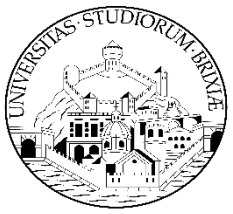

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Comparative Somatic Mutational Profiling of CD34+ Hematopoietic Precursors and Circulating Endothelial Cells in Patients With Primary Myelofibrosis

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1. INTRODUCTION

1.1 Myelofibrosis

1.1.1 Definition

Myelofibrosis (MF) is a myeloproliferative neoplasm (MPN) characterized by clonal myeloproliferation, deregulated cytokine production and bone marrow (BM) fibrosis[1,2]. It is characterize by clonal ineffective hematopoiesis and extramedullary hematopoiesis leading to progressive organomegaly, more frequently splenomegaly [1]. In addition, 10% to 20% of myelofibrosis cases may evolve in acute leukemia in the first decade after diagnosis[3].

Myelofibrosis could present de novo as primary myelofibrosis (PMF), or from phenotypic transformation of the Myeloproliferative neoplasms Polycythemia Vera (PV) or Essential thrombocythemia (ET), referred as post-PV MF or post-ET MF, respectively [4]. PMF carries a poor prognosis, with a median survival ranging from months to years, depending on its clinical and molecular features. The disease has a progressive course characterized by bone marrow failure; organ failure due to extramedullary hematopoiesis; and transformation to acute myeloid leukemia[5].

1.1.2 Diagnosis

The diagnosis of PMF is based on the recently updated WHO criteria[6] and the International Consensus classification, which involve a composite assessment of clinical and laboratory features (Table 1)[7].

According to the 2022 revision of the WHO classification of myeloid neoplasms and acute leukemia, major diagnostic criteria for overt (fibrotic) Primary myelofibrosis include (1) presence of megakaryocytic proliferation and atypia, accompanied by reticulin and/or collagen fibrosis of grades 2 or 3; (2) not meeting WHO criteria for essential thrombocythemia, polycythemia vera, BCR-ABL1 positive chronic myeloid leukemia, myelodysplastic syndrome, or other myeloid neoplasms; and (3) presence of JAK2, CALR, or MPL mutations or presence of another clonal marker or absence of reactive fibrosis. Minor criteria include (a) anemia not attributed to a comorbid condition, (b) leukocytosis ≥ $11x10^9$, (c) palpable splenomegaly, (d) lactate dehydrogenase (LDH) level above normal limit of institutional reference range, and (e) leukoerythroblastosis, all to be confirmed in two consecutive determinations.

The diagnosis of myelofibrosis requires meeting all three major and at least one minor criteria [6]. Since the update of the WHO classification in 2016, the importance of newly recognized molecular markers has been taken into consideration in diagnosis and prognostication. In patients with no mutations in driver genes (JAK2, CALR, MPL), the so-called “triple-negative” cases, the detection of one of the associated somatic mutations (eg, EZH2, TET2, IDH1/2, ASXL1, SRSF2, or SF3B1) suffices as the presence of a clonal marker for diagnostic purposes.

Furthermore, some patients with ET or PV develop a PMF-like phenotype over time, referred to as post-ET or post-PV MF. The diagnosis of post-PV or post-ET MF, also known as secondary myelofibrosis, should adhere to criteria published by the

International Working Group for MPN Research and Treatment (IWG-MRT)[4].

Characteristic bone marrow morphology is essential for diagnosis, comprising fibrosis (graded on a World Health Organization scale from 0 to 3), megakaryocytic proliferation, and megakaryocytic atypia[8]. Within the WHO MPN category, PMF is further sub-classified into "prefibrotic" and "overtly fibrotic" PMF[9,10], according to the grade of bone marrow fibrosis. Prefibrotic primary myelofibrosis (pre-PMF) can present similarly to Essential thrombocythemia but distinguishing between these disorders is vital owing to their different prognostic ramifications. Indeed, prefibrotic MF presented a decreased survival compared with Essential Thrombocythemia. Pre-MF presented a higher rate of leukemic transformation (5.8% vs. 0.7%, respectively, at 10 years), and of progression to overt PMF (11.7% vs. 2.1%, respectively, at 10 years) compared with Essential Thrombocythemia. Pathologically, PMF is characterized by thickening and distortion of bony trabeculae, deposition of reticulin and collagen fibers, and megakaryocytic hyperplasia with atypical features[11]. Unfortunately, the pathogenesis of bone marrow fibrosis in PMF is still not very well elucidated.

PMF, early/prefibrotic stage (pre-PMF)	PMF, overt fibrotic stage
Major criteria 1. Bone marrow biopsy showing megakaryocytic proliferation and atypia,* bone marrow fibrosis grade < 2, increased age-adjusted BM cellularity, granulocytic proliferation, and (often) decreased erythropoiesis 2. *JAK2*, *CALR*, or *MPL* mutation† or presence of another clonal marker‡ or absence of reactive bone marrow reticulin fibrosis§ 3. Diagnostic criteria for *BCR::ABL1*-positive CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms are not met	Major criteria 1. Bone marrow biopsy showing megakaryocytic proliferation and atypia,* accompanied by reticulin and/or collagen fibrosis grades 2 or 3 2. *JAK2*, *CALR*, or *MPL* mutation† or presence of another clonal marker‡ or absence of reactive myelofibrosis§ 3. Diagnostic criteria for ET, PV, *BCR::ABL1*-positive CML, myelodysplastic syndrome, or other myeloid neoplasms‖ are not met
Minor criteria • Anemia not attributed to a comorbid condition • Leukocytosis ≥ 11 × 10^9/L • Palpable splenomegaly • Lactate dehydrogenase level above the above the reference range	Minor criteria • Anemia not attributed to a comorbid condition • Leukocytosis ≥ 11 × 10^9/L • Palpable splenomegaly • Lactate dehydrogenase level above the above the reference range • Leukoerythroblastosis
The diagnosis of pre-PMF or overt PMF requires all 3 major criteria and at least 1 minor criterion confirmed in 2 consecutive determinations	

Table 1: 2022 International Consensus Classification (ICC) diagnostic criteria for myelofibrosis. From Arber et al. [7]

1.1.3 Epidemiology

Among the chronic myeloproliferative neoplasms, Myelofibrosis is the least frequent. The incidence is approximately 0.1-1.5 per 100,000 individuals per year[12]. It occurs mainly in middle aged and older adults. Almost 90% of the patients are older than 60 years, with patients presenting at a median age of 64 years.

Secondary myelofibrosis are reported in 10-20% of patients with Polycythemia Vera or Essential Thrombocythemia and they occur usually after 15-20 years from the first diagnosis[4].

The median survival for myelofibrosis is 2 to 5 years[2,12,13], even if the median survival varies greatly according to risk groups. The International Working

Group for Myelofibrosis Research and Treatment reported median survival of approximately 11 and 2 years for low- and high-risk patient groups, respectively, whereas median survivals of patients in the two intermediate-risk disease categories are 8 and 4 years[13].

1.1.4 Molecular characteristics

In Myelofibrosis, as for all MPNs in general, the somatic mutations are classified into "driver" and "non-driver" mutations; the former include JAK2, CALR and MPL and the latter all the somatic mutations other than the driver mutations [14,15]. It is generally believed that driver mutations are essential for the MPN phenotype, whereas the "non-driver" mutations might contribute to disease progression and leukemic transformation[16].

Driver Mutations

JAK2 mutation

JAK2 is the most common myeloproliferative neoplasm driver gene mutated. JAK2, as well as JAK1, JAK3 and TYK2, is a member of the Janus family of cytoplasmic non-receptor tyrosine kinases that are differentially activated in the response to various cytokines[17]. JAK2 serves as the cognate tyrosine kinase for the erythropoietin and thrombopoietin receptors and can also be used by the granulocyte colony-stimulating factor receptor, all of which lack an intrinsic kinase domain.

All the members of the JAK family are composed by seven homologous domains (from JH1 to JH7). JH3 and JH4 connect JH2 to JH5, JH6 and JH7, which form the "protein four-point-one, ezrin, radixin, moesin" (FERM) domain, containing the sequences necessary to promote association of the kinase with specific cytokine receptors domains. In addition, JAK2 has a dual kinase structure: a canonical tyrosine kinase domain (JH1) paired in tandem with a weakly active pseudokinase domain (JH2), which normally inhibits JH1 kinase activity in the absence of ligand binding (Figure 1).

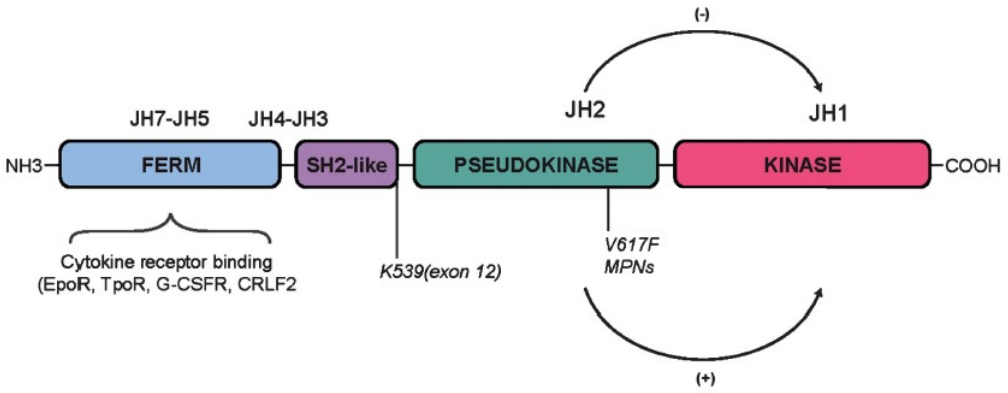


Figure 1. Domain structure of JAK2. JAK2 contains a tyrosine kinase domain (JAK homology 1 (JH1)), a pseudokinase domain (JH2), a SH2-like domain, and a domain that resembles protein 4.1, ezrin, radixin and moesin (FERM). The latter domain is responsible for attachment to the cytosolic domains of cytokine

receptors. The pseudokinase domain (JH2), functionally prevents the activation of the kinase domain, JH1 (curved arrow on the top). In contrast, mutations in the pseudokinase domain (V617F) lead to activation (arrow on the bottom) of JH1. Adapted from Vainchenker and Constantinescu, Oncogene, 2013[18].

In 2005, three different groups simultaneously discovered the *JAK2 V617F* driver mutation, in which a valine is substituted with a phenylalanine at codon 617 (V617F), due to a G to T mutation at nucleotide 1849 in exon 14 in the JAK2 JH2 pseudokinase domain. This mutation results in the impairment of the JH2 domain physiologic inhibitory influence on the JH1 kinase domain[19], leading to a constitutive increase of the JH1 kinase domain activity and, therefore, triggering constitutive activation of downstream signaling and uncontrolled cell growth. This mutation is the most frequent molecular alteration in MPN. It has been found in 95% of patients with PV and 50-60% of those with ET and PMF[20–23]. In myelofibrosis patients the median of the Variant Allele frequency (VAF) is around 50%. About two thirds of the patients are homozygous for the mutation, which results in more aggressive disease (spleen size, symptomatic symptoms, higher hematocrit level). Surprisingly, patients with a low VAF have a worst outcome for reasons not yet clear[25,26].

Nowadays, It is not yet fully understood how JAK2 *V617F* and other JH2 domain mutations alleviate JH2 inhibition, but probably they modify the JAK2 Src homology 2 (SH2)–JH2 linker region, thus altering the interface between the JH2 and JH1 domains[24]. In the heterozygous state, *JAK2 V617F* bearing receptors are still responsive to growth factors. Conversely, in *JAK2 V617F* homozygosity the receptors become autonomous with respect to growth factor, usually due to 9p uniparental disomy.

CALR mutation

CALR mutation represents the second most frequent mutation (20-30% of cases) in Myelofibrosis. In 2013, some authors have discovered that somatic mutations in exon 9 of calreticulin (CALR) gene occurred in around 20 to 25% of patients with Essential Thrombocythemia and Myelofibrosis [27,28], representing around the 70% and 88% of JAK2 negative Essential Thrombocythemia and Myelofibrosis, respectively[27,28]. CALR mutations are most commonly heterozygous and mutually exclusive with JAK2/MPL mutations[27,28].

To date, 50 CALR mutant variants have been described[29]. These have consistently found to be insertions or deletions (most commonly a 52bp deletion, “type 1 mutation”, or 5bp insertion, “type 2 mutation”) in its final exon resulting in a 1bp shift in the reading frame and a common novel C-terminal sequence[27,28]. Usually, CARL is involved in the regulation of calcium uptake and release by the endoplasmic reticulum, and acts as a chaperone, regulating folding and quality control of newly synthesized glycoproteins in the endoplasmic reticulum[30,31]. Conversely,

CALR mutations can impart TPO-independence in cell lines[32–34], retroviral and transgenic mouse models[35–37], mimicking the effect of activating MPL mutations and recapitulating their phenotype in vivo.

The respective mutational frequencies of type 1 and type 2 variants in CALR mutated PMF are reported as ≈70 and ≈15%, respectively [28,38,39]. Those CALR variants unclassifiable as type 1 or 2 are categorized as 'type 1-like' and 'type 2-like' on the basis of their structural similarities to the classical mutants[40].

Type 1, or type-1 like, and type 2, and type 2-like, mutations are associated with different clinical phenotypes. Briefly, Type 1 mutations, which are more frequent in myelofibrosis, have a more favorable outcome[40,41], while Type 2 mutations are more frequently associated with Essential Thrombocythemia and result in higher platelet counts[42]. Despite the fact that patients with type 2-like CALR mutation had the highest values for platelet count, they had the lowest risk of thrombosis, significantly lower than that of patients carrying *JAK2 V617F*[42].

Overall, CALR-mutant patients had a lower cumulative incidence of developing anemia, thrombocytopenia, and marked leukocytosis, and a longer interval to the development of large splenomegaly (>10 cm below the left costal margin) compared with other mutational subgroups[38].

MPL mutation

The MPL gene is located on chromosome 1p34 and encodes for the thrombopoietin receptor, playing, therefore, a crucial role in the regulation of megakaryocyte growth and survival. MPL is a unique type I hematopoietic cytokine receptor because it is the only one expressed in hematopoietic stem cells.

