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Title:

REVISED CYTOGENETIC FEATURES AND IMPACT OF MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs) IN ELDERLY PATIENTS AFFECTED BY MYELOPROLIFERATIVE NEOPLASMS (MPNs)

SSD MED/15

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English Summary

Actual cytogenetic risk stratification in primary myelofibrosis (PMF) is two-tiered: ‘favorable’ and ‘unfavorable’. Recent studies have suggested prognostic heterogeneity within the unfavorable risk category. In 1002 PMN patients, we performed stepwise analysis of impact on survival from individual and prognostically ordered cytogenetic abnormalities, leading to a revised three-tiered risk model: ‘very high risk (VHR)’, ‘favorable’ and ‘unfavorable’. Median survivals for VHR (n=75), unfavorable (n=190) and favorable (n=737) risk categories were 1.2 (HR 3.8, 95% CI 2.9–4.9), 2.9 (HR 1.7, 95% CI 1.4–2.0) and 4.4 years and survival impact was independent of clinically derived prognostic systems, driver and *ASXL1/SRSF2* mutations. The current study clarifies the prognostic hierarchy of genetic risk factors in PMF and provides a more refined three-tiered cytogenetic risk model. Although the recent advantage of risk classification of patients, the only curative treatment for PMF is the allotransplantation, that is proposed to select fit patients.

A total comprensive pathogenetic aspects of MPNs remain unclear, and the inflammation phenomena related to MPNs developments have been much less studied. To underline the “inflammed” and immune derangement in this setting of patients, we identified in MPNs patients, myeloid-derived suppressor cells (MDSCs), that are specialized immunosuppressor able to control the functions of other immune cells, able to suppress a strong anti-leukemia immune response, thereby supporting tumor immune escape, and preventing excessive inflammatory responses.

We recollected samples of 55 new MPNs cases and analysed the presence of MDSCs and their correlation to clinical and molecular features. We enrolled 12 PMF, 10 Polycytemia Vera (PV), and 23 Essential Thrombocytemia (ET), 5 with unclassifiable MPN. We identify that MDSCs are higher in MPNs patients than in health controls. MDSC levels were not correlated with *JAK2* status, white blood cells, Hb levels, platelet counts, splenomegaly.

In order to identify early patients who can benefit from target therapies, further studies are needed on the role of MDSCs in myeloid diseases and on the dysregulation of the lymphocyte T system in these diseases.

Italian Summary

L'attuale stratificazione del rischio citogenetico nella mielofibrosi primaria (PMF) è a due livelli: "favorevole" e "sfavorevole". Studi recenti, hanno tuttavia evidenziato l'eterogeneità prognostica all'interno della categoria di rischio sfavorevole. In 1002 pazienti affetti da malattie mieloproliferative (MPNs), abbiamo eseguito un'analisi a step, revisionando le anomalie citogenetiche e valutandone l'impatto sulla sopravvivenza, definendo un nuovo modello di rischio citogenetico a tre livelli: "very high risk" (VHR)", "favorevole" e "sfavorevole". Le sopravvivenze mediane per le categorie di rischio VHR, sfavorevoli e favorevoli sono state 1,2 (HR 3.8, IC 95% 2.9-4.9), 2.9 (HR 1.7, IC 95% 1.4-2.0) e 4.4 anni rispettivamente. L'impatto sulla sopravvivenza delle tre nuove categorie di rischio è risultato indipendente dai sistemi prognostici clinicamente derivati, dal driver mutations e dalle mutazioni ASXL1/SRSF2. L'attuale studio chiarisce la gerarchia prognostica dei fattori di rischio genetici nella PMF e fornisce un modello di rischio citogenetico a tre livelli più raffinato.

Nonostante i chiari vantaggi delle classificazioni del rischio sempre piu precisi, molti aspetti patogenetici delle MPNs rimango poco chiari e le ipotesi secondo cui tali patologie posseggano importanti substrati immunologici ed infiammatori capaci di evadere il sistema immunitario, sono oggetto di limitati studi. Abbiamo, quindi, ricercato nei pazienti con MPNs, le cellule mieloidi soppressorie (MDSC). Le MDSCs rappresentano delle cellule immunosoppressorie specializzate in grado di controllare le funzioni di altre cellule immunitarie e di sopprimere la risposta immunitaria antileucemica, supportando così la fuga immunitaria del tumore. Abbiamo identificato in tutti i pazienti con MPNs, le MDSCs, a supporto dello squilibrio infiammatorio ed immunitario di queste patologie. Sono stati arruolati 55 pazienti di cui 12 affetti da PMF, 10 da policitemia vera (PV), 23 da trombocitemia essenziale (ET) e 5 con MPN non classificabile. Le MDSCs sono risultate più elevate nei pazienti MPNs rispetto ai controlli sani. Non è stata evidenziata alcuna correlazione tra i livelli di MDSCs, lo stato mutazionale del *JAK2*, i globuli bianchi, i livelli di emoglobina, la conta piastrinica e la splenomegalia.

Al fine di identificare precocemente precocemente pazienti che possano beneficiare di terapie target, considerando che il trapianto allogenico di cellule staminali è ancora oggi l'unico trattamento curativo, gravato da tassi di mortalita elevati, sono necessari ulteriori studi sul ruolo delle MDSCs nelle patologie mieloidi e sulla disregolazione del sistema T linfocitario in queste patologie.

1. Background

1.1. Myeloproliferative neoplasms Ph negative

The World Health Organization (WHO) classification system for hematopoietic tumors was recently revised and the 2016 document recognizes several major categories of myeloid malignancies including acute myeloid leukemia (AML) and related neoplasms, myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), MDS/MPN overlap, mastocytosis, eosinophilia-associated myeloid/lymphoid neoplasms with specific mutations (e.g., PDGFR) and myeloid neoplasms with germline predisposition (Table. 1) [1],[2].

2016 World Health Organization (WHO) classification of myeloid malignancies
Myeloproliferative neoplasms (MPN)
• Chronic myeloid leukemia (CML), BCR-ABL11 • Chronic neutrophilic leukemia (CNL) • Polycythemia vera (PV) • Primary myelofibrosis (PMF) • PMF, prefibrotic/early stage • PMF, overt fibrotic stage • Essential thrombocythemia (ET) • Chronic eosinophilic leukemia, not otherwise specified (NOS) • MPN, unclassifiable • Mastocytosis
Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
• Chronic myelomonocytic leukemia (CMML) • Atypical chronic myeloid leukemia (aCML), BCR-ABL1 • Juvenile myelomonocytic leukemia (JMML) • MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) • MDS/MPN, unclassifiable
Myelodysplastic syndromes (MDS)
• MDS with single lineage dysplasia • MDS with ring sideroblasts (MDS-RS) • MDS-RS and single lineage dysplasia • MDS-RS and multilineage dysplasia • MDS with multilineage dysplasia • MDS with excess blasts • MDS with isolated del(5q) • MDS, unclassifiable • Provisional entity: Refractory cytopenia of childhood • Myeloid neoplasms with germ line predisposition

Table 1. 2016 World Health Organization (WHO) classification of myeloid malignancies. [1]

BCR-ABL1-negative MPN" is an operational sub-category of MPN that includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)[3]. The 2016 WHO classification system distinguishes prefibrotic (prePMF) from overtly fibrotic PMF [4]. PMF, PV and ET are all characterized by stem cell-derived clonal myeloproliferation.

1.2. Primary myelofibrosis, Essential Trombocytemia and Policytemia Vera

PMF is characterized by hematopoietic stem cell-derived clonal myeloproliferation that is often associated with bone marrow fibrosis [5] (Figure 1). The incidence is approximately 0.1 to 1 per 100,000 people per years, with patients presenting at a median age of 64 years. The median survival was 5 years before 1995 and increased to 6.5 years between 1996 and 2007, after introduction of Janus kinase (JAK) inhibitor, improvement of supportive care and earlier diagnosis [6].

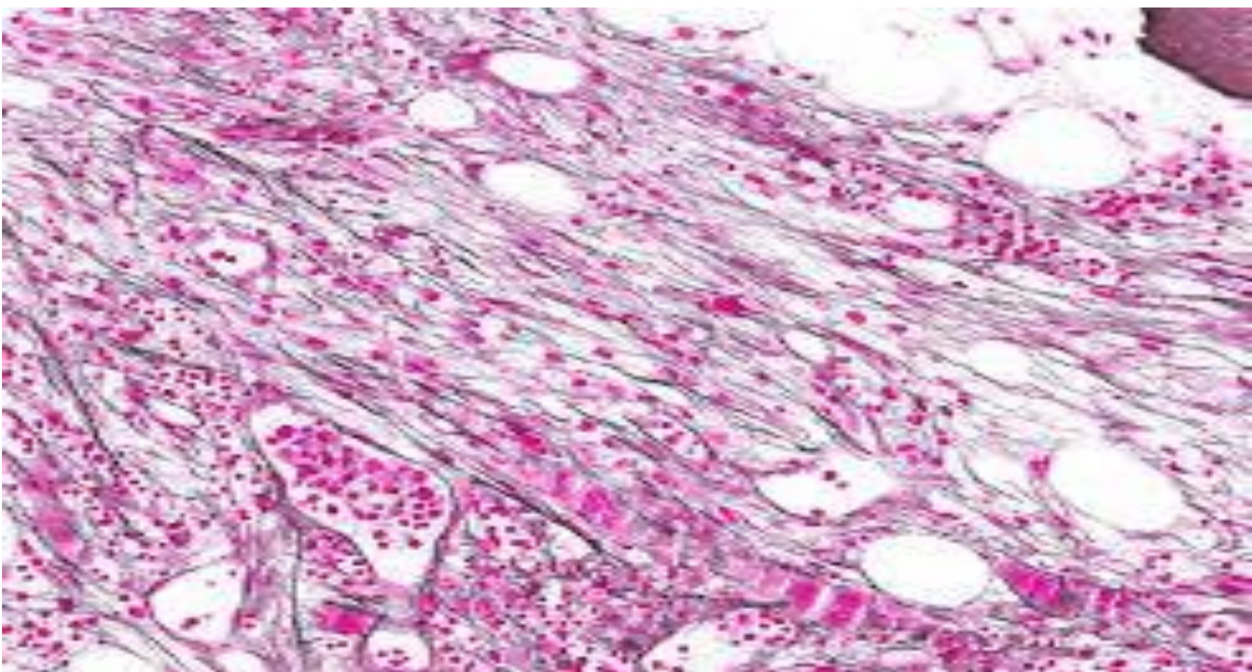


Figure 1. Bone marrow, reticulin stain shows marked increase in reticulin fiber in PMF.

Clinical manifestations in PMF include severe anemia, marked hepatosplenomegaly, constitutional symptoms (e.g., fatigue, night sweats, fever), cachexia, bone pain, splenic infarct, pruritus,

thrombosis and bleeding [7]. Ineffective erythropoiesis and extramedullary hematopoiesis (EHM) are the main causes of anemia and organomegaly, respectively. Other disease complications include symptomatic portal hypertension that might lead to variceal bleeding or ascites and non-hepatosplenic EMH that might lead to cord compression, ascites, pleural effusion, pulmonary hypertension, or diffuse extremity pain. It is currently assumed that aberrant cytokine production by clonal cells and host immune reaction contribute to PMF associated bone marrow stromal changes, ineffective erythropoiesis, EMH, cachexia and constitutional symptoms [7]. Causes of death include leukemic progression that occurs in approximately 20% of patients but many patients also die of comorbid conditions including cardiovascular events and consequences of cytopenias including infection or bleeding [8]. In approximately 90% of patients, PMF is associated with one of three mutually exclusive driver mutations, including Janus kinase 2 *(JAK2),* calreticulin *(CALR)* and myeloproliferative leukemia virus oncogene *(MPL)* [9]. Among these, *JAK2* has an estimated incidence of 65%, followed by *CALR* at 20% to 25% and *MPL* at 5% to 10%. The proteins produced from the *JAK2* and *MPL* genes are both part of a signaling pathway called JAK/STAT pathway which transmits chemical signal from outside the cell to the cell's nucleus (Figure 2) [10].

