The secondary aim was to assess whether the intensity of IL-8 and OPG immunostaining could provide any additional prognostic information.

MATERIALS AND METHODS

Patients

Files from 76 Ph (-) MPN patients were retrospectively studied. Median age was 65 years, 49 were males. 22% were diagnosed with PV, 42% with ET and 36% with MF. Diagnosis was established according to the WHO criteria

Table 1. Patients Characteristics

	ALL	PV	ET	MF
N	76	17	32	27
Male/Female (%)	63/37	76/24	50/50	70/30
Median Age	65	65	65	65
Fatigue (%)	19	24	0	37
Splenomegaly (%)	42	24	19	78
Sweating (%)	9	12	3	15
Bone pain (%)	8	18	0	7
Thrombotic events, Including cardiac events (%)	13	8	14	16
Weight loss (%)	5	0	4	9
JAK2 (+) (%)	77	77	74	83
Unfavorable karyotype* (%)	7	10	5	10
WBC ≥ 10000 (%)	39	35	38	37
HB ≤ 11.0(%)	25	0	3	63
PLTs ≥ 400(%)	66	59	97	35
Neutrophils ≥ 6600 (%)	43	35	42	46
Median overall survival (months)	54	49	60	50

*Unfavorable karyotype: complex karyotype or sole or two abnormalities that include +8, -7/7q-, -5/5q-, 12p-, inv(3), or 11q23 rearrangement.

and routine tests underwent at the time of diagnosis included a full blood count, renal, liver, bone profiles, LDH, EPO level and JAK2. The main findings of all patients studied were fatigue (19%), splenomegaly (42%), bone pains (8%), and sweating (9%), while 13% experienced a thrombotic event, 5% lost weight and 77% were JAK2 positive. Karyotype was abnormal in 32% while 7% presented unfavorable karyotypic findings such as rearrangement of chromosome 5 or 7, or >3 chromosomal abnormalities. In more detail, symptoms associated with splenomegaly were present in 24% of patients with PV, 19% with ET and 78% with MF. Additional complaints included fatigue present in 24% of patients with PV and 37% with MF. In ET no fatigue was observed. Sweating was present in 12% of patients with PV, 3% with ET, 15% with MF. Bone pains were present in 18% of patients with PV, in 7% with MF and none in ET; weight loss was present in 4% of patients with ET, and 9% in MF; weight loss was not observed in patients with PV; thrombotic events were noted in 8% of patients with PV, 14% of patients with ET and 16% of patients with MF. JAK 2 positivity was observed in 77% of patients with PV, 74% of patients with ET and in 83% of patients with MF; an unfavorable karyotype was noticed in 10% of patients with PV, in 5% of patients with ET and 10% of patients with MF. Their characteristics are shown in Table 1. Patients' median follow-up period was 55 months.

Patients' treatment consisted of red blood cell transfusions, hydroxyurea, corticosteroids and ruxolitinib in MF; Aspirin, or clopidogrel combined with or without hydroxyurea in PV and ET.

Methods

The study was approved by the local ethical committee and patients' consent was obtained.

Immunostaining for IL-8 and OPG was performed on paraffin-embedded 4μm sections of formalin fixed BM biopsies, carried out at the time of diagnosis, using the two-step peroxidase conjugated polymer technique. Grade of positivity and intensity in OPG expression was scaled in low, intermediate and high according to the percentage of positive stained megakaryocytes or myeloid cells and to the intensity of the staining respectively. Grade of positivity of IL-8 -expression was