In 2006, a somatic activating mutation in exon 10 of the MPL virus oncogene, *MPL W515L*, was described in *JAK2 V617F*-negative PMF[43]. It accounts for approximately 5–10% of JAK2 unmutated patients with essential thrombocythemia or PMF[44,45]. This mutation is characterized by a G to T transition at nucleotide 1544, resulting in a tryptophan to leucine substitution at codon 515 of the transmembrane region of MPL, inducing constitutive activation of the thrombopoietin receptor in a cytokine-independent fashion[43]. Although several substitutions have been described[46], somatic MPL mutations occur most often in exon 10. A less common mutation, S505N, in the MPL trans-membrane domain, resulting in serine to asparagine switch, can be inherited or acquired and causes essential thrombocytosis. MPL mutations force a change in receptor conformation, activating JAK2 in the absence of thrombopoietin binding. Missense mutations at codon 515 of MPL (which encodes myeloproliferative leukaemia protein, the thrombopoietin receptor itself), including M515L and M515K, have been reported and they are associated with increased STAT-3, STAT-5, ERK and AKT signalling[43,47].

"Triple negative"

Around 10-20% of patients diagnosed with myelofibrosis did not show any mutations in one of the 3 MPN driver genes (JAK2, CALR, MPL), and these cases are called "triple negatives". Usually, "triple negative" patients are older, and presented with anemia and lower platelet counts compared with patients harboring mutations in one of the three MPN driver genes[38]. This group of patients is characterized by a poor prognosis, with an estimated survival of just over 2 years.

In a retrospective study of 428 patients with PMF, median overall survival for patients with a mutation of JAK2, CALR, MPL, or triple-negative disease were 5.9, 15.9, 9.9 and 2.3 years, respectively[48]. In another study, "triple negative" patient had the worst outcome, followed by patients with JAK2 mutation, absence of type 1-like CALR mutation and MPL mutation[49]. When adjusting for age, CALR-mutant patients still have improved overall survival compared with those with JAK2 mutations or triple negative myelofibrosis[38].

Similarly, considering the leukemia free survival, triple negative patients had an highest 10-year cumulative incidence of blast transformation (34.4%), compared with those with JAK2 V617F (19.4%; p= 0.043), MPL (16.9%), and CALR (9.4%; p= 0.016) mutations[38].

Interestingly, the driver mutational profile has less impact on prognosis in those with secondary MF, based on a study of 359 patients with post–PV-MF (n=194) and post–ET-MF (n=165)[50]. Only triple negative post–ET-MF have shown a shorter survival compared with CALR-mutated post–ET-MF (P=0.01), and there was no difference between other genotypes, including type 1 vs. type 2 CALR, JAK2, and MPL-mutated secondary Myelofibrosis[50].

Non-driver mutations

Beside mutations in the so-called "MPN-driver" genes, PMF patients presented additional somatic mutations, which could be detected also in other myeloid disorders, such as primarily myelodysplastic syndromes and acute leukemias. These highly heterogeneous mutations group included genes involved in the regulation of DNA-methylation (TET2, DNMT3A, IDH1/2), chromatin modifications (ASXL1, EZH2), RNA splicing (SF3B1, SRSF2, U2AF1), and DNA repair (TP53) (see Figure 2). Mutations in these genes often represent the first mutational event in clonal evolution that results in clonal expansion, and it may be followed by the acquisition of one of the driver mutations.

These mutations are considered an additional diagnostic criterion if driver mutations are absent, as in triple-negative patients. However, the same myeloid mutations can be found in healthy individuals, constituting the so-called "clonal hematopoiesis of indeterminate potential (CHIP)"[51]. Therefore, the identification of these mutations in triple-negative patients should be carefully interpreted, considering also the other diagnostic criteria (i.e., clinical phenotype and blood cell

count, together with bone marrow abnormalities).

In order to clarify the prognostic relevance of non-driver mutations and their impact on survival, an international collaborative project analyzed the outcome of 879 patients with PMF and known mutational status[14]. 79% of the patients displayed at least one somatic mutation. In this study, Vannucchi et al[14] first described that mutation in one of the following genes is associated with increasingly poor prognosis in MF patients: ASXL1, SRSF2, U2AF1Q157, EZH2 and IDH1/2. Because of that the mutations in these genes are reported as "High molecular risk (HMR) mutations". However, in multivariate analysis only ASXL1 mutations remained significantly associated with survival in the context of the International Prognostic Scoring System (IPSS). Subsequent studies confirm the inferiors outcome related to HMR mutations[52,53]. Multivariate analysis of 641 patients reported that mutations of *ASXL1*, *SRSF2*, and *U2AF1*Q157 were associated with inferior survival in PMF[49]. In detail, ASXL1 mutations correlated with constitutional symptoms, leukocytosis, and >1% circulating blasts; SRSF2 mutations correlated with older age; and EZH2 mutations associated with >1% circulating blasts. Patients with ASXL1, EZH2, SRSF2, or IDH mutations were at risk for premature death or leukemic transformation. *ASXL1* and *SRSF2* were also independently associated with inferior Leukemia free survival (HR: 2.1 and 4.3, respectively). Instead, loss of heterozygosity for *TP53* was associated with leukemic transformation [54].

Notably, it was subsequently demonstrated that also the number of mutations may impact on patients' outcome. Indeed, the presence of 2 or more somatic mutations predicted for worse survival[53]. Another report on comprehensive mutational screening of 104 genes by NGS at diagnosis and during follow up in 197 patients demonstrated the presence of somatic mutations in 90% of cases, and 37% carried somatic mutations other than *JAK2 V617F* or CALR. Moreover, also in this report, the presence of $\geq$2 somatic mutations significantly reduced overall survival and increased the risk of AML transformation[54].

Also for the non-driver somatic mutations, the impact of mutational profile is different in patients with secondary myelofibrosis[50]. Indeed, those with post–ET myelofibrosis were more likely to have ASXL1 and EZH2 mutations, compared with those with post–PV myelofibrosis. However, in post–PV myelofibrosis, there was no association between a single-somatic gene mutation, HMR profile, or number of HMR-mutations and overall survival. In post–ET-MF, only SRSF2-mutated status correlated with shortened survival.

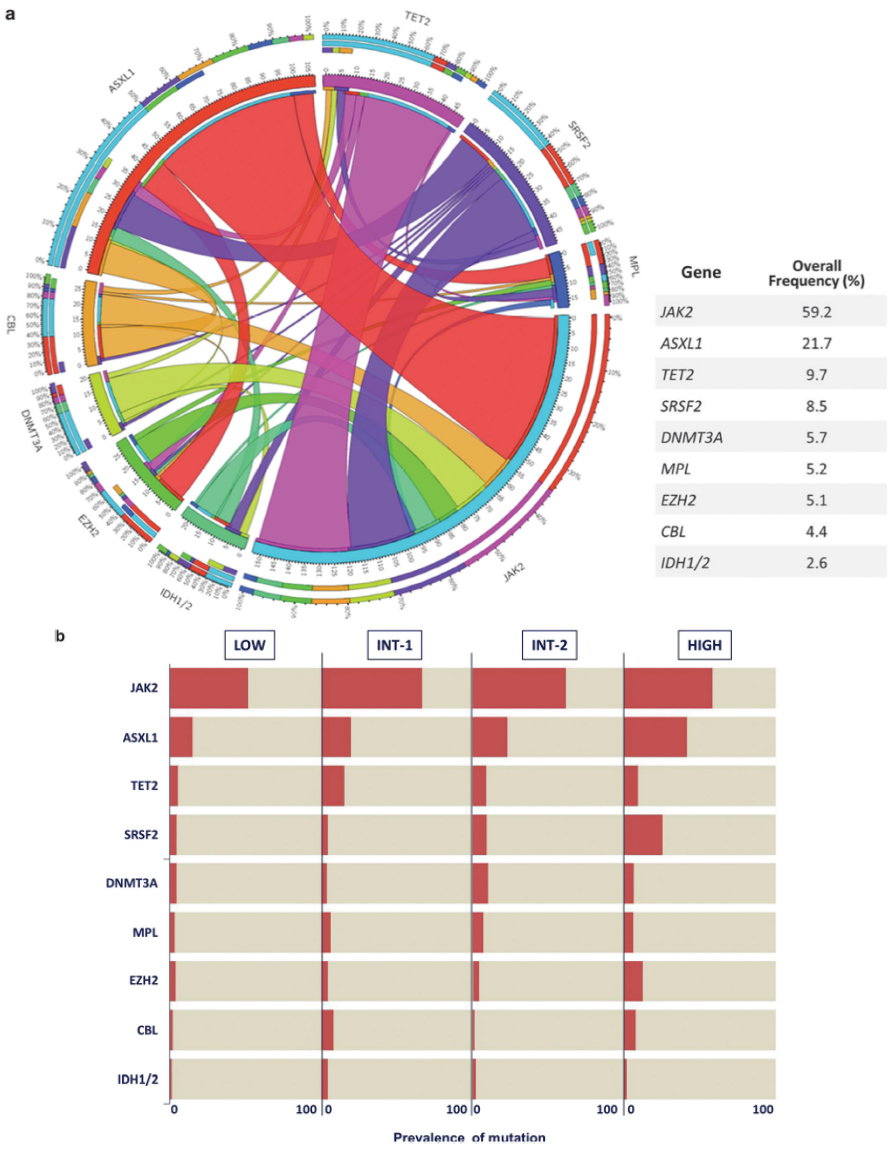


Figure 2: The frequency and the pair-wise co-occurrence of mutations in the 483 PMF patients included in the European cohort are presented by a Circos diagram in (a). Co-occurring mutations are indicated in the clockwise direction. In the Circos representation, the length of the arc corresponds to the frequency of mutation in the first gene (color coded) and the width of the ribbon corresponds to the frequency of patients who also had a mutation in the second gene. The frequency of mutations in this cohort is shown on the right side. (b) shows the prevalence proportion of individual mutations in the four IPSS risk categories. Vannucchi et al. [14]

Until now, the treatment decisions for patients with myelofibrosis are not yet driven by the presence or absence of MF-associated molecular mutations, but rather, influenced by myelofibrosis subtype, symptom burden, and risk category.

Whereas the driver mutational profile influences prognosis, there is less impact on response to JAK inhibition. In the phase 3 studies of ruxolitinib compared with placebo or best available therapy, there was no statistically significant difference in efficacy measures when comparing JAK2-mutated and WT patients[55,56]. Subsequently, a letter reported on spleen and symptom responses to the JAK inhibitor, fedratrinib, in

patients with CALR-mutated MF[57]. The clinical observation of JAK-inhibitor response regardless of mutational profile is supported by recent translational studies revealing an activated JAK2 signaling signature in MPN patients irrespective of mutational profile[58].

1.1.5 Clinical Presentations

Clinical manifestations in myelofibrosis include severe anemia, marked hepato-splenomegaly, constitutional symptoms due to inflammatory cytokine production (e.g., fatigue, night sweats, fever), cachexia, bone pain, splenic infarct, pruritus, thrombosis, and bleeding (Table 2).

Fatigue is the most common presenting symptom, and it occurs in about 50 to 70 percent of patients. Usually, it is related to inflammatory cytokine release by the PMF clonal cells. Splenomegaly and hepatomegaly are also often clinical features of PMF patients, and they are due to the marked extramedullary hematopoiesis. At least 90% of PMF patients presented splenomegaly, and around 25 to 50 % of them presented with symptoms related to it. Conversely, an enlarged liver is found in 40 to 70 % of patients. Symptoms due to splenic disease often figure prominently in PMF. Patients may note a dragging or heavy sensation in the left upper abdomen, and the spleen may compress the patient's stomach, leading to early satiety. Severe left upper quadrant pain may result from multiple and/or recurrent episodes of splenic infarction or inflammation of the tissues surrounding the spleen (i.e., perisplenitis). Portal hypertension may develop as a result of increased splanchnic flow due to splenomegaly and/or intrahepatic obstruction associated with extramedullary hematopoiesis[59]. Complications include ascites, esophageal and gastric varices, gastrointestinal bleeding, and hepatic encephalopathy. Portal vein thrombosis is a recognized complication of PMF and other chronic myeloproliferative disorders[59] and may precede the clinical onset of the disease, similar to what has been documented in polycythemia vera[60].

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, cachexia, and constitutional symptoms. Some patients note weight loss, and 5 to 20 percent experience other signs of a hypermetabolic state such as low-grade fever, bone pain, and night sweats. Pruritus could be found in less than 20% of patients and, usually, it did not correlate with plasma levels of cytokines known to be abnormally expressed in PMF[61]. Another disease complication, but less frequent, is pulmonary hypertension, which is often asymptomatic and it has been associated with reduced overall survival[62,63].

Vascular complications present a major source of morbidity and mortality in Myelofibrosis[65]. The incidence of arterial and venous thrombotic events in PMF (2 per 100 patient-years) is approximately the same as that seen in essential thrombocythemia (1 to 3 per 100 patient-years), and significantly lower than that seen

in polycythemia vera (5.5 per 100 patient-years)[66] (for more detail see the following section).

Feature	Results (%)
Median age, years (range)	65 (14 to 92)
Males	62%
Constitutional symptoms	34%
Fatigue	65%
Spleen >10 cm below left costal margin	31%
Red cell transfusions required	38%
Hemoglobin <10 g/dL	54%
White blood cell count >25,000/uL	16%
White blood cell count <4000/uL	16%
Platelet count <100,000/uL	26%
Circulating blasts ≥1 percent	56%

Table 2: **Clinical presentation of PMF patients.** Data from: Tefferi A, et al. One thousand patients with primary myelofibrosis: the Mayo Clinic experience. Mayo Clin Proc 2012; 87:25[64].

Peripheral blood leukoerythroblastosis (ie, presence of nucleated red cells, immature granulocytes and dacryocytes) is a typical but not invariable feature of PMF; prefibrotic PMF might not display overt leukoerythroblastosis.

Approximately 15 to 30 percent of patients with primary myelofibrosis are asymptomatic, and the diagnosis is made as a result of detecting splenomegaly (occurring in at least 90 percent of patients), hepatomegaly (40 to 70 percent) or checking blood cell counts for an unrelated cause.

Although secondary acute myelogenous leukemia is the single most common cause of death, the majority of patients die from PMF-related complications[13]. Indeed, the main causes of death include leukemic progression (around 20%), cardiovascular events (around 20%) and consequences of cytopenias, including infection (10%) or bleeding (5%)[13][16]. Patients with MF have a substantially reduced life expectancy, with a median survival time of only 6 years for those diagnosed with PMF[13].