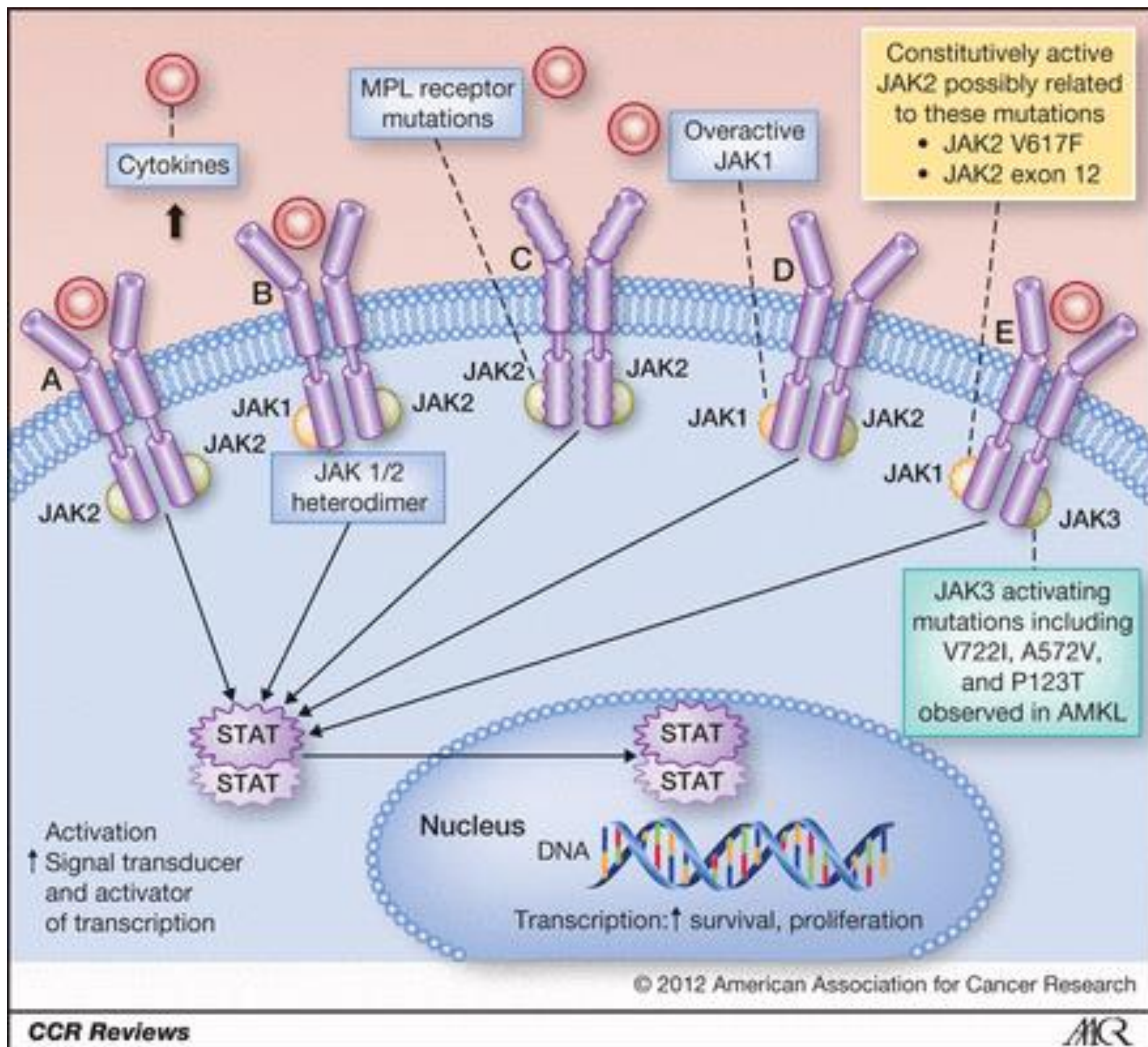


Figure 2. JAK receptor signaling and activation of STAT proteins. *Adapted from "Presentation and outcome of patients with 2016 WHO diagnosis of prefibrotic and overt primary myelofibrosis". Blood, 2017.[10]*

In addition of the prior driver mutations, 80% of patients with PMF harbor other DNA variants in myeloid genes, including *ASXL1, TET2, EZH2, SRSF2, DNMT3A, U2AF1,* and *IDH1/IDH2*, often in multiple combinations [11, 12]. In addition to their presumed pathogenetic relevance, driver and other mutations in PMF have recently been shown to influence overall survuival (OS) and leukemia free survival (LFS) [9, 12-14]. Current evidence supports prognostic distinction based on the presence or absence of type 1-like *CALR* mutations, whereas *ASXL1, SRSF2, EZH2* and *IDH1/IDH2* mutations

are considered as hight molecular risk (HMR) mutations, the prognostic relevance of which is further amplified by the number of such mutations in an individual patient [15].

Current diagnosis of PMF, ET and PV are based on the 2016 WHO-criteria and involves a composite assessment of clinical and laboratory features [1] (Figure 3).

2016 Revised WHO Diagnostic Criteria for Myeloproliferative Neoplasms

Arber et al. Blood 2016;127:2391

	Polycythemia Vera (PV)	Essential Thrombocythemia (ET)	Primary Myelofibrosis (PMF) (overt)	Primary Myelofibrosis (prefibrotic) (prePMF)
Major criteria	1 Hemoglobin (Hgb) >16.5 g/dL (men) >16 g/dL (women) or Hematocrit >49% (men) >48% (women) or ↑ red cell mass >25% above mean	1 Platelet count ≥450 x 10^9/L	1 Megakaryocyte proliferation and atypia*** and ≥ grade 2 reticulin/collagen fibrosis ***megakaryocytes with aberrant nuclear/cytoplasmic ratio and hyperchromatic and irregularly folded nuclei and dense clustering	Megakaryocyte proliferation and atypia*** and ≤ grade 1 reticulin/collagen fibrosis, Increased cellularity, granulocytic proliferation and decreased erythropoiesis
	2 Bone marrow (BM) tri-lineage myeloproliferation with pleomorphic mature megakaryocytes*	2 BM megakaryocyte proliferation with large and mature morphology and hyper-lobulated nuclei. Reticulin fibrosis grade should be ≤1	2 Not meeting WHO criteria for other myeloid neoplasm	Not meeting WHO criteria for other myeloid neoplasm
	3 Presence of *JAK2* mutation	3 Not meeting WHO criteria for other myeloid neoplasms	3 Presence of *JAK2*, *CALR* or *MPL* mutation or presence of another clonal marker or absence of evidence for reactive bone marrow fibrosis	Presence of *JAK2*, *CALR* or *MPL* mutation or presence of another clonal marker or absence of evidence for reactive bone marrow fibrosis
		4 Presence of *JAK2*, *CALR* or *MPL* mutation		
Minor criteria	1. Subnormal serum erythropoietin level	1. Presence of a clonal marker or absence of evidence for reactive thrombocytosis	1. Anemia not otherwise attributed 2. Leukocytosis ≥11 x 10^9/L 3. Palpable splenomegaly 4. Increased lactate dehydrogenase (LDH), above upper normal limit 5. Leukoerythroblastosis	1. Anemia not otherwise attributed 2. Leukocytosis ≥11 x 10^9/L 3. Palpable splenomegaly 4. Increased lactate dehydrogenase (LDH), above upper normal limit
	PV diagnosis requires meeting all three major criteria **or the first two major criteria and** one minor criterion. *BM biopsy may not be not required if Hb >18.5 g/dL in men or 16.5 in women (Hct >55.5 in men and 49.5 in women)	**ET** diagnosis requires meeting all 4 major criteria or first three major criteria and one minor criterion	**PMF** diagnosis requires meeting all 3 major criteria and at least one minor criterion	**prePMF** diagnosis requires meeting all 3 major criteria and at least one minor criterion

Figure 3. 2016 Revised WHO Diagnostic Criteria for Myeloproliferative Neoplasms . *Adapted from Arber et all. The 2016 revision to the WHO, Blood, 2016.[1]*

PMF should be distinguished from other closely related myeloid neoplasms including chronic myeloid leukemia (CML), PV, ET, MDS, chronic myelomonocytic leukemia (CMML) and "acute myelofibrosis." The presence of dwarf megakaryocytes raises the possibility of CML and should be pursued with *BCR-ABL1* cytogenetic testing. Patients who otherwise fulfill the diagnostic criteria for PV should be labeled as "PV" even if they display substantial bone marrow fibrosis [14]. Prefibrotic PMF can mimic ET in its presentation and mutation profile (both can express *JAK2, CALR or MPL* mutations) [16, 17] careful morphologic examination is necessary for distinguishing the two; megakaryocytes in ET are large and mature-appearing whereas those in prefibrotic PMF display abnormal maturation with hyperchromatic and irregularly folded nuclei; the distinction between ET and pre-fibrotic PMF is prognostically relevant [18, 19]. Polycythemia vera and essential thrombocythemia are myeloproliferative neoplasms characterized by increased rate of cardiovascular events, a varying burden of symptoms, and an intrinsic risk of evolution to secondary forms of myelofibrosis and acute leukemia; however, survival is only modestly reduced in most instances. In the last few years, following the description of driver mutations in *JAK2, MPL* and *CALR*, the diagnostic criteria for PV and ET were revised, making the identification of very early stages feasible. Scores for identifying patients at different risk of thrombosis were refined, and they largely guide therapeutic decisions. Treatment is therefore mainly focused on reduction of thrombosis risk, control of myeloproliferation, improvement of symptomatic burden, and management of disease-associated complications. New drugs recently entered the clinical arena, with the promise to improve overall patients' management. However, evidence of a disease-modifying potential is largely missing and represents a still unmet clinical need.

1.3.Risk stratification and the risk adapted therapy.

The first prognostic modeling in PMF developed the International Prognostic Scoring System (IPSS) in 2009 [20]. The IPSS for PMF is applicable to patients being evaluated at time of initial diagnosis and uses five independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dL, leukocyte count >25x10^{9}/L, circulating blasts >1% and presence of constitutional symptoms [20]. The presence of 0, 1, 2 and >3 adverse factors define low, intermediate-1, intermediate-2 and high-risk disease. The corresponding median survivals were 11.3, 7.9, 4 and 2.3 years [20].

The IWG-MRT subsequently developed a dynamic prognostic model (DIPSS) that utilizes the same prognostic variables used in IPSS but can be applied at any time during the disease course [19][21]. DIPSS assigns two, instead of one, adverse points for hemoglobin <10 g/dL and risk categorization is accordingly modified: low (0 adverse points), intermediate-1 (1 or 2 points), intermediate-2 (3 or 4 points) and high (5 or 6 points). The corresponding median survivals were not reached, 14.2, 4 and 1.5 years [22].

IPSS- and DIPSS-independent risk factors for survival in PMF were subsequently identified and included unfavorable karyotype (i.e., complex karyotype or sole or two abnormalities that include 18, 7/ 7q-, i(17q), inv(3), 5/5q-, 12p- or 11q23 rearrangement) [23, 24], red cell transfusion need and platelet count <100 3 10^{9}/L [25]. Accordingly, DIPSS was modified into DIPSS-plus by incorporating these three additional DIPSS-independent risk factors: platelet count <100 3 10^{9}/L, red cell transfusion needs and unfavorable kar- yotype [26, 27]. The four DIPSS-plus risk categories based

on the afore- mentioned eight risk factors are low (no risk factors), intermediate-1 (one risk factor), intermediate-2 (two or 3 risk factors) and high (four or more risk factors) with respective median survivals of 15.4, 6.5, 2.9 and 1.3 years (Figure 4) [27].

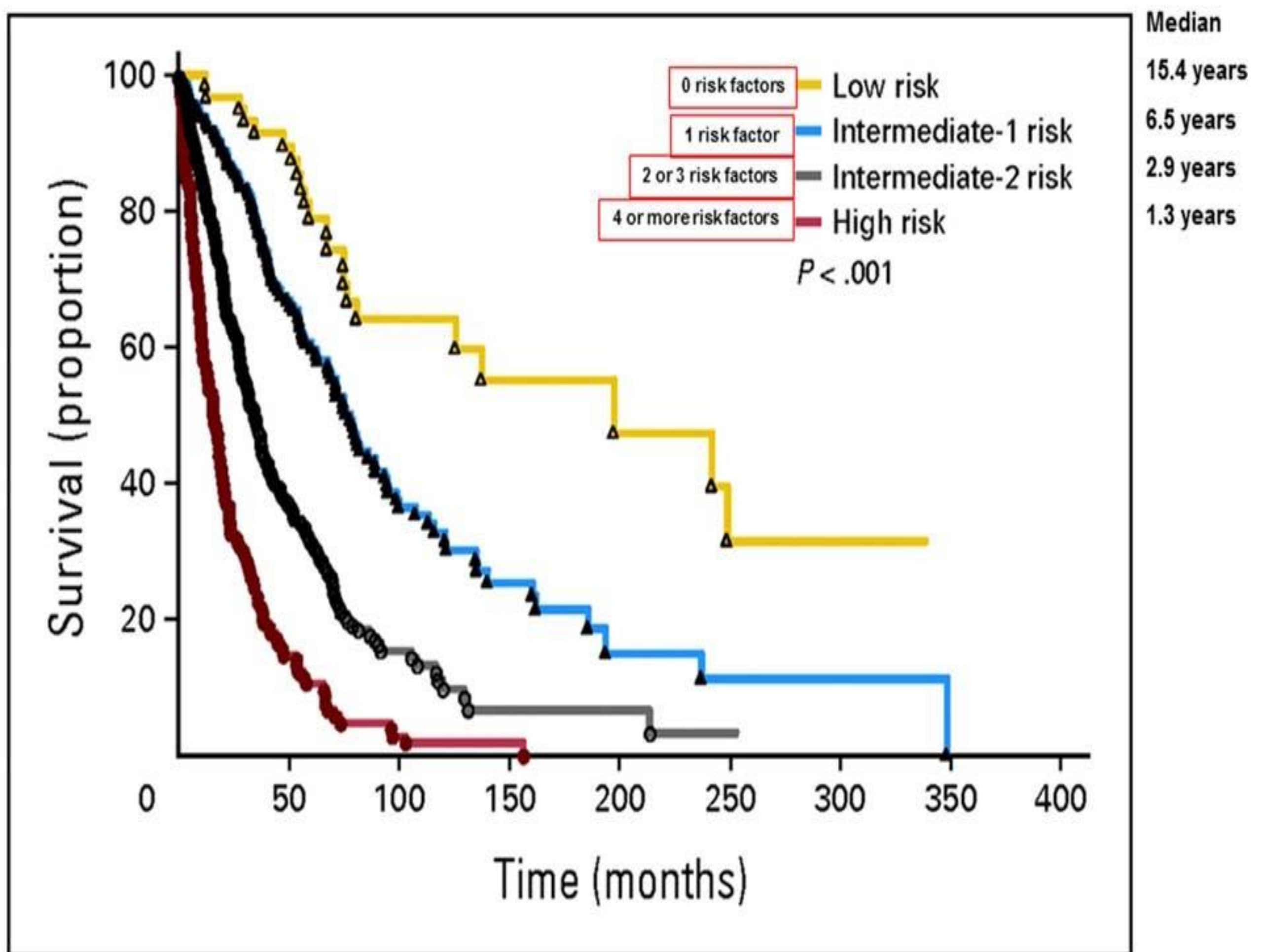


Figure 4. Survival data of 793 patients with primary myelofibrosis evaluated at time of their first Mayo Clinic referral and stratified by their Dynamic International Prognostic Scoring System (DIPSS-plus.)[4]

Since the publication of DIPSS-plus, several studies that suggest additional prognostic information have been published. [4] For example, a >80% two-year mortality in PMF was predicted by

monosomal karyotype, inv(3)/i(17q) abnormalities, or any two of circulating blasts >9%, leukocytes >40 x 10^9/L or other unfavorable karyotype [28]. Treatment of PMF includes supportive care, use of JAK2 inhibitors and other drugs, surgical removal or involved field irradiation of the spleen, and allogeneic stem-cell transplantation (alloSCT). These treatment measures, except for alloSCT, are mostly palliative and unlikely to modify the natural history of the disease [29]. Unfortunately, alloSCT carries a substantial risk of treatment-related mortality and morbidity, which underscores the need for reliable prognostic models that facilitate in otherwise transplantation-eligible patients [4].