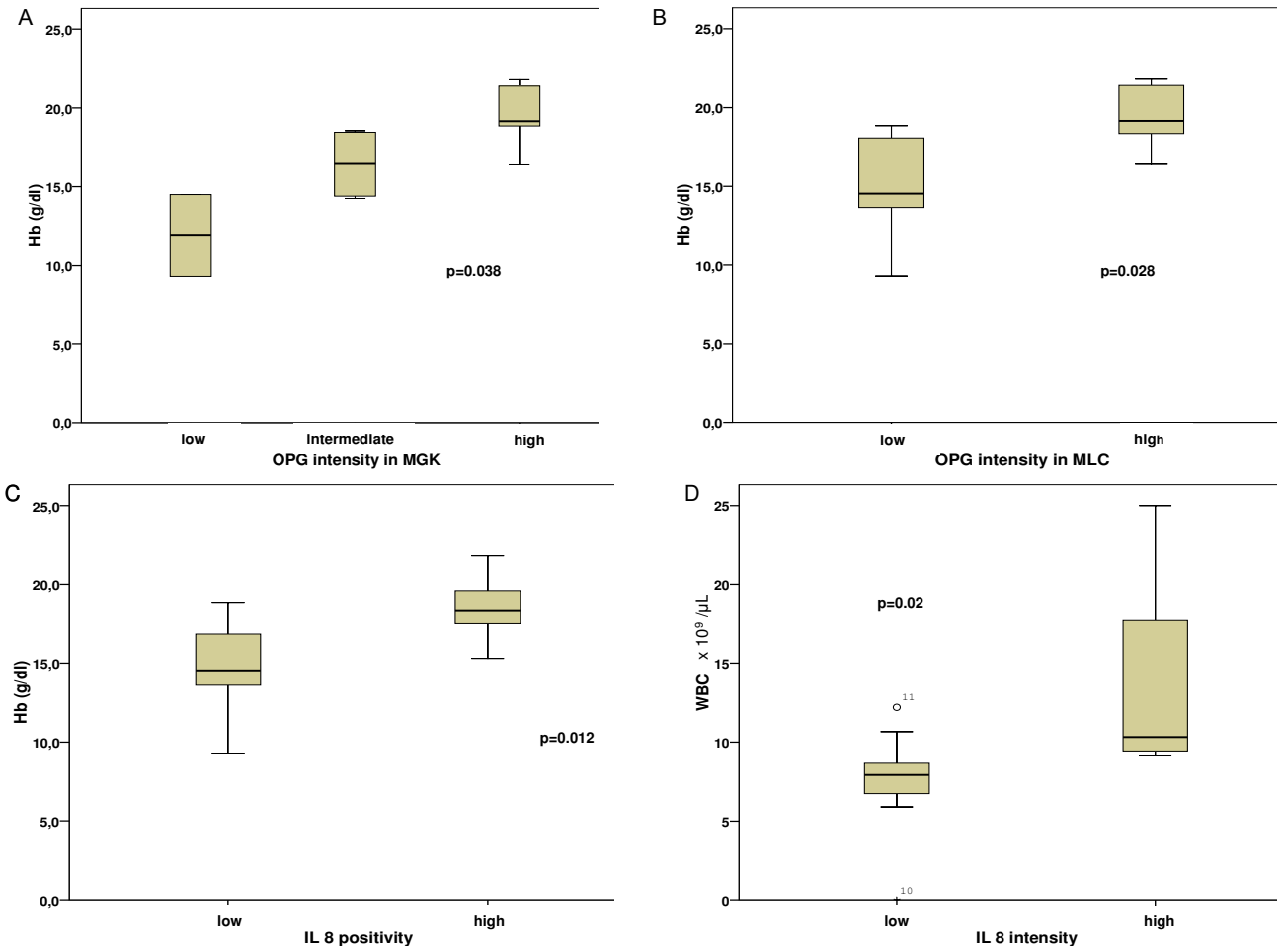

Figure 1. Correlations of OPG and IL 8 in patients with PV

scaled in low, intermediate and high or in low and high according to the percentage of positive stained megakaryocytes. Grade of intensity of IL-8 -expression was scaled in low or high.The scoring system used to evaluate bone marrow fibrosis was according to Manoharan et al 1979. Patients with no reticulin fibers demonstrable or mild presence of reticulin fibers in their bone marrows were placed in the low fibrosis category whereas patients with moderate or severe fibrosis were labeled as high fibrosis category. Statistical analysis was performed conventionally, using the SPSS version 22.0 package, and p values <0.05 were considered significant.

RESULTS

The analysis of results was performed separately in every MPN-group but also in the whole series due to the relatively low number of patients and also because the grade of expression observed in terms of intensity or positivity of both staining was similar in all MPN groups.

Subgroup Analysis

In PV patients high intensity of OPG expression in megakaryocytes, coincided with high haemoglobin levels (Figure 1A, p=0.038, Kruskal Wallis test). High intensity OPG was also expressed in cells of myeloid lineage and this correlated with high haemoglobin levels (Figure 1B, p=0.028) as well. High grade of positivity of IL-8 expression correlated with high haemoglobin levels (Figure 1C, p=0.012, Mann Whitney test) while high intensity of IL-8 expression correlated with high WBC counts (Figure 1D, p=0.02, Mann Whitney test).