1.1.6 Risk stratification

The first widely used risk stratification tool for MF was the Lille Classification which based on the white blood cell count (>30 x 10^9/L or <4 x 10^9/L earning 1 point) and the hemoglobin level (<10 g/dL earning 1 point)[67]. Requiring just a blood complete count, Lille score risk was simple to use, but not incorporate other important prognostic disease features that could better distinguish between groups of patients with differing outcomes.

Therefore, in 2009, a new score risk system was developed by the International

Working Group for MF Research and Treatment (IWG-MRT) that better discriminate prognosis, providing more confidence in therapeutic decision making[13]. The International Prognostic Scoring System (IPSS) incorporated five clinical features at the time of diagnosis, that were found to have prognostic significance in multivariate analysis: age higher than 65 years, presence of constitutional symptoms, hemoglobin <10g/dL, leukocytes >25x10^9/L, peripheral blood blasts >1%. Considering the presence of not of the adverse factors, the IPSS risk score allow to have four groups of risk: low (0 points), intermediate-1 (1 point), intermediate-2 (2 points), and high (≥3 points). These different risk groups had a median survival of 11.3, 7.9, 4 and 2.3 years, respectively[13]. Subsequently, Passamonti et al, on behalf of the IWG-MRT, proposed a dynamic prognostic model (DIPSS), which allow to apply the same prognostic variables used in IPSS at any time during PMF disease course[68]. Notably, in the DIPSS risk score more weight was assigned to anemia due to the higher prognostic power in time-dependent analysis. Indeed, DIPSS assigned two, instead of one, adverse points for hemoglobin < 10g/dL. Thus, the DIPPS groups included low (0 points), intermediate-1 (1-2 points), intermediate-2 (3-4 points), and high risk (5-6 points) with a median OS not yet reached, 14.2, 4, and 1.5 years, respectively[68]. An age adjusted (aa)DIPSS was also created for PMF patients <65 years of age that would be traditionally considered appropriate for definitive therapy with hematopoietic stem cell transplantation.

Approximately, one third of patients with PMF present with abnormal karyotype[69], but neither IPSS nor DIPSS considers cytogenetic findings in its prognostic model. It was reported that patients with complex karyotype (≥3 abnormalities), trisomy 8 and other abnormalities of chromosomes 5, 7, 17 or 12p- had a worst outcome, while sole 20q-, sole 13q- or sole +9 were known as favorable cytogenetic markers. Caramazza et al[69], refined cytogenetic-risk categorization and developed a new prognostic score, named DIPSS-plus, in which three additional DIPSS-independent risk factors were incorporated to DIPSS. The three additional negative prognostic risk factors were platelet count <100x10^9/L, the need for red blood cell transfusions, and the presence of an unfavorable karyotype (complex karyotype or sole or two abnormalities that include +8, -7/7q-, i(17q), -5/5q-, 12p-, inv(3), or 11q23 rearrangement)[70]. The DIPSS-plus stratifies patients into 4 risk groups with median OS of 15.4, 6.5, 2.9, and 1.3 years, respectively[70]. In the DIPSS-plus, High risk cytogenetic profile recognizes LR IPSS patients with worse survival. In detail, 7% of low-risk IPSS patients had an unfavorable karyotype, upgrading their risk[69].

Thereafter, through the use of second-generation sequencing new molecular abnormalities were discovered in PMF patients[14]. So, in the last years, efforts to integrate molecular information with the clinical and cytogenetic prognostication systems have led to the development of the Genetics-Based Prognostic Scoring System (GPSS), the Mutation-Enhanced International Prognostic Scoring System (MIPSS) and MIPSS70+ version 2.0 (the karyotype-enhanced MIPSS70) (Table 3).

	MIPSS70 (3-tiered)		MIPSS70+ version 2.0 (5-tiered)		GIPSS (4-tiered)
	Genetic variables *One HMR mutation* *(1 point)* *≥2 HMR mutations* *(2 points)* *Type 1/like CALR absent* *(1 point)*	Clinical variables *Hemoglobin < 10 g/dL* *(1 point)* *Leukocytes > 25 × 10(9)/L* *(2 points)* *Platelets < 100 × 10(9) /L* *(2 points)* *Circulating blasts ≥2%* *(1 point)* *Constitutional symptoms* *(1 point)* *Bone marrow fibrosis grade ≥2* *(1 point)*	Genetic variables *VHR karyotype* *(4 points)* *Unfavorable karyotype* *(3 points)* *≥2 HMR mutations* *(3 points)* *One HMR mutation* *(2 points)* *Type 1/like CALR absent* *(2 points)*	Clinical variables *Severe anemia* *(2 points)* *Moderate anemia* *(1 point)* *Circulating blasts ≥2%* *(1 point)* *Constitutional symptoms* *(2 points)*	Genetic variables *VHR karyotype* *(2 points)* *Unfavorable karyotype* *(1 point)* *Type 1/like CALR absent* *(1 point)* *ASXL1mutation* *(1 point)* *SRSF2 mutation* *(1 point)* *U2AF1Q157 mutation* *(1 point)*
Very low risk (median survival)			Zero points (*not reached*)		
Low risk (median survival)	0-1 points (*not reached*)		1-2 points (*16.4 years*)		Zero points (*26.4 years*)
Intermediate-1 risk (median survival)					One point (*8 years*)
Intermediate risk (median survival)	2-4 points (*6.3 years*)		3-4 points (*7.7 years*)		
Intermediate-2 risk (median survival)					Two points (*4.2 years*)
High risk (median survival)	≥5 points (*3.1 years*)		5-8 points (*4.1 years*)		≥3 points (*2 years*)
Very high risk (median survival)			≥9 points (*1.8 years*)		

Table 3: **New prognostic models in primary myelofibrosis.** Abbreviations: MIPSS70: mutation-enhanced international prognostic system for transplant-age patients (age ≤ 70 years); MIPSS70+ version 2.0: mutation and karyotype enhanced international prognostic system. Survival quotes are for age ≤ 70 years; GIPSS: genetically inspired prognostic scoring system. Survival quotes are for all age groups; HMR: high molecular risk mutations include ASXL1, SRSF2, EZH2, IDH1, IDH2 and, in addition, for GIPSS and MIPSS70+ version 2.0, U2AF1Q157; VHR: very high risk karyotype. Severe anemia: Hemoglobin <8 g/dL in women and < 9 g/dL in men. Moderate anemia: Hemoglobin 8-9.9 in women and 9-10.9 in men. From Tefferi et al.[3]

These new prognostic models included components that highlighted the independent prognostic contribution of driver[71] and other mutations[14,53,72–74], karyotype[75] and sex-adjusted hemoglobin levels[76]. Indeed, the mutational status of the three key drivers (JAK2, MPL, CALR) in addition to the presence of other somatic mutations (ASXL1, SRSF2, EZH2, and IDH1/2) that have shown to influence outcome were included in the construction of these newer prognostication systems[14,38]. When compared to the IPSS, the MIPSS provided refinement of the prognostic score and allowed the identification of subgroups of patients with a worse prognosis within an IPSS category. MIPSS70 was developed in patients age 70 years or younger in order for it to be relevant for transplant-age patients[77]. MIPSS70+ version 2.0 incorporated the recently revised three-tiered cytogenetic risk levels[75], U2AF1Q157 as an additional high molecular risk (HMR) mutation[74] and new sex- and severity-adjusted hemoglobin thresholds[76].

The genetics-based prognostic scoring system (GPSS) was developed by the Mayo group in a large cohort of PMF patients and validated in an independent patient cohort from Italy. The GPSS incorporates only cytogenetic and mutational prognostic data to create 4 risk groups of low, intermediate-1, intermediate-2, and high risk with corresponding OS of >17, 9, 5, and 2.2 years, respectively. It is important to point out that the dizzying array of prognostication tools that have been created in the last few

years have not been validated in postET-MF and postPV-MF and are still best utilized in the setting of determining eligibility for clinical trial enrolment and determining optimal benefit/risk ratio in the pursuit of HSCT. Recently, a new prognostic score has been developed for post ET and post PV myelofibrosis: the MYSEC (MYelofibrosis SECondary to PV and ET) score system[78]. MYSEC prognostic model, based on age >65 years, time to SMF >15 years, previous thrombosis, constitutional symptoms, hemoglobin <10 g/dL and circulating blast equal or >1%, clearly distinguishes outcome of patients with SMF and outperforms PMF risk models among those patients.

1.1.7 Treatment

The only treatment that is currently capable of prolonging survival or potential cure myelofibrosis is allogeneic stem cell transplant (alloSCT)[79]. However, just few patients are eligible to this procedure. Indeed, the median age at diagnosis (roughly 60 years) and the significant transplant-related morbidity and mortality limited the use of allo-SCT only to a minority of PMF patients[80]. Current drug therapy for MF is mostly palliative in scope and has not been shown to favorably modify disease natural history or prolong survival; specifically, JAK2 inhibitor treatment in PMF has not been shown to clearly reverse bone marrow fibrosis or induce complete or partial remissions; instead, its value is limited to symptoms relief and reduction in spleen size[81,82]. Generally, patients at low risk require only surveillance, but those at intermediate and high risk of disease progression require treatment as symptoms worsen.

The central importance of genetic alterations in the JAK-STAT signaling pathway in PMF pathogenesis provided the rationale for the clinical development of Jak kinase inhibitors in these patients, including with the FDA-approved agent ruxolitinib and fedratinib. Clinical studies have shown an improvement in splenomegaly, systemic symptoms, and overall survival due to the use of these drugs in comparison to placebo or best available therapy[83]. Ruxolitinib, a JAK1-2 inhibitor, leads also to a rapid and sustained down-regulation of cytokine levels, indicating that the Jak signaling pathway mediates this aberrant inflammatory cytokine profile[84]. Recently, Levine et al shown that inhibition of JAK-STAT signaling in both mutant and non-mutant cells is required to reduce inflammatory signaling and to achieve clinical benefits in MPNs[85]. These discoveries unveil a complex landscape implicating a mosaic of functionally diverse malignant and non-malignant cell population in the pathogenesis of MPN, highlighting the essential role of the tumor microenvironment in cancer progression. However, Jak kinase inhibitors do not eliminate or markedly attenuate the malignant clone in MPN and have little to no impact on bone marrow fibrosis[86].

Ruxolitinib was the first JAK1/2 pathway inhibitor FDA approved for patients with Myelofibrosis in 2011 and hydroxyurea-resistant or intolerant Polycythemia Vera in 2014 based on the results of phase 3 trials[55,56,87]. Treatment initiation is based

on risk stratification. FDA and EMA approved its use in symptomatic patients with high and intermediate risk disease. The optimal timing of ruxolitinib initiation has yet to be identified, and most of the data come from retrospective studies. Lower treatment response rates were reported in a large retrospective cohort of patients with high-risk disease and delay in ruxolitinib initiation[88].

Two large randomized studies comparing ruxolitinib with either placebo or best supportive care have now been published[55,56]. In the COMFORT-1 trial, comparing Ruxolitinib with placebo (n = 309)[55], the spleen response rate was approximately 42% for Ruxolitinib vs. <1% for placebo. In addition, about 46% of patients experienced substantial improvement in their constitutional symptoms. The main ruxolitinib-associated side effects were anemia (31% vs. 13.9%) and thrombocytopenia (34.2% vs. 9.3%). In the COMFORT-2 trial, which compared Ruxolitinib with the "best available therapy" (n = 219)[56], the spleen response was 28.5% with Ruxolitinib vs. 0%. Howeber, Ruxolitinb was associated with higher rate of thrombocytopenia (44.5% vs. 9.6%), anemia (40.4% vs. 12.3%) and diarrhea (24.0% vs. 11.0%). The 3-year follow-up information on COMFORT-2 suggested a 55% drug discontinuation rate and a slight but significant improvement in survival, which is however confounded by the cross-over design of the study and lack of substantial drug effect on JAK2V617F allele burden or bone marrow fibrosis[81].

Infections represent one of the major concerns regarding the utilization of Ruxolitinib in patients with myelofibrosis[56,89]. Moreover, patients with a high IPPS score and previously infections history had a higher risk of developing infection during Ruxolitinib treatment[89].

The FDA recently approved fedratinib (Inrebic; Celgene) for intermediate-2 or high risk primary or secondary myelofibrosis, based the results of a phase II and III trial (JAKARTA and JAKARTA2)[90,91], in which the drug significantly reduced symptoms compared with a placebo. Fedratinib is an oral kinase inhibitor with activity against wild type and mutationally activated Janus Associated Kinase 2 (JAK2) and FMS-like tyrosine kinase 3 (FLT3). It is a JAK2-selective inhibitor with higher potency for JAK2 over family members JAK1, JAK3 and TYK2. The most important side effect of Fedratinib are serious and fatal encephalopathies, including Wernicke's. In particular, serious cases were reported in 1.3% (8/608) of patients treated with Fedratinib in clinical trials and 0.16% (1/608) of cases were fatal. Instead, the most common adverse reactions for Fedratinib treated vs. placebo were diarrhea (66% vs. 16%), nausea (62% vs. 15%), anemia (40% vs. 14%), and vomiting (39% vs. 5%)[90,91]. Dosage interruptions due to an adverse reaction during the randomized treatment period occurred in 21% of patients who received fedratinib[90,91].

A few other JAK2 inhibitors have also been evaluated in clinical trials conducted in PMF patients. Pacritinib is a JAK2/FLT3 kinase inhibitor that has completed phase III trials in patients with intermediate/high-risk MF (PERSIST-1 and 2) [92], and it has shown efficacy and safety results in MF patients with low platelets,

as this remains a serious unmet clinical need. Momelotinib is a potent JAK1/2 inhibitor that was evaluated in a phase I/II trial. The drug was well tolerated in early studies, and it has shown a notable effect of mitigating anemia[93]. Itacitinib is a selective JAK1 inhibitor and in a phase 2 study that assessed 3 doses, it showed clinical activity in higher-risk PMF and was less myelosuppressive than previously discussed JAK inhibitors[94].

Outcomes in patients with relapsed/refractory PMF after ruxolitinib are generally poor with survival approximately 14 months.