Clinical and molecular risk stratification and risk-adapted therapy in primary myelofibrosis

	Molecular risk		
	High risk Presence of adverse mutations (e.g. *ASXL1*, *SRSF2*), and absence of type 1/like *CALR* mutation	**Intermediate risk** Not classifiable as high or low risk	**Low risk** Presence of type 1/like CALR mutation and absence of adverse mutations (e.g. *ASXL1*, *SRSF2*)
DIPSS-plus risk			
High	Stem cell transplant or Investigational drug therapy	Stem cell transplant or Investigational drug therapy	Stem cell transplant or Investigational drug therapy
Intermediate-2	Stem cell transplant or Investigational drug therapy	Stem cell transplant or Investigational drug therapy	Investigational drug therapy
Intermediate-1	Stem cell transplant or Investigational drug therapy	Observation or Investigational drug therapy	Observation
Low	Stem cell transplant or Investigational drug therapy	Observation	Observation

Figure 5. Clinical and molecular risk stratification and risk-adapted therapy in primary myelofibrosis. *(From Tefferi et al. Am J Hem .2016) [30]*

Current treatment recommendations favor alloSCT for DIPSS/DIPSS plus high- or intermendiate-2-risk disease, wherease a more conservative treatment approach migh to be considered for lower- risk disease [30] (Figure 5). Recently, Guglielmelli et al. [31] introduced Mutation-Enhanced International Prognostic Score Systems (MIPSS 70) and MIPSS 70 plus, that provide complementary systems of risk stratification for transplantation-age patients with PMF and integrate prognostically relevant cinical, cytogenetic, and mutation data.

2. "The Perfect Storm". Combining Inflammation and Specific Mechanisms of Tumor Immune Escape in MPNs.

Recently, alongside with the advances in molecular and cytogenetic characterization, there is a growing knowlwdge that inflammation plays a crucial role in MPN promoting affecting disease development and evolution. Moreover, several studies demonstrated that the immunesystem is profoundly disrupted in MPNs, to enable mechanisms of tumor escape. Therefore, further to the pharmacological inhibition of JAK-STAT, the recovery of protective specific tumor immune surveillance could be potentially exploited for therapeutic purposes. A current mainstay of cancer development has become the notion that cancer cells may proliferate and emerge as overt disease only when finding successful strategies of immune escape in a permissive tumor microenvironment (TME). In MPNs, the inflammatory TME promotes the progression of clonal myeloproliferation and provides an important immunosuppressive effect against cytotoxic T cells and other anti-tumor defenses (Figure 6) [32]. In addition, MPN-mutated hematopoietic stem cells (HSCs) themselves have been shown to exert broad pro-inflammatory effects, contributing to a vicious maintenance of the inflammatory TME, as well as to adopt different mechanisms of evasion from T cell immunosurveillance, eventually resulting in uncontrolled clonal escape. A growing set of research works is currently contributing to the depiction of the immunologically disrupted "cancer ecosystem" associated with MPN outgrowth [32].

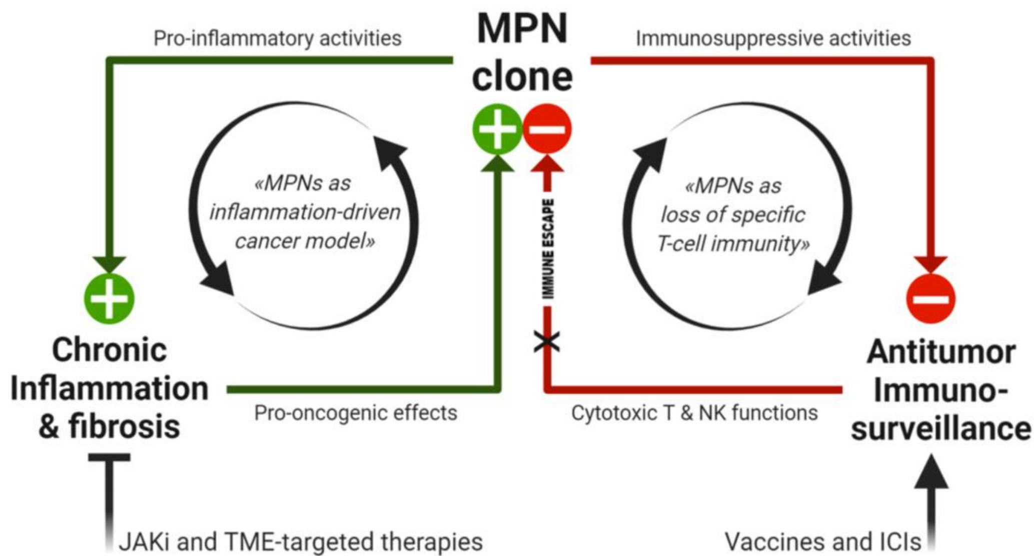


Figure 6. MPNs and inflammatory models. MPNs are a tipical inflammatory models of human cancer development, as suggested by the evidence of abnormal cytokine production and association with several inflammatory and autoimmune diasease and second cancer. Oncogenic lesion constitutively activates inflammatory pathways in HSC, eliciting the production of ROS. The accumulation of ROS in mutated cells damage DNA and favors clonal proliferation, in particular JAK2, with up regulation of cytokines IL-6, IL8, IL11, IL12-IL15, CXCL4 TNFa, NFKb.The inflammatory Tumor Micro Invironment provides an important immunosuppressive effect against cytotoxic T cell and other antitumor defence. Adopting a different mechanism of evasion from T immunosurveillance: a cancer echosistem. *Adapted from Nasillo et al, Inflammatori Microevironment ed specific T cells in Myeloproliferative neoplasm: Immunopathogenesis and novel immunotherapy. Int J Mol,Sci, 2021.[32]*

To date, robust gene expression studies by Skov et al., performing whole transcriptional analyses on blood cell from MPN cases, showed a significant down-regulation of human leucocyte antigen-I (HLA-I), HLA-II and other HLA-relatedgenes, as well as of CD40L and FAS, implying a basic impairment of tumor-antigen presentation, as well as of antigen-presenting cell (APC)-mediated costimulatory signaling and T cell cytotoxicity, respectively [33, 34]. Along with dysfunctional adaptive T cell responses, the arm of innate immunity was also found to be impaired in MPNs: lower levels of circulating natural killer (NK) cells were observed in untreated patients, compared to healthy controls, while a recovery of cytotoxic CD56-brightNK cells was associated with long-term IFN-αtherapy [105]. In addition, in a murine model a combination of deficiencies for both HLA-II and CD4+ T cells completely abrogates the emergence of MPNs, thus suggesting that the presence of “unprimed” CD4+T lymphocytes may be required for the emergence of an “MPN-permissive” TME [35]. Based on the observation, about a putative role of regulatory T lymphocytes (Tregs) in MPNs, the suppressive subset may be important in the immunopathogenesis of MPNs, due to the possibly

the direct inhibition of specific antitumor responses. However, to date, few immunological studies in MPN cases reported some unexpected and partially discordant data.

Romano et al., showed that Tregs were numerically contracted and dysfunctional, showing increased cytokine production [36]. Different cell-mediated immunosuppressive strategies have been implied in the immune escape of MPNs from specific T cell defenses [32].

Myeloid derived suppressor cells (MDSCs) have already shown relevant activities in several hematologic neoplasms and may represent a crucial link between inflammation and the inhibition of antitumor T cell immunity [37]. In MPNs, CD11b+CD14-CD33+ cells (MDSCs) were significantly more frequent in cases compared to controls and were associated with higher expression of arginase-1 (ARG1) mRNA and with specific suppressive activity against autologous T lymphocytes [37]. It has also been hypothesized that MPN-associated clonal thrombocythemia may sustain an intriguing "platelet–cancer loop", as pathologic platelets could readily suppress specific T cells [38]. The primary over activation of JAK/STAT pathways in JAK2V617F + clonal cells directly induced the overexpression of programmed cell death (PD-1) ligand 1 (PD-L1), thus supporting the idea that MPN cells exploit the PD1/PD-L1 axis to escape specific T cell immunosurveillance [39]. In addition, *JAK2* mutant cells, by inhibition of ROS-convertin genzyme through the upregulation of the PI3K/Akt pathway, can produce large amounts of ROS, which are known to negatively affect T cell effector functions. [39]. In the TME of MPNs, effects of extracellular mutated *CALR* protein may lead to the functional inhibition of the phagocytosis of cancer cells, further contributing to the escape from antitumor immunity.

Altogether, these findings suggest that the immune system is deeply dysregulated in MPNs and may be involved in MPNs develop and evolve, due to tumor immune evasion. As a matter of fact, by considering that *CALR* and *JAK2* mutations are immunogenic and that specific T cells reactive to these mutations are readily detectable in patients' peripheral blood, *CALR/JAK2* mutants must elude such T cell-mediated elimination to pathologically expand in the BM. Figure 7 provides a graphical abstract of the MPN-associated TME.

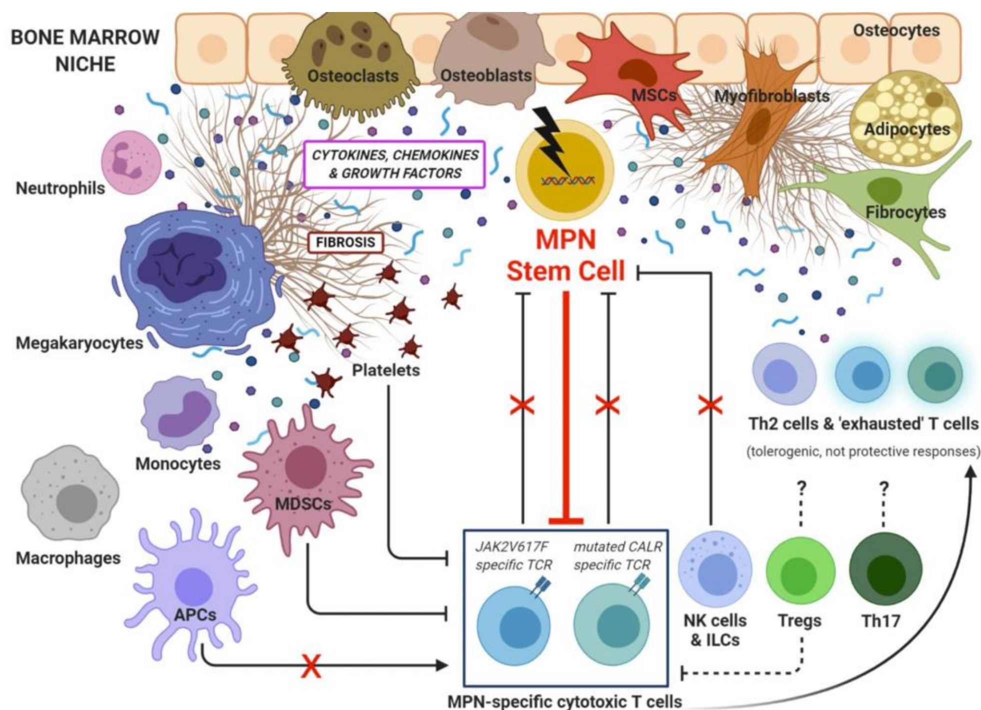


Figure 7. MPN-associated TME.The emerging cancer ecosystem in myeloproliferative neoplasms (MPNs). Several cellular players contributeto the MPN-associated microenvironment, characterized by increased cytokine signaling, fibrosis, inflammation-drivenimmunosuppression and immune escape. MSCs: mesenchymal stem cells, MDSCs: myeloid-derived suppressor cells, APCs: antigen-presenting cells, TCR: T cell receptor, Th: T helper, NK: natural killer, ILCs: innate lymphoid cells, Tregs: Tregulatory cells,⊥: inhibition, X: bloc.*Adapted by Nasillo et al.[32]*

The inflammatory microenvironment and loss of specific T cell immunity represent the emerging immunopathogenetic features of MPNs, which rely on constitutive activation of the *JAK/STAT* pathway, induced by recurrent acquired mutations [32]. Beyond JAKi, innovative therapeutic strategies addressing MPN immunological signatures are now under study. At present, by considering the growing evidence on the protective role of MPN-specific T lymphocytes, and the previous experiences describing successful of investigations on tumor-specific T cell immunity, it seems time to try exploiting the antitumor potential of MPN-specific T lymphocytes in the therapeutic management of MPN patients. Novel “T cell-based” immunotherapies may serve to hunt and eliminate residual mutated HSCs in patients with an ongoing response to “molecular” treatments against the *JAK/STAT* pathway [32]. Immune checkpoint inhibitor (CPI) therapy enhances shared neoantigen-induced T cell immunity directed against mutated calreticulin in myeloproliferative neoplasms [40].

Chronic inflammation and oxidative stress have been characteristic of MPNs [41, 42]. In murine model, tipical inflammatory cytokines, including interleukins IL-1β, IL-6, IL-8, IL-10, IL-11, IL-12, IL-15, IL-17, and IL-33, lig-and 1 (CXCL1), CXCL4, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), granulocyte macrophage-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and angiopoietin-1, have been reported to be increased [43].

The continued inflammation results in the accumulation of more ROS in cells, damages DNA, and causes clonal proliferation, driving disease progression in MPN [44, 45]. Therefore, in contrast to the clonal theory for the MPN pathogenesis with *JAK-2*, mutant clonal activation induces inflammatory changes, the accumulation of more ROS, and clonal evolution, leading to different phenotypes of MPN [45]. Shi et al. have studied the inflammatory pathway in MPN in 97 MPN patients and found that TLR2 was the predominant pattern recognition receptor (PRR), especially in PV and ET in versus PMF patients. ROS production was remarkedly higher in MF than in PV or ET, which implies that the inflammatory process in MPN involves a major role of TLR-2 and a minor role of TLR-4 in accumulating ROS. This leads to DNA damage, and, with years, the accumulation of more ROS formation, DNAdamage, and then the transformation of PV or ET into MF [46].