In ET patients, low intensity of OPG expression in megakaryocytes correlated with poor survival, compared with patients showing an intermediate and high OPG intensity expression in megakaryocytes (Figure 2A, p=0.038, Kaplan Meier test). An intermediate intensity of OPG expression in megakaryocytes was observed in patients who were JAK-2 positive (Figure 2B, p=0.023, chi square test).

In MF patients, correlations with haemoglobin, haematocrit, LDH and JAK2 did not reach statistical significance.

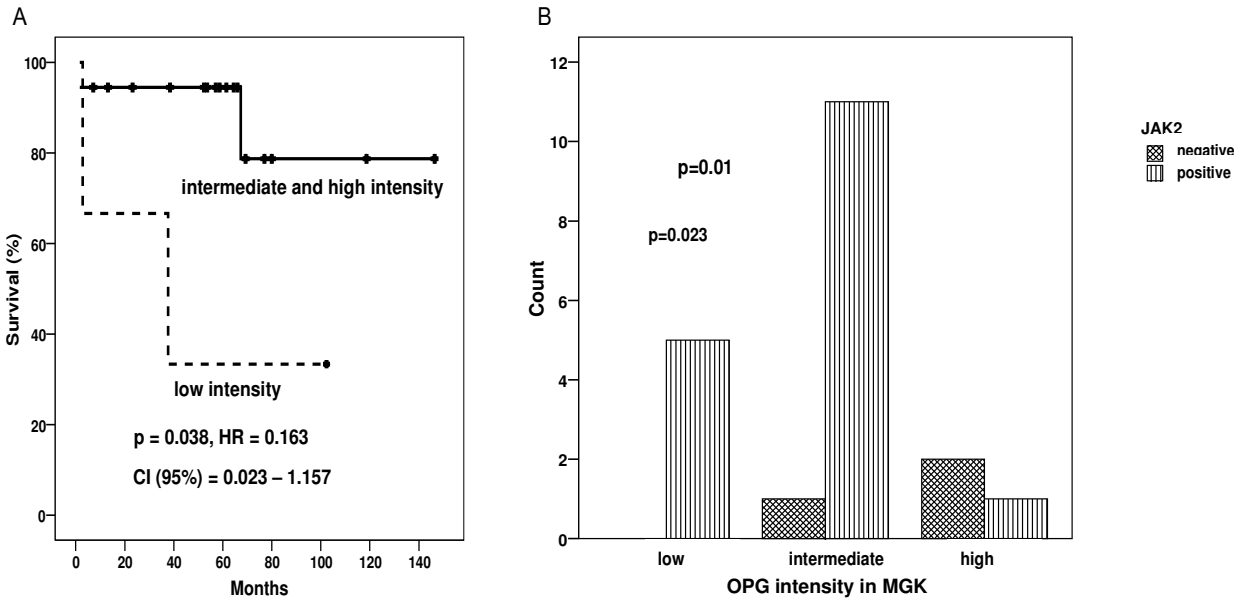

Figure 2. Correlations of OPG in patients with ET

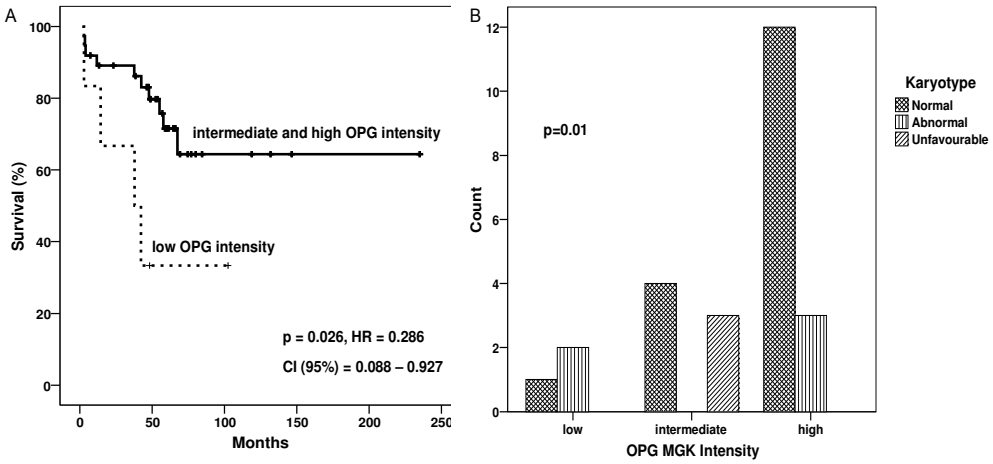

Figure 3. Survival and Karyotype findings in the whole patient series