Since inflammation contributes to constitutional symptoms, BM fibrosis, extramedullary hematopoiesis, and disease progression, detailed investigation of the mechanisms that regulate inflammatory signaling in Myeloproliferative neoplasms is of great importance. Recently, Levine and colleagues have demonstrated how inhibition of BET bromodomain proteins attenuated NF-kB signaling and reduced cytokine production *in vivo*. Most importantly, combined JAK/BET inhibition resulted in a marked reduction in the serum levels of inflammatory cytokines, reduced disease burden, and reverse bone marrow fibrosis *in vivo*[95]. Multiple non–JAK inhibitor targeted therapies are being investigated in ongoing studies, in some cases in combination with ruxolitinib. Some of them are the Histone deacetylase (HDAC) inhibitors (panobinostat), telomerase inhibitor (Imetelstat), Heat shock protein (HSP) inhibitors (HSP90), and recombinant analogue of pentraxin-2 (PRM-151). Most of these innovative drugs are still under investigation and definitive results are expected in the next years.

Historically, the first-line drug of choice for MF-associated splenomegaly was hydroxyurea, which was effective in reducing spleen size by half in approximately 40% of patients[96]. Hydroxyurea is an oral ribonucleotide reductase inhibitor, and it was the mainstay of the medical treatment of PMF before the introduction of JAK inhibitors. Spleen response to hydroxyurea lasts for an average of 1 year and treatment side effects include myelosuppression and painful mucocutaneous ulcers. Interferon(IFN)-α is of limited value in the treatment of MF-associated splenomegaly.

Allogeneic Hematopoietic Stem Cell Transplant

Allogeneic Hematopoietic Stem Cell Transplant (alloSCT) is the only potentially curative treatment for myelofibrosis, but its utility is limited by the relatively high incidence of treatment related mortality and morbidity, as well as for the old median age at diagnosis of PMF. In general, it is reasonable to justify the risk of either alloSCT or experimental drug therapy for PMF in the presence of a <5 years life expectancy or >20% 5-year risk of developing acute leukemia[97]. Those categorized as having int-2 or high-risk disease, should be considered for alloSCT if they are deemed fit according to the European Society for Blood and Marrow Transplantation/European LeukemiaNet (EBMT/ELN) International Working Group[98]. Until now, no randomized controlled trials have compared alloSCT with alternative options, while

many data are retrospective, with substantial heterogeneity in all aspects among these studies. Kroger et al, analyzed retrospectively 190 patients younger than 65 years who received alloSCT with 248 who received non-alloSCT therapies[99]. Those with DIPSS int-2 or high-risk disease had superior survival if they received alloSCT, but the risk of alloSCT outweighed the benefit in those with low-risk disease. This review did not include patients with post-PV or post-ET MF or those treated with JAK inhibitors, making it difficult to extrapolate the results to these subgroups of patients.

Optimal conditioning remains to be defined. Myeloablative conditioning (MAC) regimens are associated with an unacceptably high mortality risk, especially in those older than 45 years[100]. After adjustment for patient age, reduced-intensity conditioning has been shown to be associated with superior survival compared with MAC[101]. The stem cell source seems not affect outcome and similar outcomes have been described for matched related and unrelated donors[102]. Patients with MF may be at higher risk for hepatotoxicity after transplant, such as sinusoidal obstructive syndrome,[103] which is thought to be related to pretransplant hepatic dysfunction (from extramedullary hematopoiesis and drugs). Debated is the role of pre-transplant splenectomy[98,100]. When ruxolitinib is used as a bridge to transplant, the reduction in spleen size may also improve engraftment rates. In addition, the reduction in proinflammatory cytokines may reduce the risk for post-alloSCT graft versus host disease. The largest multicenter retrospective study of JAK inhibitor use in the peritransplant period[104] suggests continuing JAK inhibitor treatment to the time of conditioning. Survival was better and transplant-related mortality rates were lower in patients who responded to a JAK inhibitor than in those with stable/progressive disease, which may be explained by favorable disease biology in the former group.

1.2 Vascular complications

1.2.1 Epidemiology

Vascular complications are a major source of morbidity and mortality in Myelofibrosis[65]. The incidence of arterial and venous thrombotic events in PMF (2 per 100 patient-years) is approximately the same as that seen in essential thrombocythemia (1 to 3 per 100 patient-years), and significantly lower than that seen in polycythemia vera (5.5 per 100 patient-years)[66] (Table 4).

Arterial events typically include stroke/transient ischemic attack, peripheral vascular disease, coronary artery disease or acute coronary syndrome, and central retinal artery occlusion. Conversely, venous thromboses include deep venous thrombosis, pulmonary embolism, portal vein thrombosis, Budd-Chiari syndrome, and cerebral venous sinus thrombosis.

Usually, it is reported an incidence of thrombotic events of about 13% at, or prior to diagnosis, and around 11% over a median follow-up of 3-4 years[105,106]. In a study of 707 patients with PMF, fatal and nonfatal thromboses were diagnosed in 7.2% patients with a rate of 1.75% patient-years. The overall death rate due to cardiovascular events was low at 2%, accounting for 0.39 deaths per 100 patient-years. When the death from non-CV causes were considered as competing events, the estimated adjusted rate of major thrombotic events would have been 2.2% patient-years[66]. This is comparable to what is seen in Essential thrombocythemia, where the annual rate of fatal and non-fatal thrombosis was 1.9% patient-years in a series of 891 patients[107]. Notably, the rate of thrombosis in PMF could likely be obscured by other fatal and nonfatal non-CV competing events including transformation to acute leukemia. Indeed, a large Swedish population-based study reported increased 10-year probability of dying from cardiovascular and cerebrovascular diseases in young MPN (ET, PV, and PMF) patients aged 50 to 59 years (4.2% for cardiovascular disease vs. 2.1% for controls and 1.9% for cerebrovascular disease vs. 0.4% for controls), whereas no difference was observed in MPN patients versus controls aged 70 to 79 years (16.8 vs. 15.2% for cardiovascular disease and 5.6 vs. 5.2% for cerebrovascular)[108].

Venous events commonly occur in unusual sites in patients with MPN, including MF. In a series of 155 patients by Cervantes et al., out of 31 thromboembolic events, 6 (20%) were splanchnic vein thrombosis (SVT) and 1 was cerebral venous sinus thrombosis[106].

Thrombotic events are often the initial manifestation of PMF and MPN in general, or they may precede the disease diagnosis. Thrombosis are more frequent than bleeding episodes. In detail, thrombosis appears to be more common among Polycythemia Vera than Essential Thrombocythemia or Primary Myelofibrosis patients both at diagnosis[109] and during follow up[110] (Table 4). On the contrary, bleeding episodes occur primarily after the diagnosis of PMF has been established[111], while they are less frequent in Polycythemia Vera or Essential Thrombocythemia.

Disease	Molecular features	Main Phenotype	THROMBOSIS			BLEEDINGS	
			Incidence	Type	Clinical characteristics	Incidence	Clinical characteristics
PV	**JAK2 V617F (95%)** **JAK2 exon 12 (5%)** *Sub-clonal mutations in myeloid genes*	***Erythrocytosis.*** *It can be associated with leukocytosis and thrombocytosis.*	**- At diagnosis: 28.6%** **- During Follow up:** **3.8** x 100 person/year (1.5 deaths per 100 person/year)	Both arterial and venous	• **Mild microcirculatory disturbances** (*headache, itching, buzzing*) • **Major arterial and venous thrombotic events** (*ischemic stroke, peripheral artery disease, splanchnic vein thromboses, cerebral sinus thromboses, myocardial infarction, and deep vein thromboses*) • **Over-representation of thrombosis in unusual sites** (*portal system, Budd-Chiari syndrome, cerebral venous thrombosis*)	**3– 8%** *(usually **after the diagnosis**)*	- **Minor bleedings** (*e.g. ecchymoses, gingival hemorrhage, menorrhagia and epistaxis*) - **Major bleedings** (*e.g. intracranial hemorrhage, gastrointestinal bleeding, retroperitoneal bleeding*) - Extreme thrombocytosis may cause bleeding due to development of an acquired Von Willebrand syndrome - Main **cause of bleedings** are Portal hypertension with esophageal varices, the use of anti-platelet and/or anti-coagulant therapy
ET	**JAK2 V617F (60%)** **MPL exon 10 (5%)** **CALR exon 9 (20%)** **Triple negative (5-10%)** *Sub-clonal mutations in myeloid genes*	***Thrombocytosis.*** *Sometimes patients presented with normal white blood cell counts. A reduced red blood cell count could also be observed*	**- At diagnosis: 20.7%** **- During Follow up: 2-4** x 100 person/year	Mainly arterial		**3 - 18%** *(usually **after the diagnosis**)*	
MF	**JAK2 V617F (60%)** **MPL exon 10 (5%)** **CALR exon 9 (20%)** **Triple negative (5-10%)** *Sub-clonal mutations in myeloid genes. (ASXL1, DMT3A, EZH2, IDH1/IDH2, SRSF2, or TP53 are associated with a worse outcome)*	***Splenomegaly (85%); Cytopenia.*** - 2/3 of patients had anemia at diagnosis; - 40 to 50% had leukocytosis - 13-32% presented thrombocytosis	**- At diagnosis: 9.5%** **- During Follow up: 2.2** x 100 person/year	Both arterial and venous		**19 - 56%** *(~12% in patients with pre-fibrotic MF)*	

Table 4. Incidence and main clinical characteristics of vascular events in patients with PMF and other myeloproliferative neoplasms. PV=Polycythemia Vera; ET=Essential Thrombocythemia; MF=Myelofibrosis; Adapted from Farina et al., Haematologica 2021 [112]

On the other hand, also bleeding events in MF may affect survival outcomes and impact quality of life[113]. In addition, considering that a lot of MF patients presented with anemia, major bleeding episodes would, therefore, worsen pre-existing anemia and precipitate adverse outcomes. Indeed, hemorrhagic events can be fatal and are one common cause of death in PMF[13]. Bleeding events frequency seems to be higher in PMF than in ET or PV[114]. Life-threatening bleeding complications in patients with MF include variceal bleeding secondary to portal hypertension and intracranial bleeding, among others. Upper gastrointestinal bleeding was the most common cause of major bleeding among PMF patients[111]. Other major bleeding reported are intracranial hemorrhages[111], while minor bleeding manifestations in MF include ecchymosis, gingival hemorrhage, menorrhagia, and epistaxis.

1.2.2 Predisposing factors

Many features of a patient's demographics are predictive of PMF associated vascular complications[115–117] including patient age, prior vascular events, the grade of inflammatory state, and MPN-associated risk factors, such as degree of erythrocytosis, leukocytosis, and the presence of *JAK2 V617F*. Conversely, CALR mutations are associated with a reduced risk of thrombosis[110].

Considering the risk of thrombosis, only age greater than 60 years and a prior history of a thrombotic event were validated as thrombotic risk factors in MPN patients, while conflicting results have been reported for the other proposed predisposing factors[110,115,116]. Barbui et al. have shown that *JAK2 V617F* mutation and age over 60 years were the only risk factors for thrombosis in PMF, while

only a borderline association was found between leucocytosis and thrombosis[66]. Conversely, a systematic review by Lussana et al. showed a tendency towards an increased risk of thrombosis in PMF patients with JAK2 mutation, but it did not reach statistical significance for PMF, while it did for Essential Thrombocythemia [118]. On the contrary, the MD-Anderson patients analysis show that only a previous history of thrombotic events was the only predictive variable for thrombosis[105].

Notably, the conventional cardiovascular risk factor (i.e., hypertension, hyperlipidemia, diabetes and smoking) are additional variables associated with an increased rate of thrombosis.

An history of thrombosis prior to an PMF diagnosis may be attributed also to the presence in these patients of a clonal hematopoiesis of indeterminate potential (CHIP), involving JAK2 or CALR mutations prior to the development of a full blown Myeloproliferative neoplasms. Indeed, CHIP has been associated with an increased risk of coronary artery disease and stroke[119]. In particular, *JAK2 V617F+* CHIP has been most frequently associated with an increased risk of developing cardiovascular diseases, thrombosis and coronary heart disease[119]. Furthermore, Cordua et al.[120] have shown that subjects with *JAK2 V617F* or calreticulin CHIP frequently eventually develop a full blown MPN.

Considering the relationship between thrombosis and inflammation, inflammatory cytokines secreted by PMF cells, and leukocytes-derived proteases, damage the integrity of the normal vascular endothelium, leading to the acquisition of a pro-thrombotic phenotype in PMF patients[112]. Specifically, endothelial cells overexpress adhesion receptors favoring the attachment of platelets, erythrocytes, and leukocytes to the vascular wall.

Risk factors for developing hemorrhagic events are less well understood. There have been limited studies dedicated to evaluating the hemorrhagic complications of PMF. Moreover, analyses have been heterogenous in terms of number of patients, subtypes of PMF, and duration of follow-up. In conclusion, these studies failed to show a correlation between bleeding risk and leukocyte count at events presentation, as well as for the platelet count at presentation, the use of platelets aggregation inhibitors, the presence of *JAK2 V617F* mutation, gender, or prior bleeding history. One study reported the association between bleeding and older age at diagnosis[121]. However, a retrospective analysis by Wehmeier et al. of MPN patients reported that elevated platelet count and patient age were not risk factors of bleeding[122].

1.3 Endothelial cells and JAK2

1.3.1 Endothelial cells

In the 1800s, von Reckingausen established that vessels were not merely tunnels bored through tissues but were lined by cells. Subsequently, Starling's experiments and his law of capillary exchange proposed in 1896 supported the theory that the endothelium was principally a selective but static physical barrier, in contrast with Heidenhahn's description in 1891 of the endothelia as an active secretory cell system. Only more than 50 years later, in 1953, Palade could study the vessel wall with the electron microscopic and, few years later, Gowan described the interaction between lymphocytes and endothelium of post-capillary venules. All these discoveries stimulated numerous subsequent studies, that led to the current view of the endothelium as a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunologic functions[123].

The endothelial cell (EC) surface in an adult human is composed of approximately 1 to $63x10^{13}$ cells, weighs approximately 1 kg, and covers a surface area of approximately 1 to 7 m^2.[124]

The endothelium plays a pivotal role in regulating blood flow and to generate an active antithrombotic surface that facilitates transit of plasma and cellular constituents throughout the vasculature. Inflammation or high hydrodynamic shear stress affects endothelial cells activity, generating a prothrombotic and antifibrinolytic microenvironment.