In the inflamed micro-environment of MPNs, the production of different inflammatory cytokines along with elevated S100A9 results in the accumulation of MDSC in MPN [47]. The mechanism of increased MDSC could be due to (i) the inflammatory cytokine stem cell growth factor (SCF) leadingto the accumulation of MDSC [48] ; (ii) increased S100A9 levels, which inhib-ited dendric cell maturation and then increased MDSC [49], (iii) the cytokinerelease of GM-CSF, VEGF, PGE2/COX2 (prostaglandin E2/cyclooxygenase-2), and interferon (IFN)-γ. These factors are responsible for MDSC accumulation and C5a, which facilitates MDSC infiltration into tumors and enhances their suppressive abilities [50].

3. Myeloid-derived suppressor cells (MDSCs)

Myeloid cells are a tipical cellular compartment of the immune system. All myeloid cells arise from multipotent HSCs that develop into mature myeloid cells through sequential steps of differentiation. The mature myeloid cells are DC, macrophages and granulocytes and are essential for the normal functions of the innate and adaptive immune systems. The immature myeloid cells (IMCs) comprising the MDSCs (Figure 8) [51, 52].

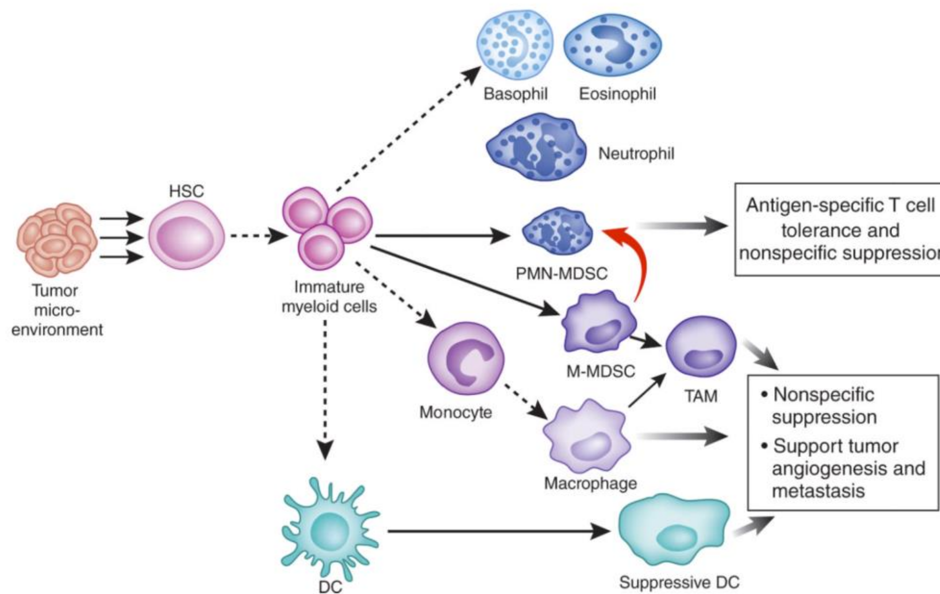


Figure 8. Myeloid derived suppressor cells differentiation. M-MDSCs differentiate into PMN-MDSCs in tumor-bearing hosts. Soluble mediators produced in the tumor microenvironment promote the aberrant differentiation of MDSCs.Dashed lines indicate the normal developmental pathway of immature myeloid precursorcells, which differentiate into DCs, monocytes-macrophages and granulocytes (basophils, eosinophils and neutrophils) in non-tumor-bearing hosts. Solid lines indicate the aberrantpathways of myeloid cell development in tumor-bearing hosts. New data (thick red line) suggest that a substantial proportion of PMN-MDSCs emerge from the M-MDSC pool. HSC, hematopoietic stem cell; TAM, tumor-associated macrophage. *Adapted from Myeloid-cell differentiation redefined in cancer, Thomas A Wynn, 2013.[53]*

IMCs are expanded in the bone marrow and subsequently, they can migrate into extramedullary sites, like the spleen, limphnodes, and inflamed tissue, where the cells can continue to proliferate, a process called "emergency myelopoiesis" [54-56]. MDSCs are a heterogenous population of myeloid cells that consist of myeloid progenitors and immature macrophages, immature granilocytes and immature dendritic cells these are increased in state of inflammation, infection and in cancer.

In cancer patients, growing tumors secrete a variety of cytokines and other molecules which are key signals involved in the generation of MDSC. Tumor cell lines overexpressing colony stimulating factors (e.g. G-CSF and GM-CSF) have long been used in vivo models of MDSCs generation. GM-CSF, G-CSF and IL-6 allow the in vitro generation of MDSC that retain their suppressive function in

vivo. In addition to CSF, other cytokines such as IL-6, IL-10, VEGF, PGE2 and IL-1 have been implicated in the development and regulation of MDSC [2]-[13]. The myeloid-differentiation cytokine GM-CSF is a key factor in MDSC production from bone marrow and it has been shown that the c/EBPβ transcription factor plays a key role in the generation of in vitro bone marrow-derived and in vivo tumor-induced MDSC. Moreover, STAT3 promotes MDSCs differentiation and expansion and IRF8 has been hypotized to balance MDSC-inducing signals. Consequently, IMCs differentiate into MDSCs through distinct activation signals like inflammatory stimuli. MDSCs can suppress immune functions by inducing the differentiation of regulatory T cells (Tregs) and regulatory B cells (Bregs) and also thay can suppress the activation of DCs natural killer, and macrophages involved in the suppression of innate and adaptive immunity [57]. It is important to note that MDSCs, that are expanded in pathological conditions like cancer, are not a defined subset of myeloid cells but rather a heterogeneous population of activated IMCs that have been prevented from fully differentiating into mature cells. MDSCs lack the expression of cell-surface markers that are specifici for monocytes, macrophages or DCs and are comprised of a mixture of myeloid cells with granulocytic and monocytic morphology [52].

3.1. Phenotipic and functional characteristics of MDSCs

MDSCs consist of two groups of cells: i) monocytic (M-MDSC) and ii) granulocytic or polymorphonuclear (PMN-MDSC) represented more than 80% of all MDSC and phenotypically and morphologically like neutrophils [58]. In mice PMN-MDSC can be defined as CD11b+, Ly6Clo, Gr+ (Ly6G) and M-MDSC as CD11b+, Ly6G-, Ly6Chi. In humans PMN-MDSC is defined as CD11b+, CD14-, CD15+/CD66b+, LOX-1 and M-MDSC as CD11b+, CD14+, HLA-DR-/low, CD15-, CD33+ [59]. Human PMN-MDSC does not express a marker homologous to mouse[55].

3.2. Mechanism of immune suppression MDSCs- mediated.

The immune suppression MDSCs- mediated depends on the following metabolic modes:

1) Arginase (Arg)-1 consuming arginine. The inhibitory activity of Arg-1 is based on its role in the hepatic urea cycke, which metabolizied L-arginine into L-ornithine. Increased accumulation of Arg-1 results in L-arginine depletion from the microenvironment that inhibits T cell proliferation by reducing T cell CD3δ expression or preventing T cells from upregulating the cell expression of the cycle regulators cyclin D3 and Cyclin-dependent kinase 4 (CD4), threby arresting the cell cycle in the G0/G1 phase [60-62];

2) Inducible nitric oxide synthase (iNOS) producing nitric oxide (NO). NO can react with superoxide to form Peroxynitrite (PNT) and then directly can inhibit T cells by nitrifying T cell receptor (TCR). This process reduces the affinity of TCR for antigen Major histocompatibility complex (MHC) presented by cancer cells and blocks the migration of T cells by nitrating T – cell specific chemokines [63].

3) Reactive Oxigen Species (ROS), including the superoxide anion (O2-), hydrogen peroxide (H2O2), and PNT (ONOO-). These molecules are intrinsically involved in the activation of transcriptional and metabolic reprogramming of MDSCs and influence their differentiation and maintenance. The principal subpopulations of MDSCs exploit different mechanisms to inhibit T cell proliferation [64]. PMN-MDSC express high levels of ROS and low leves of NO, whereas M-MDSC produce large amounts of NO and immunosuppressive cytokines and both subpopulations express arginase [64]

3.3. MDSCs immunosuppressive functions in the tumour microenvironment (TME).

MDSCs expansion and suppressive mechanisms are mainly regulated by the signal transducer and activator of transcription (STAT) signalling pathway. Many tumours exploit STAT signalling through the secretion of Tumorur-derived factors (Figure 9). This hijacking of STAT signalling plays an important role during cancer initiation, progression and in maintaining an immunosuppressive tumour microenvironment (TME), for example by inducing accumulation of MDSCs or by

stimulating their suppressive capacity [51]. The activation of STAT3 can inhibit apoptosisi in myeloid cells and prevent these cells from differentiating into mature cells. Tumour-derived factors, like G-CSF, GM-CSF and VEGF, induce STAT3 signalling, resulting in increased expression of proliferation inducing and anti-apoptotic proteins, including c-Myc, Bcl-XL and ciclyn D. These proteins promote the proliferation of immature myeloid cells while preventing apoptosis and differentiation into mature cells, resulting in increased MDSCs frequencies [65]. In addition, STAT3 directly regulates MDSC suppressive mechanism by inducing NOX2 expression, and arginase production [66]. STAT3 also induces the gene expression and protein level of the pro-inflammatory protein S100A9 in myeloid progenitors. The overexpression of S100A9 prevents differentiation into mature myeloid cell types by directly facilitating ROS production, resulting in the expansion of MDSCs. Furthermore, S100A9 binds to CD33 on MDSCs and induces production of IL-10, TGF-β , arginase and ROS [67] , INF-y and IL-1B regulate STAT1 activation, which induces proliferation and suppressive capacity by regulating iNOS and arginase-I activity [65]. STAT5 induces MDSCs expansion by reducing differentiation into mature myeloid cells through inhibition of interferon regulatory factor-8 (IRF-8) [68]. STAT6 abduces MDSCs proliferation, survival and enhnances arginase-I activity in MDSCs [69].

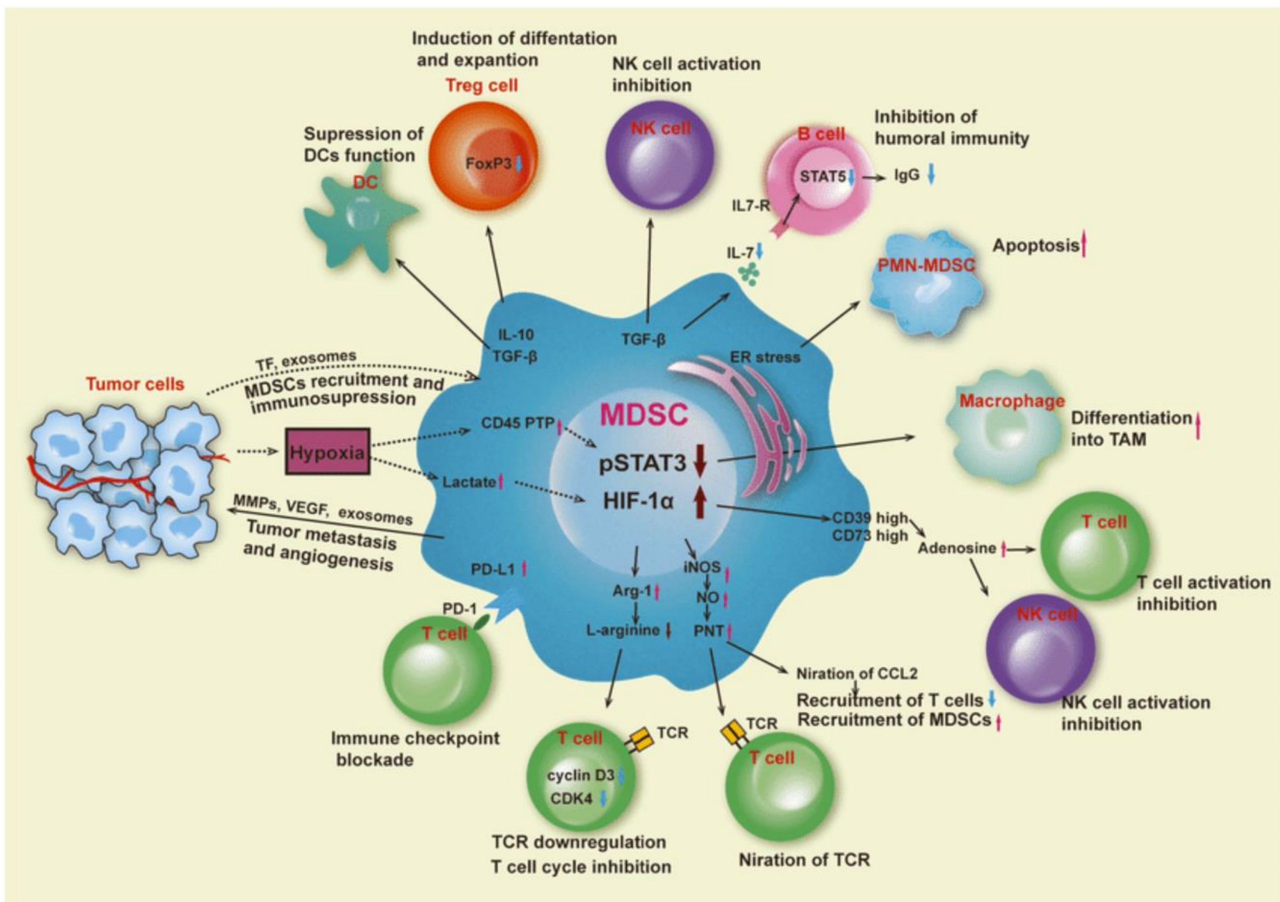


Figure 9. Immunosuppressive functions of MDSCs in the tumor microenvironment. DCs: dendritic cells; TAM: tumor-associated macrophage; ER: endoplasmic reticulum; Arg-1: arginase 1; iNOS: inducible nitric oxide synthase; HIF-1α: hypoxia-inducible factor-1α; STAT3: signal transducer and activator of transcription 3; VEGF: vascular endothelial growth factor; TF: tissue factor. In the tumor microenvironment, MDSCs are exposed to hypoxic conditions. It also produces IL-10 and TGF-β, etc., which attract Treg cells to the tumor site and enhance their immunosuppressive functions, while suppressing the functions of B cells, NK cells, and DCs. PMN-MDSCs die quickly due to ER stress. Factors released by dying cells can promote immunosuppressive mechanisms. At the same time, MDSCs can promote tumor angiogenesis and metastasis by producing VEGF, MMPs, and exosomes. Tumor tissue-derived exosomes can also affect MDSC recruitment and immunosuppression. *Adapted from Myeloid-derived suppressor cells-new and exciting players in lung cancer, Zhenzhen Yang et al, 2020.[70]*

The MDSCs released exosome are involved in immunosuppression, increased angiogenesis, and metastasis via miR-126a+ MDSC-derived exosomes in cancer. The exosomes from tumour cells also contribute to the function of MDSCs [71] . A study conducted by Ridder K et all. found that the expression of PD-L1 in MDSCs could be increased after tumour-derived exosomes were transferred from tumour cells to MDSCs in glioma models. This expression was related to the increased expression of Arg1 in MDSCs, the production of TGF-β and the strengthened immunosuppressive activity of these cells [68, 72].