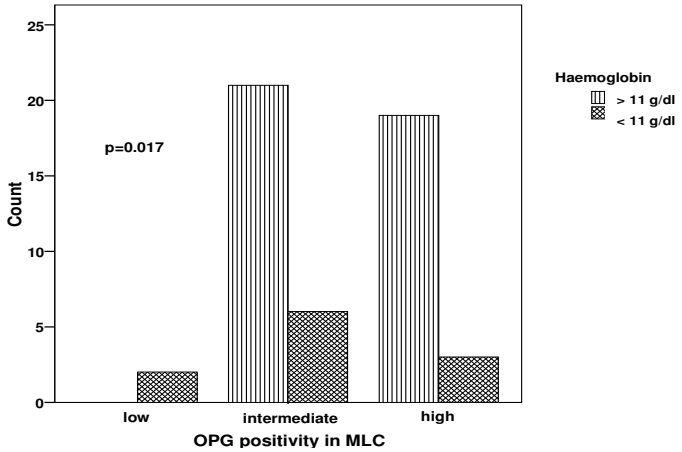

Figure 4. Correlation of OPG positivity and Haemoglobin in Myeloid cell lines

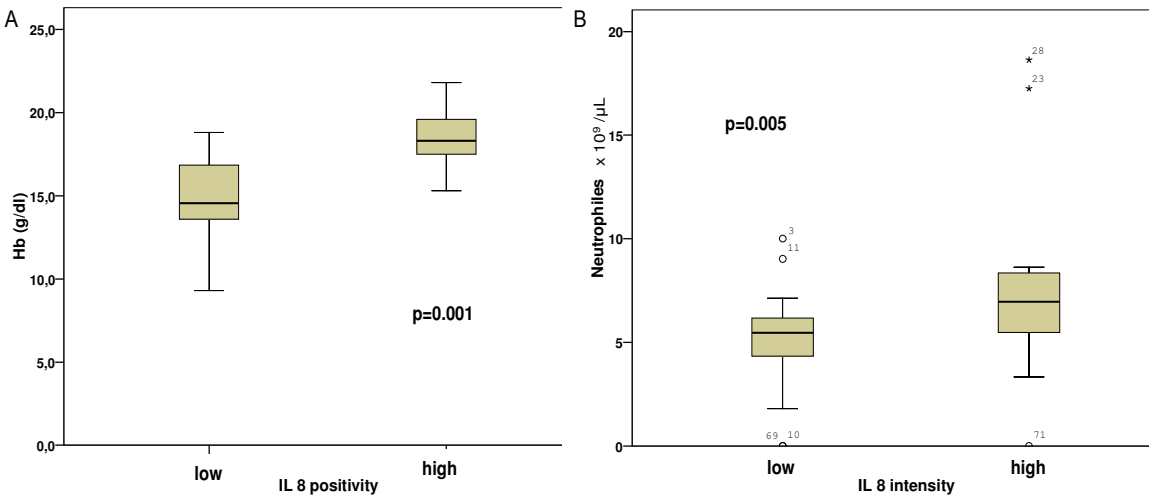

Figure 5. Correlations of IL 8 in the whole patient series

Analysis of the whole series

In the analysis of the whole series, patients with low intensity of OPG expression in megakaryocytes presented poorer overall survival (Figure 3A, p=0.026, Kaplan Meier test) whereas high intensity in OPG expression in megakaryocytes correlated with a normal karyotype (Figure 3B, p=0.01, chi square test). Furthermore, increased OPG expression in myeloid cell lineage correlated with haemoglobin levels >11.0 g/dl (Figure 4, p=0.017, chi square test).