Starting from the 1970s studies on endothelial function became more feasible thanks to the development of techniques for culturing endothelial cells in vitro[125–127]. Nowadays, we know that ECs lay the inner face of blood vessels, acting as gatekeepers controlling the passage of solutes, molecules and cells through blood vessels. This fine regulation is fulfilled by a trans-cellular system of transport vesicles[128] and by cell-to-cell specialized structures called junctions, which connect ECs one to each other[129] and are composed by transmembrane molecules. The latter are linked with intracellular molecules that, in turn, mediate anchorage to actin cytoskeleton thus stabilizing the entire junction[130]. Juctions were used by ECs also to communicate cell position, inhibit cell growth and apoptosis, control permeability and maintain apical-basal polarity to finally regulate vascular homeostasis

According to the specificity and functions of the organ where they are expressed, EC phenotype varies not only in terms of junction composition but also in terms of cell morphology, gene expression, antigen composition and functions. In summary, the endothelium is able to modify its structure and function according to the local tissues, adapting to different microenvironments in order to balance the physiological requirements of the tissues and the need of self-survival[131].

1.3.2 Assays for studying endothelium

A growing number of assays have been utilized to study endothelial cells or their progenitors. Below is a summary of the main currently used assay for studying the endothelium.

Endothelial Progenitors Cells

Which is the cell of origin of endothelial cells is still a matter of debate. Endothelial progenitor cells (EPCs) originated in bone marrow and then circulate in the peripheral blood[132–134]. EPCs are considered cells which have the capacity to proliferate, migrate, and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature endothelial cells.

EPCs are very rare peripheral blood cells (0.0001% of circulating nucleated cells)[132]. In both animal models and humans, they have been reported to play a role in vascular repair and neo-angiogenesis[132]. Asahara et al[132] initially reported the isolation of a putative EPC from human peripheral blood, on the basis of cell surface expression of CD34 and Flk-1 (receptor for vascular endothelial growth factor 2, VEGF2), as well as for their capability to generate *de novo* blood vessels. Subsequently, Urbich and Dimmeler[135] defined EPC as progenitors of endothelium that were capable of clonal expansion with stem cell like characteristics and had the capacity to differentiate into endothelial cells. Since these initial observations, there has been a great deal of debate concerning the definition and characterization of these progenitor cells. In addition, a variety of methods have been used to detect and characterize EPC which has led to disparate results[136].

Three main approaches have been used to identify and isolate EPCs (Table 5 and 6):

(1) One approach is based on the detection of surface antigen on circulating cells by Flow Cytometry (Table 5). Usually, the main markers used to identify EPC are the following: CD34, VEGFR2 (human KDR and mouse Flk-1) and CD133[137]. Unfortunately, they do not unequivocally identify EPC[137]. This approach allows to distinguish EPCs from mature circulating endothelial cells (CEC), since CD133 is a stem cell marker expressed by EPC, but not by mature endothelial cells[138].

(2) Another approach consists in plating human peripheral blood or cord blood low density mononuclear cells in culture dishes coated with fibronectin in a commercially available culture medium rich in endothelial cells growth factors and fetal calf serum[139]. After 4-5 days the non-adherent cells are removed, and the adherent cells are examined for their ability to bind acetylated low-density lipoprotein (AcLDL) and Ulex europaeus agglutinin 1 (UEA-1, a plant lectin). The putative EPCs identified are called circulating angiogenic cells (CAC). However, these markers lack specificity (numerous blood cells express the integrin receptors for fibronectin)[140] and these cells fail in forming

endothelial cells colonies in vitro[141]. EPCs identified with this method are thought to contribute in neoangiogenesis by secreting angiogenic factors (paracrine route)[141].

(3) A third method to identify EPC is based on the in vitro colony forming capacity of cultured CD34+ cells. Two classes of EPC have been described in this way, which are termed "colony forming unit-endothelial cells" (CFU-EC) and "endothelial colony-forming cell" (ECFC), respectively. CFU-EC are assayed by in plating CD34+ cells for 48h in fibronectin-coated dishes and then replating the non-adherent cells and monitoring for the emergence of the EPC-derived colonies. However, CFU-EC fail to display any postnatal vasculogenic activity and are thought ultimately be the cellular progeny of myeloid cells[140]. Since this assay includes the adhesion of mononuclear cells in vitro, this approach may select for monocytes, expressing "endothelial-specific" markers[142]. Conversely, ECFC assay allow to identify cells able to generate large colonies of human CD45− cells after 1–3 weeks of incubation[140]. The cells within these colonies are thought to be of endothelial origin because of their morphology, which resembles the ones of EC; the expression of EPC/EC-related markers (CD31, CD105, CD144, CD146, vWF, and KDR)[143], and the ability to spontaneously generate human blood vessel tubes *in vitro*[144] and *in vivo* (postnatal vasculogenesis)[145]. The ability of ECFCs to display spontaneous vasculogenic properties and to remodel into arteries and veins in vivo allow to distinguish ECFCs from all other endothelial cells precursor previously described[140]. ECFCs are likely the cell population that represents a true lineage restricted EC progenitor cell.

	CAC	EPC CFU-EC	ECFC	CEC
Immunophenotype	CD34+/- * VEGFR2+ * CD133+ CD31+ CD146- CD45+/-	CD34+ * VEGFR2+ * CD133+ CD31+ CD146- CD45+/-	CD34+ * VEGFR2+ * CD133+ CD31+ CD146- CD45-	CD34+ * VEGFR2+ * CD133- CD31+ CD146+ CD45-
Origin	BM	BM	EC?/BM?	EC
Proliferative capacity	-	-	+	-/-/+
Replating ability	-	-	+	-/+
In vitro tube formation	+/-	+/-	+	-
In vivo de novo formation	-	-	+	-
Paracrine augmentation of angiogenesis	+	+	+/-	NA
Phagocytosis of bacteria	+	+	-	-

Table 5. Biological characteristics and immunophenotype of EPCs and CECs. The main differences between Endothelial Precursors Cells (EPC) and Circulating Endothelial Cells (CEC) are shown in red. **In*

common with hematopoietic stem cells. CAC= circulating angiogenic cells; CFU-EC=colony forming unit–Endothelial Cells; ECFC= endothelial colony-forming cell; VEGFR2=vascular endothelial growth factor receptor 2; EC=endothelial cells; BM=bone marrow; NA=not applicable. From Farina et al., Haematologica 2021 [112]

Abbreviation	Definition
EPC = endothelial progenitor cell	Endothelial progenitors that differentiate into endothelial cells and may become part of the newly formed vessel wall or favor angiogenesis by secretion of pro-angiogenic factors (paracrine effect). There are several *ex vivo* assays for EPC.
ECFC = endothelial colony-forming cells	Among the EPC, ECFC originate from peripheral blood mononuclear cells and are able to form large colonies of human $CD45^-$ cells after 1–3 weeks of incubation (once called late outgrowth endothelial cells, OEC), which have phenotypic and functional properties of endothelial cells. Indeed, they are able to generate new vessels *in vivo* and to generate endothelial colonies *ex vivo*, and are now considered the true precursor cells of endothelial cells.
CFU-EC = colony-forming unit-endothelial cells	These are assayed by plating $CD34^+$ cells for 48 h in fibronectin-coated dishes and then replating the non-adherent cells and monitoring for the emergence of the EPC-derived colonies. Because of the brief period of incubation *ex vivo* they were once called early outgrowth endothelial cells, (EOC). They were initially included as endothelial precursors, but they do not possess any postnatal vasculogenic activity and, therefore, are no longer considered true EPC.
CAC = circulating angiogenic cells	Bone marrow-derived immune cell populations (T cells and certain subsets of monocytes) that stimulate vascular regeneration and angiogenesis through a paracrine mechanism.
CEC = circulating endothelial cells	Mature endothelial cells circulating in the peripheral blood, which are shed from vessel walls as a result of pathophysiological conditions that affect the endothelium.
ELC = endothelial-like cells	Monocytes that closely resemble endothelial cells and acquire endothelial cell surface markers.

Table 6. Main abbreviations referring to endothelial progenitor cells and mature endothelial cells, and brief definitions of the types of cells. From Farina et al., Haematologica 2021[112]

Circulating Endothelial Cells

The endothelial contribution to human disease development cannot be inferred in vivo, because vascular tissue cannot be accessed non-invasively and because there are few specific markers. To solve this problem, some researchers started to study circulating endothelial cells (CEC), which are mature differentiated endothelial cells, that are shed from vessel walls as a result of pathophysiologic turn-over of endothelial liar [146].

CEC were first identified in the 1970s although more user-friendly techniques to isolate CECs have only recently become available[147]. Endothelial cells constitute an active system, which fits to local specific stimuli, as proinflammatory cytokines, growth factors, infectious agents, lipoproteins, and oxidative stress. However, prolonged or exaggerated endothelial activation by these environmental stress leads to dysfunction and to irreversible loss of endothelium integrity with cell detachment, apoptosis and necrosis, resulting in an increased release of CECs in peripheral blood[147] (Figure 3).

In healthy individuals, the endothelial layer lining blood vessels is continuously renewed at a low replication rate of 0–1% per day[148], since normal laminar flow suppresses endothelial cells apoptosis. Thus, the detection of CECs in a healthy adult is a rare event, and as few as 0–10 CECs/mL (by immunomagnetic separation) are seen in healthy donors[149]. On the contrary, elevated levels of CECs have been reported in various pathologic situations, including cardiovascular disorders [150–153], infectious diseases[154–157], immune disorders[158–160], diabetes, chronic kidney

disease[161,162], post-transplantation [163–165] and cancer [166–168]. Several pioneering studies showed that CEC elevations could be associated also with tumor stage, tumor characteristics and prognosis[169–172], and to monitor response to therapy[168]. In addition, Circulating endothelial cells have been proposed to be a non-invasive marker of angiogenesis[166,173,174]. Recently, CECs were also reported as a reliable marker of endothelial damage in patients undergoing hematopoietic stem cell transplantation[165]. Therefore, both in neoplastic and inflammatory diseases, the number of CECs in peripheral blood is increased and the greater percentage of cells have an activated phenotype with abnormal expression of pro-adhesive and procoagulant molecules[166,174–176].

In contrast to EPC, which are a proposed marker of regeneration and vessel proliferation, CECs serve as a marker of endothelial damage/dysfunction and reflects a pro-thrombotic tendency[163].

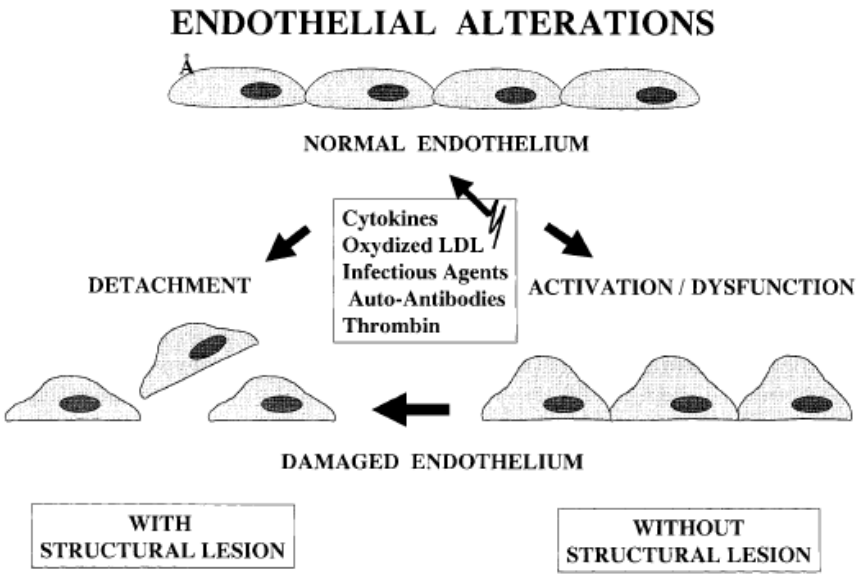


Figure 3: Schematic representation of endothelial alterations from Dignat-Gorge et al[147]

Initially, CECs were first identified using morphologic criteria. Subsequently, objective methods to identify CECs with the application of immunofluorescence, and the use of antibodies against various endothelial cells markers, were introduced. Indded, the two main methods of quantification of CECs are based on the immunobead and flow cytometry methods, which – until recently – have been mutually exclusive. For both, the detection of CECs ultimately depends on the availability of specific endothelial markers. However, the endothelium is a highly dynamic structure, closely involved in haemostasis, inflammation, regulation of vascular tonus, and angiogenesis. In all these different state, the endothelium may express different superficial markers[177]. Therefore, for enumeration of the total CEC number, and not a particular subpopulation, it is necessary to identify markers that are specific for and are constantly expressed by all CEC. For decades, no

consensus was reached on reliable endothelial cell specific markers[178]. Recently a consensus definition of CECs has been reached[52], which defines CECs as large (> 10 μm in length) CD146+ cells. CD146 (MUC18) is expressed by circulating endothelial cells, but not by monocytes, granulocytes, platelets, megakaryocytes, T or B lymphocytes[53]. It is of note that the expression of CD146 on endothelial cells is prominent, in contrast to the lower antigen density on T lymphocytes (from 10-fold and up to 1000-fold)[179], giving rise to the concept of CD146bright and $CD146^{dim}$/$CD146^{low}$ cells.

One of the most widely used method to isolate CEC is CD146 immunomagnetic separation, which use magnetic beads coupled to a monoclonal antibody (mAb) targeting CD146, as first described by Dignat-George[180]. Immunomagnetic separation, originally developed to detect rare events in peripheral blood, has been long used for CEC quantification, and it is reported as one of the preferable method in a recent guideline[181]. Since endothelial cells are characterized by strong expression of CD146, they would attract more immunomagnetic beads than CD146low T lymphocytes, and, therefore, CECs would have a better chance of being separated out by a magnet. This approach has been widely applied to detect CECs in different clinical situations [182]. The endothelial origin of CD146-positive cells isolated by immunomagnetic selection in peripheral blood was validated by staining with other endothelial markers (UEA-1, von Willebrand factor, CD31, etc.) and by the demonstration of Weibel–Palade bodies[181]. CD146-based immunomagnetic isolation has some limits for the use in clinical practice. Indeed, the technique is time-consuming and operator-dependent for the variable cell morphology and characteristic (necrotic, apoptotic, or viable).