The Long non-coding (lnc) RNAs and MicroRNA (miRNA) networks regulate the differentiation, expansion and suppression function of MDSC in the tumour microenvironment through different

signalling pathway [73]. The higher expression of lncRNA increased the expression of ARG-1 in MDSCs. Micro RNA, miR-155 and miR-21 can promote the proliferation and immunosuppressive functions of MDSCs via targeting SHIP-1 and phosphatase and tensin homolog, respectively, leading to STAT3 activation [74].

3.4. Chemotherapy effecting on MDSCs.

Cancer promotes the differentiation of myeloid progenitors in MDSCs, that in turn influences tumour growth, angiogenesis, and metastasis. Chemotherapeutics have the potential ability to inibith MDSCs. Low dose of chemotherapy has been shown to be effective in eliminating MDSCs population in tumour-bearing mice; treatments with chemotherapy as 5 fluorouracil (5-FU), paclitaxel, cisplatin and gemcitabine were found to deplet MDSCs and enanche anti tumour immune activity. Few chemotherapy drugs as cyclophosphamide (CTX), doxorubicin and melphalan, can induce the expansion of MDSCs, through the action of inflammatory mediators including GM-CSF, G-CSF, IL-1b, IL6 and CCL2. These therapy-induced MDSCs are highly proliferative and express high levels of C -chemochine receptor type 2 (CCR2)[75, 76]. Expression of CCR2 on MDSCs induced their migration to sites of early tumour cell metastases to promote tumour spread and counter regulate antitumour immune responses. Effector T cells can amplify chemotherapy- induced MDSCs. These chemotherapy- induced MDSCs suppress T-cell activation in a PD-1 dependent manner. Chemotherapy involved in granulocytes expansion contributes to tumour rejection and suppress the antitumor activity [77].

3.5. Targeting MDSCs in cancer immunotherapy.

Immune suppression has a crucial role in promoting tumour progression and it is correlated with the failure of cancer immunotherapies. Successful cancer immunotherapy strategies could require the elimination of tumour microenvironment immune suppressive factors. These approaches include: 1) depleting MDSCs populations through low-dose chemotherapy and tyrosin kinase inhibitors: 2)

preventing MDSCs recruitment and migration of MDSCs; 3) attenuating the immunosuppressive mechanism of MDSCs by down regulating the expression of Arg-1, iNOS and reducing ROS generation; 4) inducing the differentiation of MDSCs into mature myeloid cells to reduce the MDSC population and remove their immunosuppression [78]

4. Aims of the study.

The objectives of this project are:

i) In a retrospective cohort, review the cytogenetic features and identify the clinical and molecular aspects of MPNs able to impact the survival of elderly patients, candidates for complex therapies.

ii) In a prospective cohort, identification of MDSCs in aged patients affected by MPNs using flow cytometry.

5. Material and methods.

i) We designed a reprospective, monocentric study. All Mayo Clinic patients with WHO World-defined MPNs constituted the core study group.

After approval from the Mayo Clinic institutional review board, clinical and laboratory data, including cytogenetic information, were collected from patients at the time of diagnosis or referral to the Mayo Clinic. Diagnoses of PMF and leukemic transformation were according to WHO criteria [79]. Cytogenetic analysis and reporting were done according to the International System for Human Cytogenetic Nomenclature (ISCN) criteria [80]. Cytogenetic analysis in all instances was performed on fresh bone marrow aspirates, placed in hypotonic trypsin-colcemid solution, and processed

according to standard techniques for chromosome analysis using using GTL banding with trypsin and Leishman stain. Thrombolytic agents were added to clotted bone marrow specimens to improve success rates. From approximately the year 2000, the clinical laboratory has transitioned from manual cutting of chromosomes from Kodachrome prints to digital computer imaging.

Chromosomal abnormalities were considered clonal if the same structural abnormality or extra chromosome appears in at least 2 and monosomy in at least 3 metaphases [80]. For the purposes of the current study, a minimum of 10 metaphases was analyzed before assigning a normal karyotype status. A complex karyotype was defined as the presence of 3 or more distinct structural or numeric abnormalities. Monosomal karyotype was defined as 2 or more distinct autosomal monosomies or single autosomal monosomies associated with at least one structural abnormality [46]. Previously described methods were used to screen for PMF-relevant mutations; driver mutational status was classified into favorable and unfavorable category based on the presence or absence of type 1/like *CALR* mutations [81]; high molecular risk (HMR) mutations studied in the current report included *ASXL1* and *SRSF2* [9] .

We followed a stepwise approach, to prognostically assign specific cytogenetic abnormalities into distinct risk categories. The process first considered sole abnormalities occurring in at least 10 incident cases and normal karyotype as the reference for survival data comparisons. Other notable but less frequent abnormalities were then considered in the context of both single and multiple abnormalities, to overcome statistical limitations from inadequate sample size. Overall and leukemia-free survivals were calculated from time of referral to the Mayo Clinic, commensurate with time of cytogenetic analysis [2].

Standard statistical methods were used to determine significance of differences among groups in the distribution of continuous or nominal variables. Overall survival data were prepared by the Kaplan–Meier method and compared by the long –rank test. Cox proportional hazard regression model was applied for multivariable analysis. *P*-value <0.05 was considered significant. The Stat View (SAS Institute, Cary, NC, USA) statistical package was used for all calculations.

ii) Prospective and monocentric study. All University of Eastern Piedmont (UPO) patients with new diagnosis of WHO-defined MPNs constituted the core study group and included PMF, ET and PV.

We recollected whole blood sample from the elderly patients (more than 65 years). All patients have been enrolled at the time of diagnosis (from June 2020 to March 2022) and all are affected by MPNs. All patients signed a consent form. Samples was collected in a 10 ml tube with EDTA from the division of Hematology of Azienda Ospedaliera-Universitaria Maggiore della Carità, UPO in Novara. We used two groups of samples, the patients affected, and the control samples obtained from healthy donor. We research the MDSCs in all cases and analized clinical, molecular, and cytogenetic features.

Blood processing Plasma and Isolation of PBMC

Whole blood samples with EDTA are processed within 4 hours after collection. The blood sample was diluited in a 50 ml falcon tube with 30 ml physiological solution, then 10-15 ml of Ficoll-Paque (Lympholyte, Miltenyi Biothech Bergish Gladbach, Germany) solution was added into the tube by avoiding the mixing of blood and fill. The tube was then centrifugate for 20 min at 2200 rpm with an accelleartion (AC) value of 7 and a break (DC) value 0. The PBMCs were collected from the inferfhase between diluited plasma and separation medium (Figure 10), wasched twice and resuspended in physiological solution in the appropriate volume. The cells were then counted in Burked chamnber by diluiting 1:10 with TURK solution.

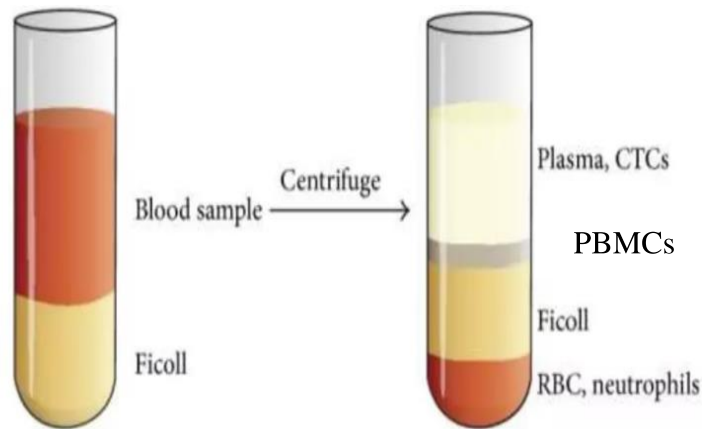


Figure 10. Schematic figure of a density gradient centrifugation. Isolation PBMCs. Peripheral blood is layer over the Ficoll-Paque and, following centrifugation, the blood components are separated into plasma, lymphocytes, monocytes, platelets and granulocytes, red blood cells (RBC). The lymphocytes, monocytes and platelets layer can be extracted, centrifugated toremove platelets with the residual cells representing the peripheral blood mononuclear cell population.

MDSCs Phenotiping

Previously isolated PBMCs were stained for Fluorescence -Activated Cell Sorter (FACS) analysis with CD45 (BD Bioscience), CD33 (APC), HLA-DR (BV605), CD14 (BV650), CD15 (BV510), CD16 (BD Bioscience) and LOX-1 (PE). Stained cells were analyzed on a flow cytometer (BD Bioscience) and data were analyzed using FlowJo (last version) software. MDSC subpopulation phenotypes were defined according to Bronte et al. [59] follows:

Mo-MDSC: HLA-DR-/low CD33+ CD15- CD14+

PMN-MDSC: HLA-DR-/low CD33+ CD15+ CD14-

LOX-1- PMN-MDSC: HLA-DR-/low CD33+ CD15+ CD14-LOX-1+.

Statistical analisis was performed using nonparametric Mann Whitney tes and Spearman test. Data were expressed as mean and Standard Deviation (SD) and were considered statistically significant when p values were <0.05 (Graph Pad prism v.8 was used for the statistical analysis).

6. Results

i) *Review cytogenetic findings in Mayo Clinic MPN patients*

A total of 1,002 patients with PMF (median age 65 years; 62% males) and available cytogenetic information were considered. The presenting clinical and laboratory features of the study population, stratified by the presence or absence of abnormal karyotype and by the most frequent sole abnormalities are outlined in table 2. DIPSS risk distribution was 11% high, 43% intermediate-2, 33% intermediate-1 and 13% low.[20] Driver mutational information was available in 637 patients and included 66% *JAK2*, 15% *CALR* type 1/like, 4% *CALR* type 2/like, 5% *MPL* and 10% triple-negative. On informative cases, *ASXL1* was mutated in 38% and *SRSF2* in 14% (Table 2). Median follow-up was 3.1 years, during which time 748 (73%) deaths, 75 (7.5%) leukemic events and 52 (5.2%) AlloSCT were recorded. Treatment was consistent with what was considered standard of care at the time. Abnormal karyotype was reported in 449 (45%) patients. Compared to normal karyotype (*n*=553; 55%), abnormal karyotype was associated with older age (p=0.02), lower hemoglobin level (p=0.001), higher red cell transfusion requirement (p=0.03), lower leukocyte count (p=0.007), lower platelet count (p<0.001), higher circulating blast count (p=0.001), higher risk DIPSS (p=0.002), and lower incidence of *ASXL1* mutations (p=0.01) (Table 2).