In terms of IL-8 in the whole series, high positivity was observed in all patients with high haemoglobin levels (Figure 5A, p=0.001, Mann Whitney test). Furthermore, high IL-8 intensity expression was observed in patients with high neutrophil counts (Figure 5B, p=0.005, Mann Whitney test).

DISCUSSION

The present study is the first where IL-8 and OPG were studied by immunohistochemistry in BM samples. With regard to IL-8, it was expressed at high intensity in all MPN patients with high neutrophil counts, which is considered an indicator of tumor aggressiveness (Tefferi A et al., 2010) and the level of positivity was higher in patients with high haemoglobin levels. Its degree of expression was not found correlated with other disease characteristics. IL-8 in MPN has been studied in the serum only and immunohistochemistry findings are herein shown for the first time to the best of our knowledge. Emadi et al studied IL-8 serum levels in patients with MF not receiving chemotherapy at the time of the study, and found them significantly increased, contributing to the myeloproliferative process. It is worth mentioning that in Emadi's study, IL-8 expression by flow cytometry was observed in various haematopoietic cell types, including granulocytes, monocytes, megakaryocytes, platelets and CD34+ cells, derived from unmobilised normal blood. Emadi et al showed that the level of IL-8 is significantly increased in the serum of patients with MF and that various haematopoietic cells including platelets, participate in its production. Increased serum levels of IL-8 were observed in patients with PV and ET while IL-8 was also found to enhance formation of erythroid colonies in vitro and were suggested to promote extramedullary haematopoiesis in the liver and spleen (Corre-Buscail I et al., 2005); various haematopoietic cells including platelets were shown to contribute to IL-8 production. Tefferi et al found that IL-8 is also associated with elevated levels of circulating blasts and with the presence of constitutional symptoms in MPN. In addition, IL-8 promotes endothelial cell proliferation, capillary tube organization and matrix metalloproteinase expression in endothelial cells (Li.A et al., 2003).

Regarding OPG, we showed that MPN patients with low intensity of OPG in megakaryocytes by immunostaining have poor survival. High intensity of OPG MGK expression in all patients was related with a normal karyotype. OPG expression compared to karyotypic findings has not been mentioned in the literature yet and this will require further studies as it could be used as a prognostic marker in the future. There are a few other studies on OPG in myeloproliferative neoplasms in medical literature; Bock et al showed that osteosclerosis in MF is associated with increased endothelial OPG expression. Immunohistochemical detection of OPG protein revealed strong labeling of endothelial cells within proliferating vessels in fibrotic IMF and heterogeneously labeled megakaryocytes and fibroblasts. Even though, the cellular origin of OPG in patients with MPN has not been elucidated, Bock et al

showed that bone marrow areas with fully developed fibrosis and osteosclerosis, exhibited predominant endothelial OPG expression (Bock O et al., 2005). Increased production of OPG, provokes impairment of osteoclast formation, thereby inducing osteosclerosis, but also can contribute to endothelial growth and neoangiogenesis (Collin Osdoby P., 2001). Endothelial cells have been identified as potential producers of OPG (Kneipe H et al., 2012). Besides a role in bone homeostasis, OPG may be important for endothelial cell proliferation and survival (Malyankar UM et al., 2000).

In conclusion, our study supports that low intensity of OPG MGK expression by bone marrow immunohistochemistry in patients with MPN is associated with poor survival. Furthermore, high intensity IL-8 immunohistochemical expression in MGK was also observed in all MPN patients with neutrophilia which is an unfavorable prognostic factor. These findings certainly warrant further investigation as both biomarkers are potential therapeutic targets. To the best of our knowledge, these findings have not been reported yet. It is evident that the clinical phenotype of MPN is the result of both clonal myeloproliferation and a secondary inflammation process characterized by bone marrow stromal changes. The development of novel more effective therapies largely depends on a better understanding of the disease pathogenesis. Increasing knowledge of the bone marrow microenvironment will hopefully establish curative strategies for MPN. It is worth mentioning that in all MPNs but most commonly in the advanced myelofibrosis state, autoimmune and chronic inflammatory diseases have been described, linked to elevated biomarkers of inflammation (Barbui T et al., 2011; Hasselbach H., 1990; Kristinsson SY et al., 2010). Chronic inflammation may be both an initiator and a driver of clonal evolution in patients with MPN, as elegantly proposed by Hasselbach (2012).

Declaration of interest: None

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