Flow Cytometry is the other well-recognized method for CECs selection [146,183]. A battery of markers are now used to identify cells of endothelial origin, including CD31, CD105, CD141[184]. The use of multiple targets allow to better discriminate CECs from other cell populations (e.g. lymphocytes or immature endothelial progenitors). Notably, the absence of CD133 may also be used to distinguish CECs from EPCs[185]. In addition, Flow Cytometry allows to evaluate the expression of functional markers, such as E-selectin, intracellular adhesion molecule-1, and vascular cell adhesion molecule-1. However, flow cytometric enumeration of CECs is itself far from being a standardized technique, as results from various studies with a high degree of variability among centers[183,186]. In addition, Flow cytometry assays in whole blood are at risk to overestimate CEC by enumerating false-positive cells. Indeed, the normal range for the immunomagnetic approach does not exceed 10 cells mL/1 of blood, while the number of CECs reported according to different flow cytometric protocols may vary by thousands[182]. One possible explanation for this is the superior sensitivity of flow cytometry for the detection of cells with low expression of CD146 (i.e. CD146dim). At the same time, it cannot be excluded that higher levels of CECs may derive from false positives.

Notably, in 2008, Widemann et al[146] reported a new hybrid assay that incorporated an algorithm combining immunomagnetic selection of CD146+ cells with flow cytometric quantification. In parallel, Terstappen[187] developed a semi-automatic method for the detection of CECs, also using a combination of iron microbeads and monoclonal antibodies. Terstappen's method uses CD105 and a nuclear stain (DAPI) in addition to CD146. The reason for choosing a semi-automatic method was the attempt to overcome the standardization problems of the traditional methods used up to then (i.e., immunomagnetic separation and flow cytometry detection). In fact, the lack of standardized assay methods, the lack of consensus on the definition of a CEC[174,188,189] and disease heterogeneity have led to a wide variation in the reported range of CECs in the literature (1–5,700 per mL). These assays allow to overcome the lack of standardization and the variability in CECs detection associated with the methods previously described. Moreover, the true endothelial nature of the CECs obtained used this technology was confirmed by gene expression profiling studies[190].

1.3.3 Endothelium involvement in PMF

A significant increase in marrow and splenic micro-vascular density (MVD)[191] has been reported in Myeloproliferative neoplasms, particularly in PMF[192,193]. Moreover, massive neo-angiogenesis in marrow[193,194] and spleen[195] is a hallmark of PMF. Whether neo-angiogenesis in PMF is an epiphenomena of the PMF pro-inflammatory milieu or primarily associated with the pathophysiology of this myeloproliferative diseases, due to endothelial cells dysregulation by the same pathogenic mechanism causing hematopoietic cell proliferation[196], remains controversial. Notably, these two mechanisms are not mutually exclusive and could be operating in concert.

In addition, PMF patients shown increased serum levels of proangiogenic factors, such as vascular endothelial growth factor (VEGF) [197] and FGF-2[198]. It has been suggested that autocrine and paracrine signaling pathways lead to increased levels of VEGF which may not only contribute to accelerated hematopoietic cell growth but also act as an important contributor to the PMF associated thrombotic risk[199].

The increased marrow and splenic micro-vessel density and neoangiogenesis, together with the high incidence of vascular complications, has led some authors to hypothesizes direct involvement of endothelial cells by the malignant process in the PMF. Notably, PMF patients presented an average percentage of Endothelial precursors cells (EPC), which is significantly higher than in the healthy controls and in other MPNs[199]. Moreover, the number of circulating EPCs directly correlates with the phase of the disease. Indeed higher levels are associated with an early PMF stage[199]. So far, the cause of this massive mobilization from bone marrow to the peripheral circulation is still not yet known. However, a strong pro-inflammatory

microenvironment, enriched in TGF-β, PDGF, G-CSF in the BM of patients with myelofibrosis, is supposed to promote exaggerated proliferation and mobilization of endothelial progenitors into the bloodstream and to extramedullary sites as spleen and liver. In addition, CEC levels are increased in MPN patients, regardless of their driver mutational status[200], highlighting the involvement of endothelium in these chronic hematological neoplasms.

To better investigate the role of endothelial cells in myelofibrosis some authors decided to research the *JAK2 V617F* PMF driver mutation in endothelial cells and their precursors, since endothelium cannot be easily sampled from patients due to ethical reasons.

1.3.4 Endothelial cells and Jak2 mutations

In endothelial cells, JAK2 physiologically regulates vascular smooth muscle cells growth, vascular tone balance and integrity, playing a crucial role in maintaining endothelial-vascular homeostasis[201]. Moreover, JAK2 deficiency is reported to significantly inhibit endothelium-dependent response to vasodilators, decrease endothelial angiogenic function, and to reduce post-perfusion recovery after hindlimb ischemic injury, both in vitro and animal studies[201]. Furthermore, previous studies revealed that abnormal JAK2 activation caused by hyperglycemia is detrimental to endothelial function[202], and JAK2 is indispensable for angiotensin II-induced hypertension[203,204].

A classic concept in tumor angiogenesis is that the blood vessels in tumor contain stable and genetically normal endothelial cells, while the tumor cells characteristically exhibit genetic instability and may have different genomic alterations. However, recent studies, have changed this paradigm through various observations, showing that, in some cases, endothelial cells derived from the tumor itself and that the "tumor-derived endothelial cells" harbored the same genetic alterations of malignant cells from which they had been originated. This has been proven in some hematologic malignancies, such as in chronic myeloid leukemia[205,206], in lymphomas[207], in chronic lymphoblastic leukemia[208], in myelodysplastic syndromes[209], in multiple myeloma[176]; and solid tumors, such as neuroblastoma[210], the glioblastoma[211,212] and melanoma[213].

Considering the fundamental role of *JAK2 V617F* in MPN, and he high frequency of vascular complications in MPN patients, some authors have tried to identify the *JAK2 V617F* mutation also in endothelial cell (Figure 4).

Since the difficulties in analyzing endothelium, authors have tried firstly to detect the *JAK2 V617F* mutation on circulating endothelial precursors cells (EPC). However, the results were in contrast. Indeed, assayable MPN CFU-ECs (Yoder et al.[137], Piaggio et al.[214], Soxer et al.[215]) were first shown to be JAK2+, while ECFCs from these same patients were found to be JAK2V617F-. Only 3% of the ECFC colonies analyzed by Yoder were JAK2V617F+. Interestingly, these mutated-ECFC were

derived from the same patient, who presented with a thrombotic event and only later developed classic hematologic signs of Polycythemia Vera. Notably, increased numbers of both CFU-ECs[196,199,215] and ECFC[216] have been found in the blood of patients with MPN, regardless their mutational status. The absence of the JAK2 mutation in ECFC from MPN patients was recently confirmed by Guy and colleagues [217]. At variance with these observations, Teofili et al have shown that ECFCs from patients with myeloproliferative disorders can carry the *JAK2 V617F* mutation[218] (Figure 4). Almost half of the MPN patients studied were reported to have MPN like genetic abnormalities in their ECFCs, including either SOCS gene hypermethylation or the presence of *JAK2 V617F*. Notably, mutated ECFCs were detected only in patients with a history of thrombotic events[218]. Moreover, the presence of JAK2 mutation or other evidence of clonality in ECFCs was associated with JAK/STAT pathway activation and significantly greater adhesion of mononuclear cells to mutated ECs than normal E-CFCs[218].

In 2009 Sozer et al., for the first time, reported that mature endothelial cells captured by laser microdissection from the lumen of hepatic venules harbored the *JAK2 V617F* mutation in three Budd-Chiari syndrome patients, a disease characterized by the occlusion of the hepatic veins. Rosti et sl. further confirmed the presence of *JAK2 V617F* in micro-laser dissected ECs from the splenic vein in MPN patients [219].

For some authors[220], all this studies explored the hypothesis that the oncogenic lesion could hit a common endothelial and hematopoietic progenitor cell, the so-called "hemangioblast", which results in mutated EC and myeloid cells in a subpopulation of patients with MPNs. The mutated endothelial cells would, therefore, induce endothelium dysfunction and be primarily responsible for the pathogenesis of the vascular damage (for more detail, please see the next section 1.3.6 on this topic).

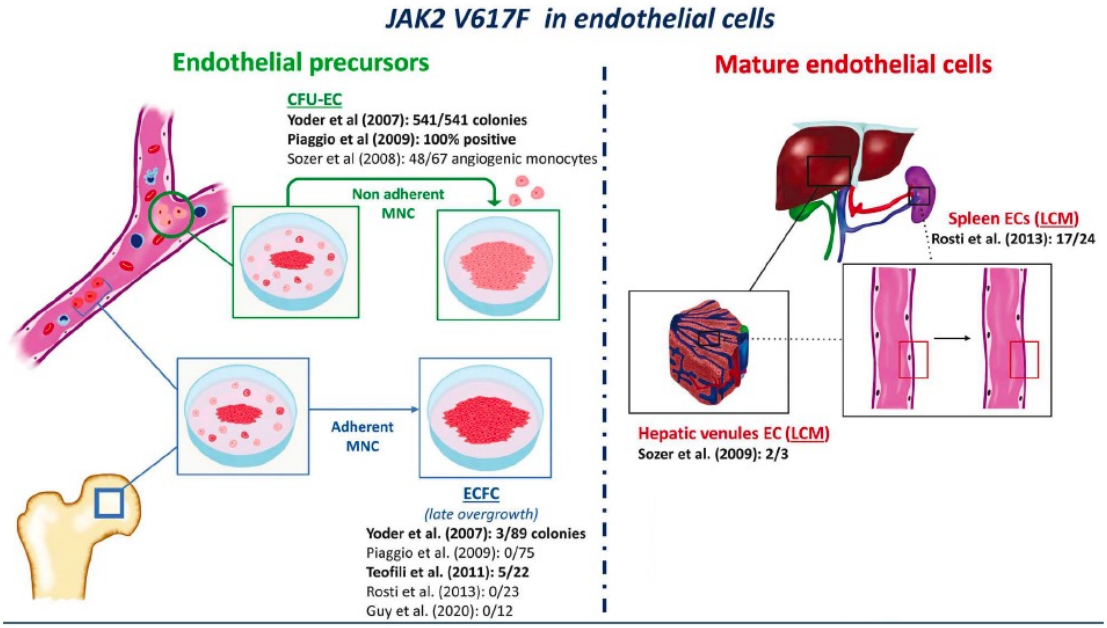


Figure 4: Evidence for JAK2 mutated endothelial cells in patients with myeloproliferative neoplasms. JAK2 V617F has been detected in both endothelial progenitors and mature endothelial cells. Studies in

which a JAK2 mutation has been detected in endothelial progenitor cells or mature endothelial cells are shown in bold. Positive colonies or patients are expressed on the total number of colonies or patients analyzed. CFU-EC: colony forming unit-endothelial cells, derived from nonadherent mononuclear cell culture (see the text); MNC: mononuclear cells; EC: endothelial cells; ECFC: endothelial colony-forming cells, derived from long-term adherent MNC culture (see the text); LCM: laser-capture microdissection; CEC: circulating endothelial cells; adapted from Farina et al., Haematologica 2021[112].

1.3.5 Effects of the JAK2 V617F mutation expression in Endothelial cells on MPN hematopoiesis and vascular complications

The evidence of *JAK2 V617F* mutation in human endothelial cells stimulated several studies to explore the role of this mutation in endothelial cells using both in vitro and animal models.

Etheridge et al. firsts described the critical role of *JAK2 V617F* mutated ECs in the development of the bleeding abnormalities in murine models[221]. They used FF1 transgenic mice to express *JAK2 V617F* in different cell lineages. Specifically, in their model the JAK2 mutation was exclusively expressed in endothelial cells, resulting in dysfunctional hemostasis in response to injury, resembling the bleeding diathesis observed in MPN patients[221]. One of the potential mechanisms proposed by Etheridge and colleagues was related to von Willebrand Factor (vWF) regulation.

More recently, using both an "in vitro" model of human *JAK2 V617F* mutated ECs and an "in vivo" model of mice with endothelial-specific *JAK2 V617F* expression, Guy et al[222] wanted to evaluate whether vascular EC expression of *JAK2 V617F* is sufficient to promote a pro-thrombotic state or not. With their models, they have shown that *JAK2 V617F*+ ECs, in the absence of similarly mutated hematopoietic cells, had a higher thrombotic rate due to a pro-adhesive phenotype, as a result of increased endothelial P-selectin exposure, secondary to degranulation of Weibel-Palade bodies[222]. Interestingly, these mice displayed a higher propensity for thrombosis, despite having normal blood counts and normal rates of thrombin generation[222]. By contrast, they presented with EC characterized by increased surface expression of P-selectin and von Willebrand factor (VWF), both of which are contained within Weibel-Palade bodies. Moreover, this thrombotic tendency was accentuated by the creation of a pro-inflammatory milieu due to the administration of low doses of tumor necrosis factor alpha[222]. The pro-adhesive properties of the *JAK2 V617F* mutated ECs were reversed by treatment with either a P-selectin blocking antibody or hydroxyurea[222].

In addition, Poisson et al showed an increased degree of arterial contraction in mice with *JAK2 V617F*+ Hematopoietic stem cells and endothelial cells in response to agents that promote vasoconstriction[223]. Furthermore, Castiglione et al[224] have reported in a murine model of MPN that when *JAK2 V617F* was expressed by both hematopoietic and ECs, the mice developed an MPN phenotype as well as a spontaneous age-related dilated cardiomyopathy with an increased risk of sudden death, as well as a pro-thrombotic and vasculopathic phenotype. By contrast, mice expressing solely *JAK2 V617F* in blood cells did not demonstrate any evidence of

cardiac dysfunction or thrombosis, suggesting that the expression of the MPN driver mutation in ECs is required for the development of the cardiovascular disease phenotype. In addition, the authors demonstrated that the *JAK2 V617F*$^{+}$ ECs was associated with the development of a pro-inflammatory milieu. Finally, JAK2 mutated ECs have been reported to respond to shear flow in a different manner than wild-type ECs, leading to upregulation of EC adhesion molecules (PECAM and E-selectin).

Guadall et al[225] have provided additional evidence that *JAK2 V617F*+ ECs possess pro-thrombotic properties. Using *JAK2 V617F*+ and JAK2 wild-type-induced pluripotent stem (iPS) cells generated from an MPN patient and redirecting these iPS cells towards the endothelial lineage, the authors observed that *JAK2 V617F*+ ECs had an increased proliferative capacity when compared with Wild-type ECs. In addition, the numbers and fluorescence intensity of Weibel–Palade bodies as well as expression of vWF and P-selectin were significantly greater which was accompanied by accumulation of P-selectin at the cell surface of *JAK2 V617F*+ ECs as compared to wild-type ECs. The transcriptomic profile of these mutated cells revealed over-expression of transcripts for genes that were involved in inflammation and cell adhesion, extracellular matrix regulation, the generation of glycoproteins, and a variety of processes that are involved in venous stenosis and thrombosis.