Variables	All patients (*n*=1,002)	Normal karyotype (*n*=553; 55%)	Abnormal karyotype (*n*=449; 45%)	P value*	Sole 20q- (*n*=74; 7.4%)	Sole 13q- (*n*=56; 5.6%)	Sole +8 (*n*=26; 2.6%)	Sole +9 (*n*=14; 1.4%)	P value**
Age in years; median (range)	65 (19-92)	65 (19-89)	65 (30-92)	**0.02**	69 (30-83)	63 (37-87)	68 (30-85)	69 (46-80)	**0.03**
Age >65 years; *n* (%)	523 (52)	277 (50)	246 (55)	0.13	46 (65)	24 (43)	16 (62)	10 (71)	**0.03**
Males; *n* (%)	625 (62)	337 (61)	288 (64)	0.3	50 (70)	33 (59)	14 (54)	9 (64)	0.5
Hemoglobin, g/dl. median (range)	10 (5-16.7)	10.3 (5-16.1)	10 (5.2-16.7)	**0.001**	9.9 (6.7-15)	11 (6.6-14.9)	10 (6.2-13)	11.2 (7.8-14)	0.1
Hemoglobin <10 g/dl; *n* (%)	514 (51)	260 (47)	254 (57)	**0.003**	45 (64)	23 (41)	16 (62)	6 (43)	**0.04**
Transfusion -requiring; *n* (%)	367 (37)	186 (34)	181(40)	**0.03**	45 (64)	40 (71)	14 (54)	9 (64)	0.6
Leukocytes, x 10^9/l. median (range)	9 (1-236.1)	9.9 (1-236.1)	8 (1-218.5)	**0.007**	6.1 (1-71.5)	10 (2.2-176)	7 (1.3-142)	10.9 (2.6-40)	**0.001**
Leukocytes >25 x 10^9/l; *n* (%)	162 (16)	89 (16)	73 (16)	0.9	6 (8)	12 (21)	3 (12)	2 (14)	0.3
Platelets, x 10^9/l. median (range)	204.5 (6-2466)	245 (8-2466)	153 (6-2282)	**<0.0001**	159 (12-1921)	246 (14-1043)	158 (17-684)	172 (38-769)	**<0.0001**
Platelets <100 x 10^9/l; *n* (%)	259 (26)	108 (20)	151 (34)	**<0.0001**	23 (32)	8 (14)	9 (35)	4 (29)	**0.03**
Circulating blast %; median (range)	1 (0-18)	1 (0-15)	1 (0-18)	**0.001**	0 (0-6)	1 (0-13)	1 (0-18)	0 (0-4)	0.2
Circulating blasts ≥1%; *n* (%)	538 (54)	279 (50%)	259 (58)	**0.02**	29 (41)	32 (57)	14 (54)	6 (43)	0.4
Constitutional symptoms; *n* (%)	333 (33)	177 (32)	156 (35)	0.4	23 (32)	13 (23)	7 (27)	7 (50)	0.4
DIPSS† risk Distribution				**0.002**					0.2
High; n (%)	112 (11)	54 (10)	58 (13)		8 (11)	3 (5)	2 (8)	0 (0)	
Intermediate-2; n (%)	431 (43)	217 (39)	214 (48)		38 (54)	24 (43)	13 (50)	8 (57)	
Intermediate-1; n (%)	334 (33)	199 (36)	135 (30)		17 (24)	20 (36)	11 (42)	5 (36)	
Low; n (%)	125 (13)	83 (15)	42 (9)		8 (11)	9 (16)	0 (0)	1 (7)	
Driver mutational status *"N" evaluable* =637				0.6					0.1
JAK2; n (%)	419 (66)	235 (64)	184 (68)		34 (81)	16 (50)	7 (78)	11 (100)	
CALR type 1/like; n (%)	100 (15)	58 (16)	42 (16)		2 (5)	10 (31)	0 (0)	0 (0)	
CALR type 2/like; n (%)	23 (4)	16 (4)	7 (3)		1 (2)	1 (3)	0 (0)	0 (0)	
MPL; n (%)	33 (5)	21 (6)	12 (4)		2 (5)	3 (10)	0 (0)	0 (0)	
Triple-negative; n (%)	62 (10)	39 (10)	23 (9)		3 (7)	2 (6)	2 (22)	0 (0)	
ASXL1-mutated; *n* (%) *"N" evaluable*=436	165 (38)	108 (43)	57 (31)	**0.01**	5 (20)	3 (12)	1 (14)	2 (25)	**0.004**
SRSF2-mutated; *n* (%) *"N" evaluable*=423	61 (14)	36 (15)	25 (14)	0.8	7 (29)	1 (4)	1 (13)	1 (13)	0.2

†DIPSS, Dynamic International Prognostic Scoring System-plus uses five independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dL, leukocytes >25 x 10^9/L, circulating blasts ≥1% and constitutional symptoms.
*P value for comparison of normal vs abnormal karyotype
**P value for comparison of five groups: normal karyotype vs sole abnormalities of 20q-, 13q-, +8 and +9

Table 2: Clinical and laboratory features of 1,002 patients with primary myelofibrosis, stratified by normal vs abnormal karyotype and the most frequent sole abnormalities. [2]

Among the 449 (45%) cases with abnormal karyotype, 320 (32%) harbored sole, 68 (7%) two and 61 (6%) three or more abnormalities; by definition, therefore, 61 (6%) patients had complex karyotype and of these 25 (2.3%) were classified as monosomal karyotype. The most frequent sole abnormalities were 20q- (*n*=74;~7%), 13q- (*n*=56;~6%), +8 (*n*=26;~3%) and +9 (*n*=14; 1.4%); less frequent sole abnormalities included 7q- (*n*=12), -Y (*n*=9) and a sex chromosome abnormality other than –Y (*n*=10). Phenotypic correlative studies involving the most frequent sole abnormalities showed significant associations between older age and 20q-, +9 and +8 (p=0.03); lower hemoglobin level and 20q- and +8 (p=0.04); higher leukocyte count and +9 and 13q- (p=0.001); higher platelet count and 13q- (p<0.001); and *ASXL1* mutations and +9 and 20q- (p=0.004) (Table 2)

Infrequent sole abnormalities included monosomy 7 (*n*=7), 5q- (*n*=6) +21 (*n*=5), 12p-/12p11.2 (*n*=5), 11q-/11q23 (*n*=4), i(17q) (*n*=4) and inv(3)/3q21 (*n*=3) abnormalities.

A stepwise approach was undertaken, to prognostically classify specific cytogenetic abnormalities. Single abnormalities with at least 10 occurrences were initially considered: 20q- (*n*=74), 13q- (*n*=56), +8 (*n*=26), +9 (*n*=14) and 7q- (*n*=12). Amongst these, 20q-, 13q- and +9 were associated with survival data that was not significantly different from that of normal karyotype (*n*=553) the results remained unchanged when analysis was adjusted for age. In contrast, survival of patients with either +8 or 7q- was significantly worse than that seen with normal karyotype Age-adjusted survival data were similar for 20q- vs 13q- vs +9 and for +8 vs 7q- . Furthermore, each one of 20q-, 13q- and +9, when compared to the combined +8/7q- group, was associated with significantly longer survival (Table 3). Accordingly, sole abnormalities of +8 and 7q- were grouped together, to serve as the initial template

for "unfavorable" risk category, and sole abnormalities of 20q-, 13q- and +9 were classified with normal karyotype to form the initial template for the prognostically superior "favorable" risk category (age-adjusted p=0.007; HR 0.6, 95% CI 0.4-0.8).

We next focused on specific abnormalities that have historically been marked as being particularly detrimental for survival, including monosomy 7, inv(3)/3q21, i(17q), 11q-/11q23 and 12p-/12p11.2 [21,25,48]. Considering the relative rarity of sole occurrences involving these abnormalities, they were considered in the setting of both single and multiple abnormalities, in order to assemble eighteen cases of monosomy 7, eleven of inv(3)/3q21 (*n*=5) or i(17q) (*n*=6), eleven of 12p-/12p11.2 and thirteen of 11q-/11q23 (Table 3). Monosomy 7 was chosen over monosomal karyotype after preliminary analysis confirmed its primary prognostic contribution to "monosomal" karyotype (HR 3.4, 95% CI 1.3-8.8, compared to monosomal karyotype without monosomy 7).

As expected, each one of the above-listed high risk cytogenetic categories was associated with significantly worse survival, compared to normal karyotype, with more than 5-fold hazard ratio for inv(3)/3q21/i(17q) (HR 6.6, 95% CI 3.6-12.2), monosomy 7 (HR 6.3, 95% CI 3.8-10.4) and 12p-/12p11.2 (HR 5.6, 95% CI 2.9-10.5) (Table 3). These latter three, but not 11q-/11q23 abnormalities, were also associated with significantly shorter survival, compared to the +8/7q- unfavorable risk category template (Table 3); significance in all instances was retained when analysis was adjusted for age. Accordingly, single/multiple abnormalities of inv(3)/3q21, i(17q), monosomy 7 and 12p-/12p11.2 were grouped together and assigned to an operational very high risk (VHR) category, and displayed similar survival data when compared to each other (p=0.8).

We had established three risk category templates: favorable (normal or sole abnormalities of 20q-, 13q- or +9; *n*=697), unfavorable (sole abnormalities of +8 or 7q-; *n*=38; HR 2.2, 95% CI 1.5-3.2) and VHR (single or multiple abnormalities of -7, inv(3)/3q21, i(17q) and 12p-/12p11.2; *n*=40; HR 6.1, 95% CI 4.3-8.6).

Each one of the above-listed operational cytogenetic groups was subsequently compared to normal, unfavorable and VHR cytogenetic risk templates, in order to determine its best-fit category. Based on results obtained, risk allocations were straightforward for sole chromosome 1 translocations/duplications (favorable risk), sole sex chromosome abnormalities including –Y (favorable risk), sole translocations not involving chromosome 1 (unfavorable), other sole abnormalities not otherwise classified (unfavorable) and non-monosomal and complex karyotypes without VHR abnormality (unfavorable) (Table 3).

Specific abnormalities	Total N=1,0002	Survival comparison vs. Normal karyotype (N=553) P value (HR; 95% CI)	Survival comparison vs. "Unfavorable" category template (N=38) †† P value (HR; 95% CI)	Survival comparison vs. "VHR" category template (N=40) † P value (HR; 95% CI)
Sole 20q-	74	0.1 (1.3; 0.9-1.7)	**0.02 (0.6; 0.4-0.9)**	**<0.0001 (0.2; 0.1-0.3)**
Sole 13q-	56	0.08 (0.7; 0.5-1.0) *	**<0.001 (0.3; 0.2-0.6)**	**<0.0001 (0.1; 0.06-0.2)**
Sole +9	14	0.7 (0.9; 0.4-1.7)	**0.02 (0.4; 0.2-0.8)**	**<0.0001 (0.1; 0.04-0.3)**
Sole sex chromosome abnormality, including -Y	19	0.6 (0.9; 0.5-1.4)	**0.03 (0.5; 0.3-0.9)**	**<0.0001 (0.1; 0.06-0.3)**
Sole chromosome 1 translocations/duplications	21	0.7 (1.1; 0.7-1.9)	**0.01 (0.4; 0.2-0.8)**	**<0.0001 (0.1; 0.05-0.3)**
Sole translocations not involving chromosome 1	25	**0.03 (1.7; 1.1-2.6)**	0.27 (0.7; 0.4-1.3)	**<0.0001 (0.2; 0.09-0.4)**
Sole +8	26	**<0.001 (2.2; 1.4-3.3)**	-----	**0.0009 (0.4; 0.2-0.7)**
Sole 7q-	12	**0.009 (2.3; 1.2-4.3)**	-----	**0.008 (0.4; 0.2-0.8)**
Sole autosomal trisomies, other than +9 or +8	15	**<0.0001 (3.8; 2.1-6.6)**	0.19 (1.6; 0.8-3.1)	0.12 (0.6; 0.3-1.1)
Sole abnormalities not otherwise classified	31	**0.008 (1.7; 1.1-2.5)**	0.33 (0.8; 0.5-1.3)	**<0.0001 (0.3; 0.2-0.5)**
Two abnormalities without VHR abnormality	52	0.07 (1.3; 1.0-1.8)	0.05 (0.6; 0.4-1.0)	**<0.0001 (0.3; 0.2-0.4)**
Single/multiple 5q- abnormalities	11	0.28 (1.5; 0.7-3.0)	0.3 (0.6; 0.3-1.5)	**0.003 (0.2; 0.08-0.6)**
Single/multiple monosomy 7 abnormalities	18	**<0.0001 (6.3; 3.8-10.4)**	**0.005 (2.5; 1.3-4.7)**	**-----**
Single/multiple 12p-/12p11.2 abnormalities	11	**<0.0001 (5.6; 2.9-10.5)**	**0.03 (2.2; 1.1-4.6)**	-----
Single/multiple 11q-/11q23 abnormalities	13	**<0.0001 (3.0; 1.7-5.3)**	0.3 (1.4; 0.7-2.7)	0.2 (0.6; 0.3-1.3)
Single/multiple i (17)/inv (3) abnormalities	11	**<0.0001 (6.6; 3.6-12.2)**	**0.008 (2.8; 1.3-6.0)**	-----
Monosomal karyotype without VHR abnormality	13	0.19 (1.5; 0.8-3.0)	0.38 (0.7; 0.3-1.5)	**0.002 (0.2; 0.1-0.6)**
Non-monosomal complex without VHR abnormality	27	**0.02 (1.7; 1.1-2.6)**	0.36 (0.8; 0.4-1.3)	**<0.0001 (0.3; 0.2-0.5)**

*Trend favoring 13q-; †Ver high risk (VHR) category template included single or multiple abnormalities of -7, inv (3), i(17q) and 12p-/12p11.2

Table 3: Cytogenetic abnormalities among 1,002 patients with primary myelofibrosis and corresponding comparisons of survival.[2]

Based on the above elaborated survival analysis, the following cytogenetic abnormalities were classified into the revised “favorable” cytogenetic risk category: normal karyotype and sole abnormalities of 20q-, 13q-, +9, chromosome 1 translocation/duplication and sex chromosome abnormality including –Y (*n*=737); the following abnormalities were assigned to the revised “VHR” cytogenetic risk category: single/multiple abnormalities of -7, inv(3)/3q21, i(17q), 12p-/12p11.2, 11q-/11q23 and autosomal trisomies (e.g. +21, +19) other than +8 or +9 (*n*=75); all other abnormalities were assigned to the “unfavorable” risk category (*n*=190).

Survival data stratified by the new revised cytogenetic risk stratification are depicted in figure 11 and demonstrate the adverse impact of VHR, compared to both unfavorable (HR 2.2, 95% CI 1.6-3.0) and favorable (HR 3.8, 95% CI, 2.9-4.9; $p<0.0001$) risk categories, and that of unfavorable, compared to favorable (HR 1.7, 95% CI 1.4-2.0; $p<0.0001$) risk category (Figure 11). Multivariable analysis confirmed the independent prognostic contribution of the revised cytogenetic risk model in the context of DIPSS (HR 2.9; 95% CI 2.2-3.7 for VHR and 1.6, 1.3-1.9 for unfavorable), driver mutational status (HR 4.5, 95% CI 3.2-6.4 for VHR and 1.6, 1.3-2.1 for unfavorable) and HMR (i.e. *ASXL1*/*SRSF2*) mutations (HR 4.3, 95% CI 2.8-6.7 for VHR and 2.3, 1.7-3.0 for unfavorable); an all-inclusive multivariable analysis found the revised cytogenetic risk model, DIPSS, driver mutational status and HMR mutations to predict shortened survival, independent of each other: HRs (95% CI) were 4.1 (2.6-6.5) for VHR, 2.3 (1.7-3.1) for unfavorable, 2.6 (1.9-3.6) for absence of type 1/like *CALR*, 1.9 (1.5-2.4) for HMR mutations, 7.7 (4.3-13.9) for DIPSS high, 5.8 (3.5-9.7) for DIPSS intermediate-2 and 3.1 (1.9-5.2) for DIPSS intermediate-1.

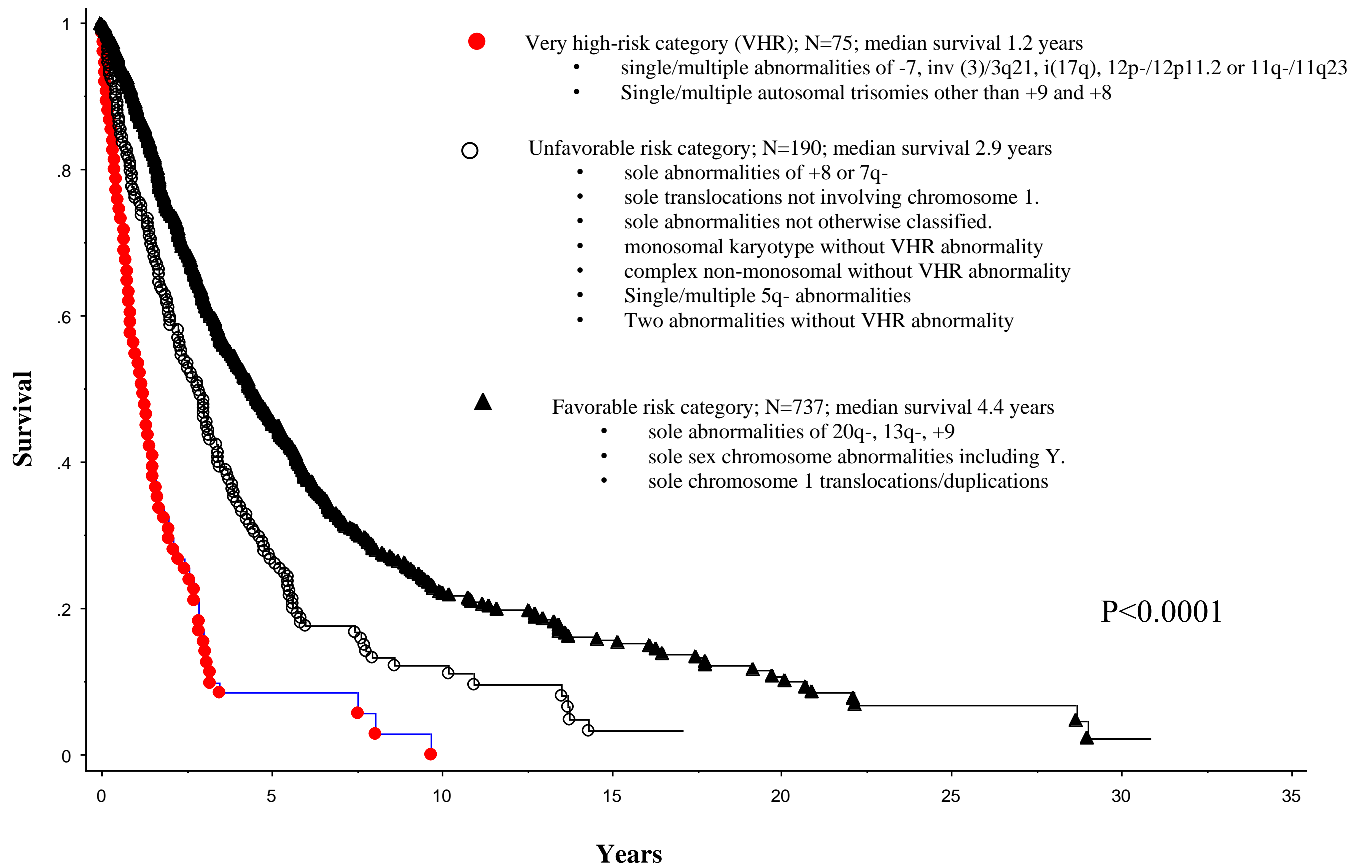


Figure 11: Overall survival of 1,002 patients with primary myelofibrosis stratified by the revised three-tiered cytogenetic risk model. *Tefferi and Nicolosi, Leukemia 2019.* **[2]**

ii) <u>Identification of MDSCs in UPO MPNs patients</u>

We recollected samples of fifty-five new MPNs patients. All samples of new diagnosed patient's prospective analysed have been stored and has been evaluated the presence of MDSCs to figure out the correlation between MDSCs and immunosenescence, in the MPN setting of patients, occurs at the diagnosis. We enrolled 55 patients including 12 PMF, 10 PV, and 23 ET, 5 with unclassifiable MPN and 5 lymphoproliferative diseases that subsequent we excluded from cytogenetic and molecular analyses. All patients have been enrolled at the time of diagnosis (from June 2020 to March 2022). Median age was 73 years old; 35 cases presented *JAK2* positive, 3 *MPL*, and 9 *CALR* mutation.

At the time of first referral, 11 (5%) of 55 patients with PMF displayed cytogenetic abnormalities. In the latter study, sole abnormalities were the most frequent (52%), while 43% presented with complex karyotype, and 3% with monosomal karyotype; the most frequent sole abnormalities were 20q-

(15%), 13q- (8%), +8 (6%), +9 (6%) and chromosome 1 translocations/duplications (3%). Although the prevalence of abnormal karyotype in PMF was not affected by driver mutational status, significant associations between +9 and *JAK2* mutations and 13q- and *CALR*/*MPL* mutations were reported; of note, among 8 triple-negative patients, 33% displayed abnormal karyotype and the majority of those with normal karyotype harbored other mutations. Cytogenetic risk classification showed a favourable risk in 35, unfavourable in 6, very high risk in 5 patients and in 4 patients we didn't get mitoses. Clinical features, molecular and cytogeneitic finding are summarized in table 4 and table 5.

Variables	**All patients (*n*=55)**	**Normal karyotype *(n=37*; 67%*)***	**Abnormal karyotype *(n=18*; 33%*)***	**P value***
Age in years; median (range)	71 (65-87)	71 (65-84)	71 (65-87)	**0.02**
Males; *n* (%)	35 (63)	22 (59)	13(41)	0.3
Hemoglobin, g/dl. median (range)	10 (5-16.7)	10.3 (5-16.1)	10 (5.2-16.7)	**0.001**
Hemoglobin <10 g/dl; *n* (%)	35 (63)	19 (51)	16 (49)	**0.003**
Transfusion -requiring; *n* (%)	22 (40)	14 (38)	8 (44)	**0.03**
Leukocytes, x 10^9/l. median (range)	9 (1-236.1)	9.9 (1-236.1)	8 (1-218.5)	**0.007**
Leukocytes >25 x 10^9/l; *n* (%)	12 (22)	4 (11)	8 (44)	0.9
Platelets, x 10^9/l; median (range)	204.5 (6-2466)	245 (8-2466)	153 (6-2282)	**<0.0001**
Circulating blast %; median (range)	1 (0-18)	1 (0-15)	1 (0-18)	**0.001**
Constitutional symptoms; *n* (%)	33 (60)	15 (40)	17 (51)	**0.04**
Driver mutational status *"N" evaluable =55*				0.6
JAK2; n (%)	35 (65)	20 (54)	15 (83)	
CALR type 1/like; n (%)	4 (7)	4 (11)	0 (0)	
CALR type 2/like; n (%)	5 (10)	3 (10)	2 (11)	
MPL; n (%)	3 (3)	0 (0)	3 (12)	
Triple-negative; n (%)	8 (15)	2 (5)	6 (33)	
ASXL1-mutated; *n* (%) *"N" evaluable=12*	6 (50)	1 (10)	5 (42)	**0.01**
SRSF2-mutated; *n* (%) *"N" evaluable=12*	3 (14)	1 (10)	2 (16)	0.8

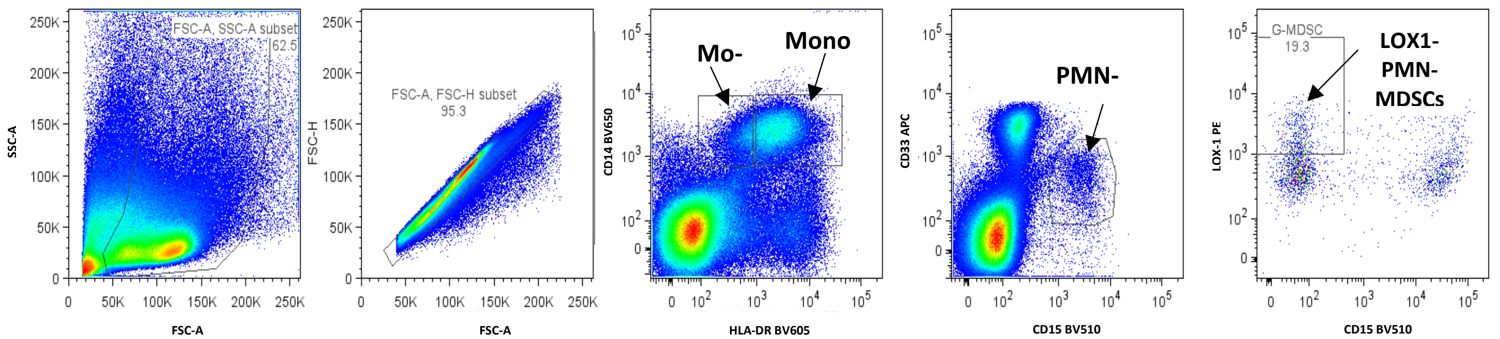
Table 4 Clinical, molecular, and cytogenetic features of 55MPNs patients. *Abbreviation:* †DIPSS, Dynamic International Prognostic Scoring System-plus uses five independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dL, leukocytes >25 x 10^9/L, circulating blasts ≥1% and constitutional symptoms.*P value for comparison of normal vs abnormal karyotype**P value for comparison of five groups: normal karyotype vs sole abnormalities of 20q-, 13q-, +8 and +9.[2]

Specific abnormalities	**Total N=55**
Sole 20q-	8
Sole 13q-	3
Sole +9	2
Sole sex chromosome abnormality, including -Y	2
Sole chromosome 1 translocations/duplications	1
Sole translocations not involving chromosome 1	5
Sole +8	2
Sole 7q-	3
Sole autosomal trisomies, other than +9 or +8	2
Sole abnormalities not otherwise classified	1
Two abnormalities without VHR abnormality	6
Single/multiple 5q- abnormalities	3
Single/multiple monosomy 7 abnormalities	1
Single/multiple 12p-/12p11.2 abnormalities	3
Single/multiple 11q-/11q23 abnormalities	2
Single/multiple i (17)/inv (3) abnormalities	3
Monosomal karyotype without VHR abnormality	2
Non-monosomal complex without VHR abnormality	6

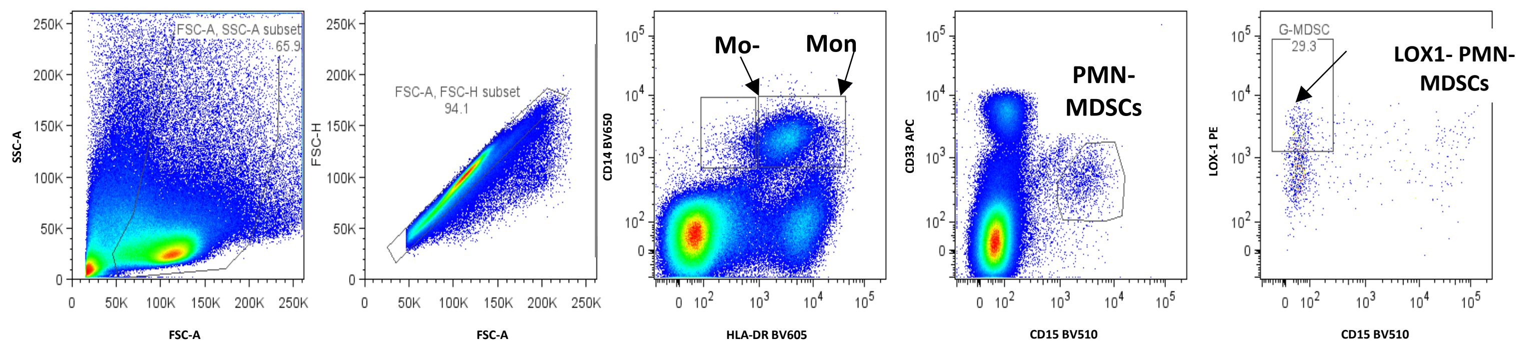
Table 5. Characterization of cytogentic abnormalities in 55 MPNs patients

Gating strategy and characterization of Mo-/PMN-/LOX1-PMN-MDSC in PBMCs

Percentage of total MDSC, PMN-MDSC, M-MDSC, LOX1- PMN-MDSC in PBMCs was determined based on the cell surface marker expression. Our gating strategy began with the elimination of dead cells using forward (FSC-A) and side scatters (SSC-A), and further discrimination of doublets by FSC-H vs FSC-A (Figure 12). Monocyte (mono) were gated as HLA-DR+ CD14+ and Mo-MDSC as HLA-DR-/low, CD14+, PMN-MDSC were gated as, CD15+CD33int. We used, LOX-1, to identify a subgroup of PMN-MDSCs, that is LOX1-PMN-MDSCs (Figure 12).