Furthermore, some studies explored the role of JAK2 mutated endothelial cells on hematopoiesis and myeloproliferative neoplasms development. *JAK2 V617F*-bearing endothelial cells have been reported to promote the proliferation of *JAK2 V617F* hematopoietic stem and progenitors cells (HSPC) over the JAK2-wild type HSPC in vitro, likely through a critical role of the TPO/MPL signaling axis[226]. Subsequently, Zhan et al[227] confirm “in vivo” the evidence that the *JAK2 V617F*-bearing vascular niche promotes *JAK2 V617F* clonal expansion, while inhibiting WT hematopoiesis. This data support the previous reports showing that the bone marrow microenvironment in myeloid malignancies may impair normal hematopoiesis and instead promote malignant stem cells expansion[228,229]. In addition, Zhang has shown how the JAK2 V617F-mutant HSPC transplanted in a WT recipient mice are either insufficient to develop a MPN phenotype in the absence of additional disease-promoting mechanism, as for example a JAK2 V617F-mutated bearing vascular niche, or require a longer period of time to develop the disease phenotype in WT environment than in mutant environment[227]. Therefore, in this model MPN myeloproliferation requires *JAK2 V617F* expression by both HSPCs and ECs. However, there are evidence in mouse models indicating the presence of the JAK2 mutation in HSPCs alone is sufficient to induce an MPN[54].Another study exploring the impact of *JAK2 V617F* mutation in endothelial cells reported as JAK2 mutated HSPC are protected by *JAK2 V617F*-bearing vascular niche from the otherwise lethal irradiation administered during conditioning for bone marrow transplantation[230].

All these discoveries emphasize the relationship between endothelial and hematopoietic cells in MPNs. In particular, the presence of *JAK2 V617F* MPN driver

mutation in endothelial cells may have a role favoring both clonal hematopoiesis and vascular complications. In addition, all these results support the ancient theory that ECs and hematopoietic cells could derive from a common precursor, called "hemangioblast".

Effects of JAK2 V617F expression in Endothelial Cells

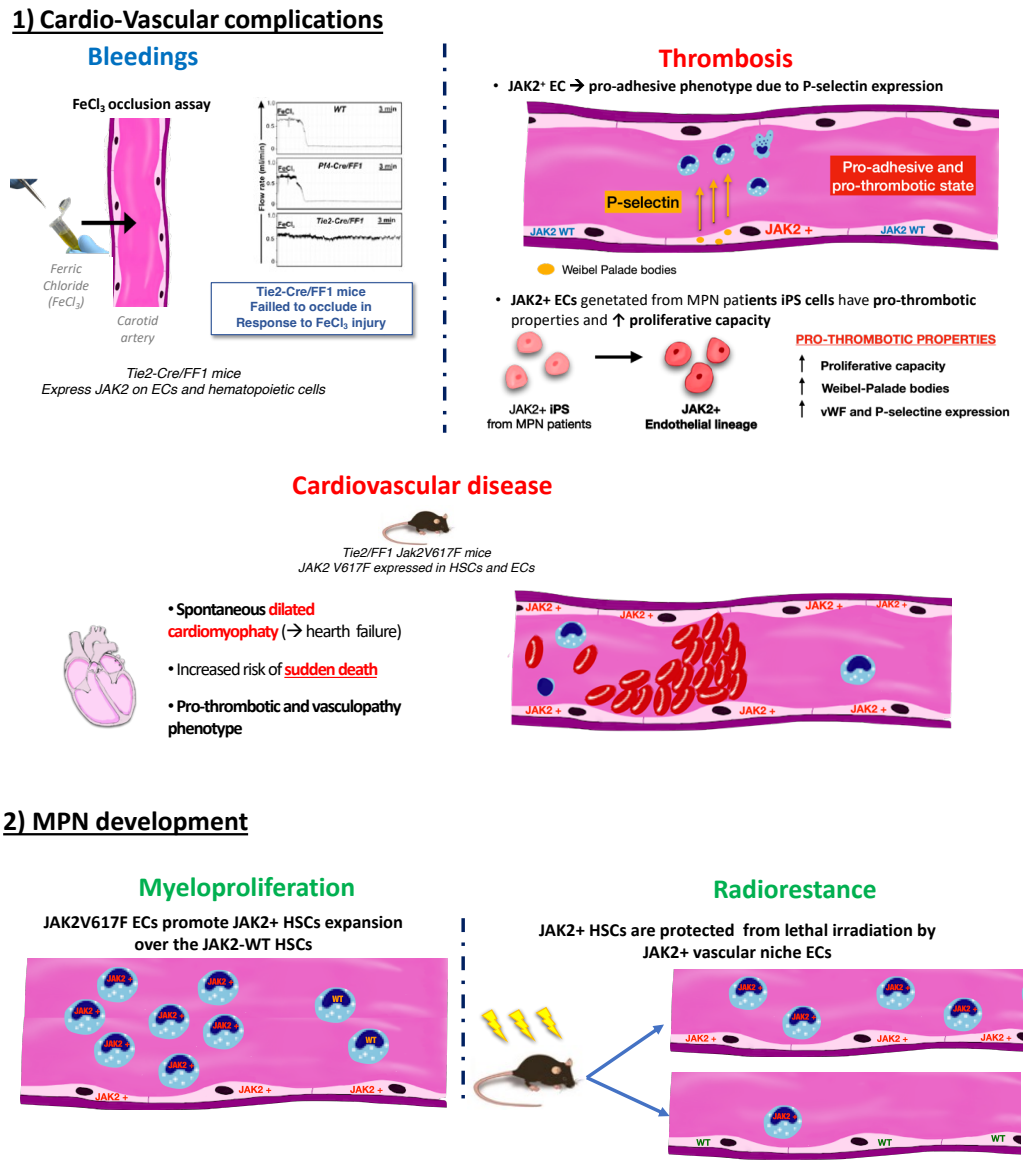


Figure 5: Effects of JAK2V617F expression in endothelial cells. The presence of the *JAK2 V617F* mutation in endothelial cells (EC) has an impact on both (1) vascular complications and (2) the development of myeloproliferative neoplasms (MPN). Specifically, it affects bleeding[221] (the carotid arteries of Tie2-Cre/FF1 mice expressing JAK2 mutations on both EC and hematopoietic stem cells (HSC) failed to occlude in response to ferric chloride, which normally induces occlusive thrombosis in murine carotid arterial); thrombosis (both in a mice model, due to enhanced P-selectin expression[222], and in an in vitro model of induced pluripotent stem cells[225]) and cardiovascular disease (mice expressing *JAK2 V617F* had spontaneously dilated cardiomyopathy and an increased risk of sudden death[224]). Finally, JAK2-mutated EC affect MPN development, promoting JAK2 HSC expansion[226], and radio-resistance[230]. FeCl3: ferric chloride; EC: endothelial cells; MPN: myeloproliferative neoplasms; iPS: induced pluripotent stem cells; HSC: hematopoietic stem cells; WT: wild type. From Farina et al. Haematologica 2021[112]

1.3.6 The hemangioblast

During embryonic development, hematopoietic stem cells and endothelial cells derive both form the mesodermal germ-cell layer; but exactly how is debatable. Several authors proposed that both hematopoietic and endothelial arise from a mesoderm-derived common precursor called "hemangioblast". The term ''hemangioblast'' was initially coined by Murray in 1932[231] and referred to a mass of cells derived from the primitive streak mesoderm that contain both endothelium and blood cells. This was meant to complement and contrast the term ''angioblast,'' which was thought to be the source of vessels and endothelium[232]. However, the hemangioblast, as originally described by Murray, was not a clonal mesoderm precursor giving rise to both blood and endothelium. Only in the late 1990's, the concept of the hemangioblast as a common clonal precursor was developed, deriving from the observations that single mesodermal cells isolated from in vitro differentiating mouse ESCs could give rise to both blood cells and endothelium[233,234]. That was possible through the development of the BL-CFC in vitro assay. This assay allows clonal (single-cell) analysis of blast colony-forming cells (BL-CFCs), which are derived from differentiating mouse embryonic stem (ES) cells[233].

Interestingly, in many species HSPCs appear as clusters attached to the endothelium that lines the ventral wall of the abdominal aorta during embryonic development; this observation has long implicated the endothelium as the source of developing blood cells. Indeed, when endothelial cells isolated from mouse embryos are grown in culture, a subset has the potential to develop into mature blood cells such as erythroid, myeloid and/or lymphoid cells[234]. Lineage-tracing markers in mice have identified that definitive HSPCs arise in the aorta-gonad-mesonephric region of embryos from hemogenic endothelium which gives rise, by asymmetric division, to resident ECs and HSPCs which are released into the blood and subsequently colonize the liver[235]. The Peault laboratory subsequently described the presence of definitive HSPCs in the aorta-gonad-mesonephric region of human embryos that were capable of colonizing adult xenografts and reported that definitive HSPCs were derived from hemogenic endothelium that resemble those observed in mouse embryos[236]. More recently, the relationship between HSPCs and hemogenic endothelium has been clarified[237]. The authors proposed that hematopoietic stem cells form a subset of early endothelial cells known as hemogenic endothelium[237–239]. This concept is based on continuous single cell imaging which indicated that freely moving cells expressing blood-specific markers (CD45, CD41, CD11b) were generated from ECs expressing vascular endothelial cadherin (VE-cadherin, also known as Cdh5)[240].

Moreover, the reports discussed above showing that the *JAK2 V617F* driver mutation[137,214,218,219,241,242] may be present in both hematopoietic cells and ECs in MPN patients have reinforced the evidence, supporting the existence of a

common precursor cell for both EC and hematopoietic cells. In addition, some authors have recently provided evidence that *JAK2 V617F* may be acquired in utero[243] or during childhood[244] by MPN patients where *JAK2 V617F* was the only or the first driver mutation. This data supports that the acquisition of *JAK2 V617F* in MPN patients can occur in utero and is at least chronologically consistent with involvement of "hemangioblast" by MPN driver mutations. Since the period when ECs are hemogenic may be very brief and occurs very early during embryogenesis, the "hemangioblast" may acquire the MPN driver mutation in a limited group of patients. These assumptions would support the observation that not all *JAK2 V617F* MPN patients possess mutated ECs[112].

Some authors have reported that monocytes isolated from MPN patients resemble endothelial like cells (ELC), accounting for the detection of MPN driver mutations in endothelial and hematopoietic cells (figure 6). Leibundgut et al.[196] initially reported that CD14+ monocytes were capable of generating JAK2V617F+ ECs in vitro. Subsequently, Sozer and colleagues[245] showed that also human CD34+ cells were capable of generating normal and JAK2V617F+ ELCs in vivo. These reports suggest that JAK2 mutated-CD34+ cells and CD14+ monocytes (both elevated in MPN) may both transform to JAK2V617F+ ELC. These observations have led to considerable confusion, suggesting to some investigators that monocytes can transition to EPC[141] and then acquire an endothelial like phenotype. However, a more plausible hypothesis is that monocytes can serve as circulating regulators of the angiogenic response and play a crucial role in neoangiogenesis during wound healing, tissue ischemia, and tumorigenesis by secreting pro-angiogenic factors rather than by directly participating in neo-vessel formation or endothelial turnover[246,247].

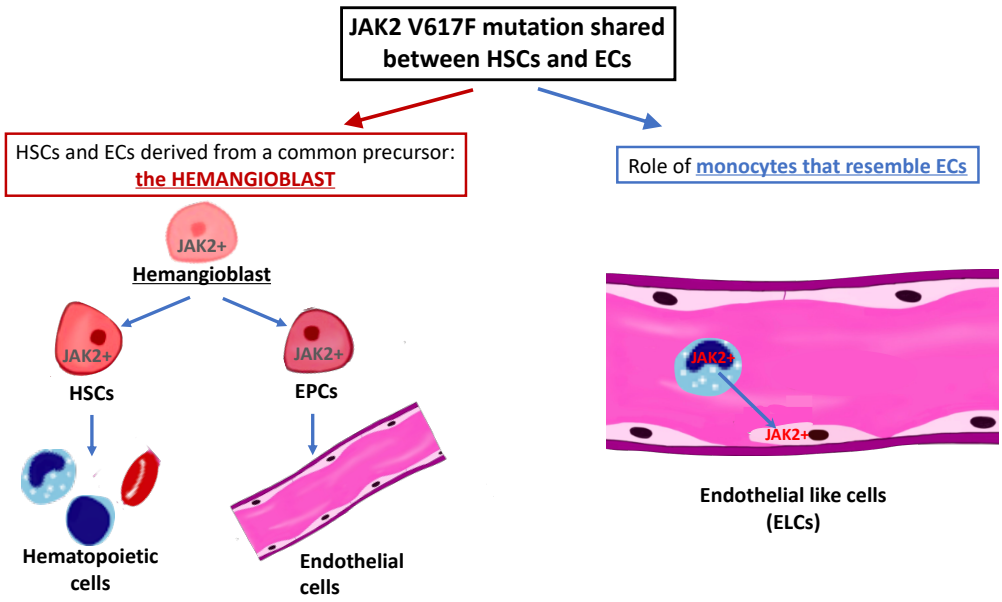


Figure 6: Possible origin of JAK2-mutated endothelial cells. The documentation of JAK2 myeloproliferative neoplasm driver mutations in myeloid cells and endothelial cells (EC) suggests that in some individuals both cell types originate from a "hemangioblast", which might serve as the cell of origin

for myeloproliferative neoplasms during embryogenesis. On the other hand, JAK2-positive EC may be derived from monocytes that resemble EC (endothelial-like cells) as well. EC: endothelial cells; EPC: endothelial progenitor cells; HSC: hematopoietic stem cells; ELC: endothelial-like cells. From Farina et al., Haematologica 2021[112]

Finally, the lack of conclusive evidences demonstrating in higher vertebrates the existence of a common precursor between hematopoietic and endothelial cells, has led some authors to the proposition that hemangioblast may be a state of competency that is never fulfilled in vivo due to the restriction and constraint imposed by the microenvironment[248] (Figure 7).