Panel a) Patients samples

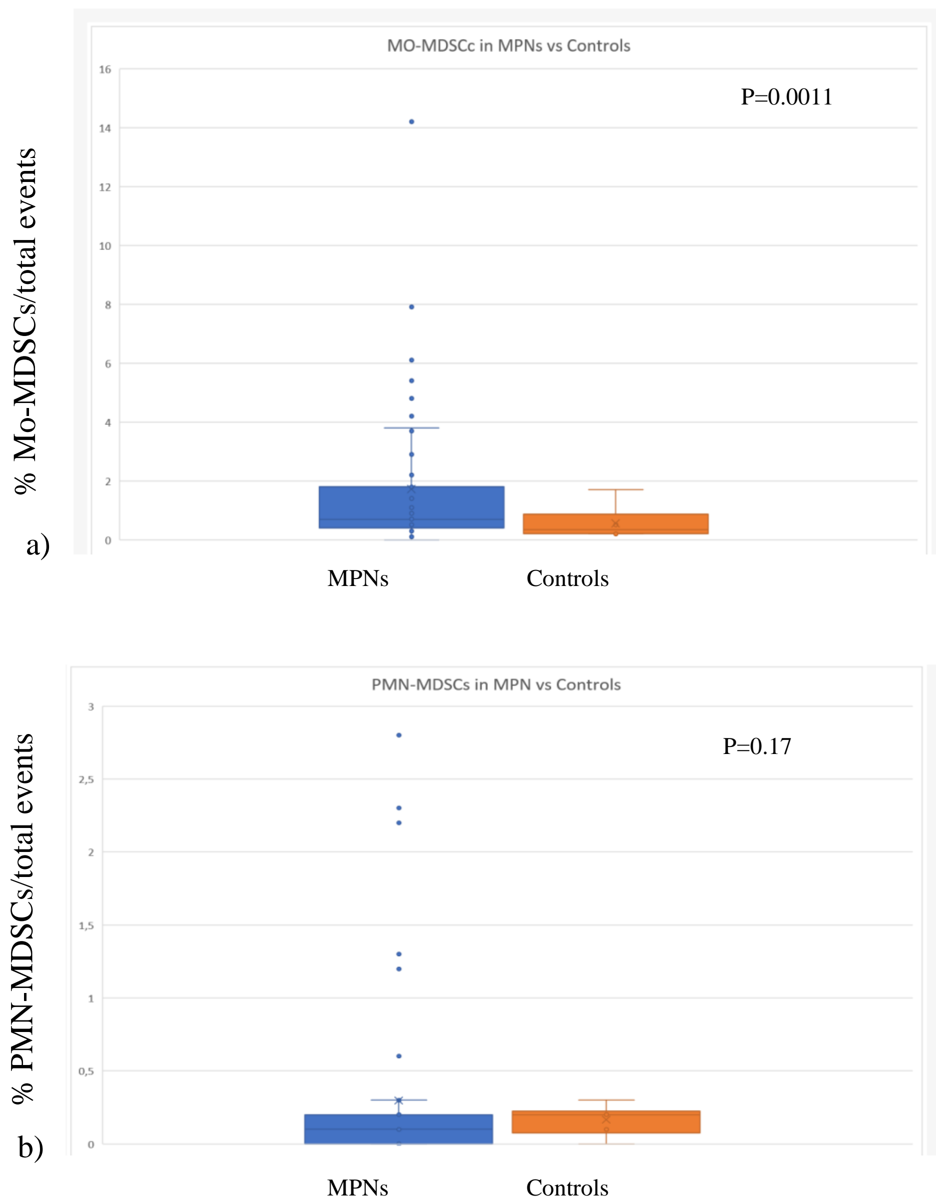


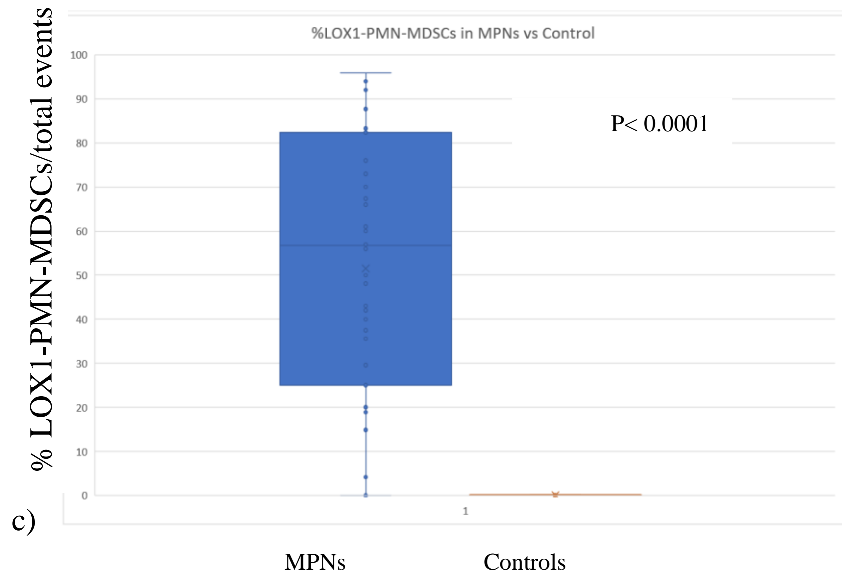
Panel b) Healthy control samples

Figure 12 Gating strategy for MO-/PMN/LOX1-PMN-MDSCs in PBMC, based on cellular surface markers: (Panel a) representation of samples from patients affected by MPN and (Panel b) representation of controls samples.(A)Dot plot for side scatter (SSC-A) and forward scatter (FSC-A).(B) Dot plot of FSC-H (Y-axis) and FSC-A (x-axis) representing the singlet in the population.Dot plot for (C) Mono and Mo-MDSC, (D) PMN-MDSCs and (E)LOX1-PMN-MDSCs identification.

Evaluation of Mo-/PMN-/LOX1-PMN-MDSCs in PBMC in MPN patients

By flow cytometry we determined the percentage of Mo-MDSCs, PMN-MDSCs, and LOX-1-/PMN-MDSCs in 55 sample of patients and 10 healthy controls. Mo-MDSCs, PMN-MDSCs, and LOX-1-/PMN-MDSCs were found in significantly higher pertentage of patients than in health control MO-MDSC (mean±CNT vs MPN: 0.5±0.58 vs 1,7 ±2,6) P=0.011 PMN-MDSCs (mean±CNT vs MPN: 0.16± 0.1 vs 0,3 ±0,6) and LOX1- PMN-MDSCs (means±SD CNT versus MPNs: 0,1± 0,04 vs 51± 32) were 85%, 80% and 95% respectively (p=0.0011, p=0.17 and p<0.00001) (Figure 13)





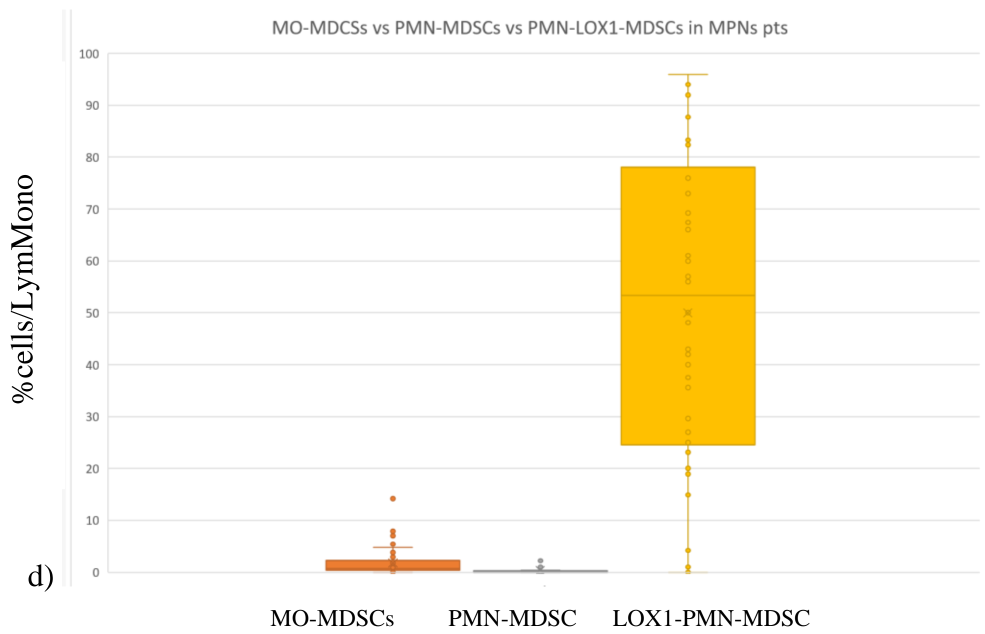


Figure 13. Percentage of MDSCs in MPN patients versus control; a) MO-MDScs; b) PMN-MDSCs; c) LOX1-PMN-MDSC; c) Comparison of Mo-MDSCc, PMN-MDSCs and LOX1-PMN-MDSCs in all MPN patiens.

In all fifty-five patients were identified the MDSCs using flow cytometry: no differences in MDSC levels among different MPNs categories. We did a multivariate analysis to compare MDSC levels and clinical cytogenetic and molecular information of patients. MDSCs levels were not correlated with *JAK2* status, white blood cells, Hb levels, platelet counts, splenomegaly, degree of bone marrow fibrosis, cytogenetic or molecular information. No information about overall survival has been requested, due to the status of new diagnosis.

7. Discussion

Current prognostication in PMF relies on information from clinical variables, karyotype, and mutations. The prototype international prognostic scoring system (IPSS) was first described in 2009 and was designed to predict survival in newly diagnosed patients. In 2010, IPSS was modified into the dynamic IPSS (DIPSS) , to allow prognostication of patients seen at any time during their clinical course [21]. Both IPSS and DIPSS employed five clinical risk factors, including older age, anemia, leukocytosis, constitutional symptoms and circulating blasts, in order to construct a four-tiered risk categorization system. In 2011, DIPSS-plus was introduced in order to account for IPSS/DIPSS-independent risk factors, including thrombocytopenia, red cell transfusion need and karyotype. The inclusion of cytogenetic information to clinically-derived risk models for PMF has boosted their performance, especially in predicting leukemia-free survival [21].

The prognostic value of karyotype in myeloid malignancies is widely recognized and integrated into formal prognostic models. Prognostically-relevant cytogenetic abnormalities in other myeloid malignancies do not always signify similar risk in PMF. For example, 11q- and 12p- abnormalities have been associated with good or very good risk disease in myelodysplastic syndromes (MDS) [52], while they were flagged as VHR abnormalities in PMF. Similarly, other VHR abnormalities in PMF, including i(17)(q10), +21 and +19, were classified as being intermediate risk in MDS [2]. On the other hand, monosomy 7 and inv(3)(q21.3q26.2)/t(3;3)(q21.3;q26.2) were associated with VHR/poor risk and +8 with unfavorable/intermediate risk disease, in both PMF and MDS, as well as in acute myeloid leukemia [52,53]. The current study suggests that broad categories such as “complex” or “monosomal” karyotype do not necessarily imply dismal outcome if one were

to account for more specific abnormalities associated with very high risk disease, which in the case of PMF included monosomy 7, i(17)(q10), inv(3)/3q21 and 12p-/12p11.2.

The current study is unique regarding its sample size (*n*=1,002), maturity of survival data (73% of patients were followed till time of death) and availability of other genetic information, which allowed assessment of prognostic interaction between karyotype and mutations. The current study clarifies the hierarchy of prognostic contribution from genetic markers and confirms the inter-independent prognostic contribution of a newly revised three-tiered cytogenetic risk stratification, driver mutational status, HMR mutations and clinically derived prognostic scoring systems.

Despite the great efforts to improve the caracterization of the MPNs, not least the revised cytogentic risk classification, the perfect patogenesis aspects of these disease remains unclear. Myeloid tumors were found to exhibit a variety of strategies to successfully undergo immune evasion making effective immunotherapy difficult. It is known that chronic inflammation induces immunosuppression which inhibits both adaptive and innate immunity in different disease as in MPNs. Inflammatory factors promote the recruitment of immunosuppressive cells into inflamed tissues, where they suppress persistent inflammation and restore homeostasis in inflamed tissue. Myeloid-derived suppressor cells were previously described as being able to suppress a strong anti-leukemia immune response in patients, thereby supporting tumor immune escape. MDSCs are specialized immunosuppressor that can control the functions of other immune cells, thus preventing excessive inflammatory responses. MDSCs can suppress the activity and the anti-leukemia immune response of T-cells through a variety of mechanisms either via direct cell–cell contact or by the release of soluble factors.

The best understood mechanisms include increased production of ROS, an increased expression of arginase-1 and inducible nitric oxide synthase (iNOS). The inhibition of inflammation prevents the recruitment of MDSCs into tumour and subsequently block tumour growth. Multiple studies are ongoing to evaluate novel treatment strategies aiming to overcome the immunosuppressive mechanisms and to enhance an anti-leukemia immune response. Patients with MPN, especially PMF, are characterized by their association with auto-immune phenomena and aged correlation features. In this study we found that MDSCs were increased in MPNs patients compared with normal healthy control suggesting a role in the bone marrow of MPN patients in mediating leukemia immune escape. We identify 3 tipe of MDSC, and we used LOX-1 as a marker to individuate PMN-MDSCs population. It was reported that LOX-1 was a novel marker for polymorfonuclear PMN-MDSC in whole blood of head and neck cancer patients. We also demonstrated that this subpopulation of cells is increased in elderly MPN patients.

The presence of MDSCs, capable of evading the T system, could represent in this setting of patients, the aging part, also present in young patients. As is well known, both elderly and young patients with MPNs do not yet have curative treatments available except for allogeneic stem cell transplantation which is burdened with a high mortality rate. The presence of MDSCs capable of evading the T system and the presence of PDL / PDL1 could represent a possibility of early identification of disease, together with the other parameters already identified and validated, useful to the clinician to offer targeted therapies early. The interesting preliminary results that we obtained in this study, allow us to think to go head in this project, recollecting a larger number of samples.

8. Conclusion and Future perspectives

The current study clarifies the hierarchy of prognostic contribution from genetic markers and confirms the inter-independent prognostic contribution of a newly revised three-tiered cytogenetic risk stratification, driver mutational status, HMR mutations and clinically derived prognostic scoring systems. A deep stratification of cytogetinics features may help clinicians to stratify the aging patients better and early, not only in terms of "years" but in terms of "aged" features, identifing the worst cases. For the future may be a good option to integrate this "three tired model " in the clinical practise.

The presence of higher MDSCs versus healty patients, capable of evading the T system, underwents the inflammed scenario of MPNs. These cells and the presence of PDL/PDL1 as suggested by differents studies, could be a possibility of early identification of disease, together with the other parameters, as cytogenetics, already identified and validated, useful to the clinician to offer targeted therapies early. The interesting preliminary results that we obtained in this study, allow us to think to go head in this project. For the future it coul be reasonable, to recollect a larger number of samples, to confirm these results and analyzed the correlation between MDSCs and senescence cells in terms of presence of P16 and P21. Due to the historical COVID era, the number of new diagnoses of MPNs has been dramatically downgrade.

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