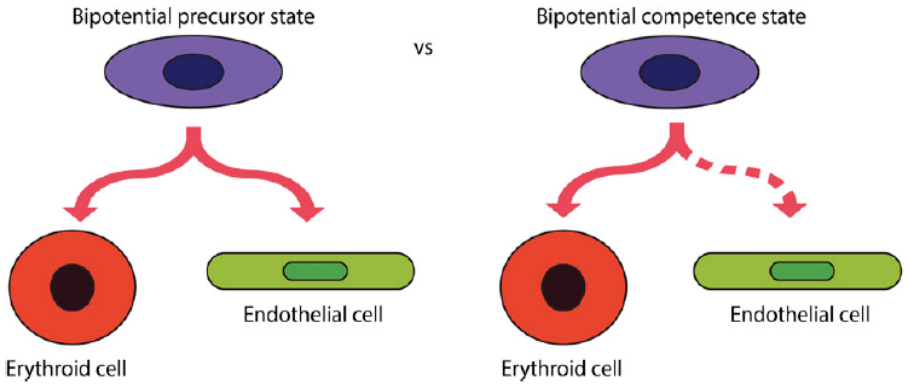


Figure 7: Is the hemangioblast a bipotential precursor state or a bipotential competence state, which reveals its latent developmental potential only under experimental conditions? From Amaya, Blood 2013[248].

In conclusion, open questions remain about the existence of a mesoderm derived common precursor with long-term proliferative potential and with both hematopoietic and endothelial differentiation capacity. In the near future, a new experimental approach or the identification of a specific marker for this mesodermal subset will be needed to demonstrate whether the hemangioblast is indeed an in vivo mesodermal precursor or just a state of competency [249].

2. AIMS

The main objective of this research project was to study “ex vivo” the molecular profile of endothelial cells in patients with Primary myelofibrosis and to compare it with the ones of paired CD34+-hematopoietic stem and progenitor cells (HSPC). In this way we aimed to investigate whether myeloid-associated mutations could be harbored by endothelial cells and if these mutations could be shared between the two different cell lines, thus exploring the theory of a “neoplastic” vascular niche in PMF patients, as well as the hypothesis of a common precursor cell between endothelial and hematopoietic cells.

For studying “ex vivo” the endothelium molecular profile, we decide to identify and isolate the mature circulating endothelial cells (CEC) in PMF patients, in order to overcome the anatomical difficulties in studying endothelium.

Therefore, in this present study we seek to achieve the following aims:

- **Aim 1.** “Ex vivo” study of the circulating endothelial cells (CECs) and their molecular profile in patients with Primary Myelofibrosis
- **Aim 2.** “Ex vivo” study of the CD34+ Hematopoietic stem and progenitors cells (HSPCs) molecular profile in patients with Primary Myelofibrosis
- **Aim 3.** To compare the CECs molecular profile with the ones of paired CD34+ HSPC, exploring the role of the “neoplastic vascular niche” in PMF and eventually the existence of a common precursor between hematopoietic and endothelial cells (hemangioblast), which may act as “cell of origin” of myelofibrosis/myeloid malignancies. In addition, for those patients who will subsequently underwent allogenic stem cell transplantation, the molecular profile of these two cells population will be investigate and compared even after the transplant itself.
- **Aim 4.** The study of the impact of CECs levels and their molecular profile on clinical outcome (i.e., vascular events, disease progression) in PMF patients.

For CECs identification and collection, we decide to use a well-recognized technology with a high specificity and sensitivity, combining the two traditional methods for CECs selection (i.e., immunomagnetic separation and flow cytometry detection), and which is the only one to have been approved by the FDA: the CellSearch and DEEPArray technologies. Our purpose was, indeed, to reach the highest grade of purity as much as possible, trying to avoid contaminations. Moreover, the use of the DeepArray sorting system allows us to have a gentle sorting system, which results in a lower cells biological damage rate, compare with the classical cytometric sorting systems. In addition, using a semi-automated system we would like

to avoid as much as possible the operator-dependent bias and to use a reproducible and standardized procedure.

Studying mature CECs, we would like to overcome, on one hand, the discussions on which cells may be considered the true circulating endothelial precursors cells; and, on the other hand, the technical and ethical issues of study the endothelium through mature endothelial cells captured by laser microdissection.

Considering the Aim 1, we decide to use a 54 PMF-related genes custom panel for studying the molecular profile of CECs by NGS analysis. To the best of our knowledge, this is the first time that myeloid-associated somatic mutations besides the *JAK2 V617F* MPN driver mutations are investigated in the endothelial compartment.

Then, the CECs genomic profile will be compared (Aim 3) with the ones of paired CD34+HSPC. In addition, for those patients who subsequently underwent allogenic stem cell transplantation (alloSCT), the molecular profile of the two cells populations will be compared before and after alloSCT, in order to evaluate if previous mutated CECs will be eventually maintained after alloSCT, or if CECs will acquire new mutations, maybe in relationship with the molecular profile of paired CD34+HSPCs, which will be predominantly deriving from the alloSCT donor and, therefore, different from the originally patient HSPCs. All these data will help in better investigate the role of "neoplastic vascular niche" and, eventually, of the possible existence of a common precursor between hematopoietic and endothelial cells.

Indeed, this study will help to understand the relationship between endothelial cells and hematopoietic cells in myeloproliferative neoplasms, in particular myelofibrosis, through the following possible scenario:

1.1. The presence of PMF-associated somatic mutation in CECs will reinforce the hypothesis of a "neoplastic" vascular niche in PMF patients (as it has been described for the mesenchymal cell[250] in acute myeloid leukemia) and the potential role in vascular complications and disease development

1.2. The co-presence of genetic alterations in both HSPCs and CECs would reinforce the hypothesis of a common precursor shared between CD34+HSPCs and EC in PMF patients, which could potentially act as the cell of origin of Primary myelofibrosis.

1.3. The co-presence of genetic alterations in both HSPCs and CECs after alloSCT, different from the hypothetical mutations harbored before transplant, would support the hypothesis of a common precursor

1.4. If HSPC and CECs will not share any mutations, the hypothesis of a common precursor becomes unlikely.

Finally, we will correlate the CECs number and their molecular profile, as well as the presence of mutations shared between the two cells populations, with the clinical characteristics of our patients and with the disease development (Aim 4).

3. STUDY DESIGN

Study Design

The MyCEC study plan is summarized in Figure 8. Each patient with Primary Myelofibrosis enrolled in the study, and each healthy control were subjected to two levies of peripheral blood: one for CECs isolation, and the other one for HSPCs selection. For those patients who subsequently underwent allogenic stem cell transplantation (alloSCT), two additional samples of peripheral blood, one for CECs isolation and the other one for HSPCs, were collected at the following time points: begore the start of conditioning regimen (T1) and one week after hematological recovery (T2).

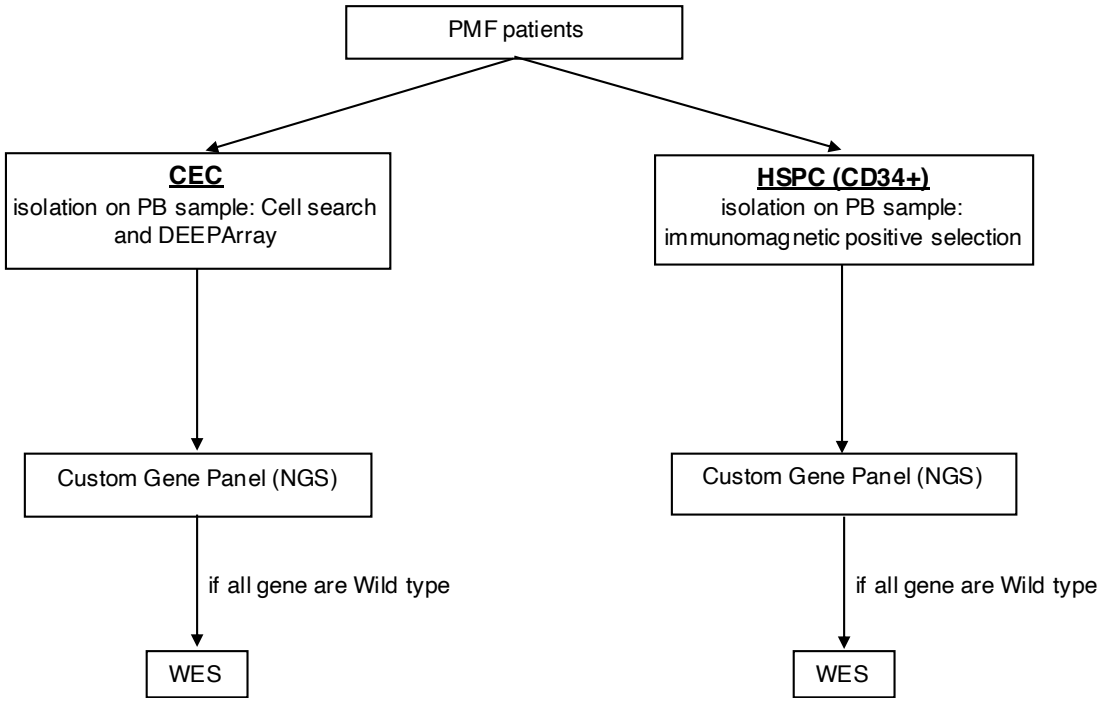


Figure 8: Study plan. Two samples of peripheral blood (10 ml each) will be collected for CECs and HSPCs isolation. CECs will be detected with the Cell search system, while they will be collected by DEEPArray system. Conversely, HSPCs will underwent CD34+ immunomagnetic positive selection. DNA from both CECs and HSPCs will be then investigate with a custom gene panel. If all the investigated genes will be wild type, then a whole exome sequencing will be performed.

From one peripheral blood sample, CECs were detected using the CellSearch system, which is based on immunomagnetic selection incorporating ferrofluid nanoparticles and fluorophore-labelled antibodies. CECs were defined as CD146+CD105+CD45-DAPI+ cells. Then, the putative CECs were sorted using the DEPArray system, using a combination of di-electrophoresis technology and high-quality image-based cell selection to manipulate individual cells. The largest possible number of CECs were collected to have a representative sample of the entire population of circulating endothelial cells.

From the other peripheral blood sample, HSPCs were isolated thanks to immunomagnetic positive selection (CD34+) from the mononuclear cell layer obtained after Ficoll centrifugation.

Thereafter, DNA was extracted from both CECs and HSPCs and amplified. Subsequently, it was analyzed by Next Generation Sequencing technology with a54-gene custom panel focused on genes mutated in PMF[14,38,50,53,251–253] (Table 7). If no mutations were detected, then Whole Exome Sequencing (WES) would have been performed only for PMF patients.

Mutational analysis of CECs in PMF patients and in MPN patients in general, to the best of our knowledge, has never been performed before. In contrast with previous studies, which investigate only the presence of the *JAK2 V617F* mutation in endothelial cells or in their progenitor, in the MyCEC trial we have investigate, for the first time, a large number of PMF-associated genes in cells related to the endothelium compartment.

The discovery of clonal mutations in the endothelial cells in patients with myelofibrosis would help in understanding the pathogenesis of the disease, as well as helping in the development of new therapeutic strategies based on the endothelial involvement, and potentially predicting the occurrence of vascular side effects (g.e. pro-thrombotic risk).

4. PATIENTS AND METHODS

4.1 Patients and samples

Patients to be enrolled in the MyCEC study must met the following inclusion criteria: (a) subjects must be over 18 years old, (b) have a Performance status greater or equal to 2 (ECOG score), (c) have been diagnosed with Primary myelofibrosis according to WHO classification, (d) and have not been treated with JAK2 inhibitors (Hydroxyurea treatment alone was permitted). These inclusion criteria were thought to avoid any possible bias or confounding factors deriving using JAK2 inhibitors or by a previous history of Polycythemia Vera or Essential Thrombocythemia.

The disease status at the time of samples collection was evaluated using the Dynamic International Prognostic Scoring System (DIPSS)[254].

The healthy controls must be over 18 years old and have not a previous history of malignant disease or cardiovascular diseases, including both thrombotic and bleeding events.

The MyCEC study protocol was approved by the Local Research and Ethics Committee and all donors provided written informed consent prior to participation in the trial, in accordance with the Helsinki II Declaration[255].

Between July 2019 and October 2022, we prospectively evaluated 17 patients with primary myelofibrosis followed at the Hematology and Bone Marrow Transplantation Units at ASST Spedali Civili di Brescia, along with 5 healthy subjects, as controls.

Subjects enrolled in the study were subjected to two levies of 10 ml peripheral blood each: one sample for CECs detection, and the other one for HSPCs selection (see Figure 8). For those patients who subsequently underwent allogenic stem cell transplantation, two additional samples of peripheral blood (one for CECs isolation and the other one for HSPCs) were collected at the following time points: before the start of conditioning regimen (T1) and one week after hematological recovery (T2).

For CECs analysis, the 10 mL of blood were collected in dedicated tubes containing a cell preservative (CellSave Preservative Tubes; Veridex LLC, Raritan, NJ, USA). All samples were stored at room temperature, shipped via overnight express courier to a referral Laboratory (Menarini Silicon Biosystems Laboratory, Bologna, Italy until August 2021, and then the Circulating Tumor Cells Laboratory at Istituto Oncologico Veneto), and processed within 96 h. The second patients' blood samples were used for CD34+HSPCs collection by immunomagnetic positive selection from the mononuclear cell layer obtained after Ficoll centrifugation (Lymphosepar I; IBL, Gunma, Japan) at CREA Laboratory in Brescia. More details in the following sections.

4.2 CEC identification and collection

4.2.1 CellSearch CECs identification

In our research project, CECs were identified using a system which combines immunomagnetic selection and flow cytometry quantification: the CellSearch system (Janssen Diagnostics, Raritan, NJ, USA). Originally, this system was developed for studying Circulating Tumor Cells (CTC), and, currently, it is the only US FDA-approved method for the clinical detection of CTC from cancer patients for its recognized high specificity and sensibility compared with others technologies. Subsequently, the same system was used to detect other rare cells in peripheral

blood, such as the Circulating Endothelial Cells.

The CellSearch system used for endothelial cell detection consists of (1) CellSave tubes for blood collection, (2) Cell-Tracks AutoPrep, which is a fully automated sample preparation system, the (3) Endothelial Cell Reagent Kit, and the (4) CellSpotter Analyzer II, a semi-automated fluorescence microscope. Therefore, the CellSearch system consists of two instruments (Autoprep and Analyzer) and dedicated reagents (Circulating Endothelial Cell Kit and Control kit), which allow to standardize the entire process, with high reproducibility, specificity and sensitivity. The CellSearch Circulating Endothelial Cell Kit contains a ferro-fluid reagent for the CECs selection, as well as immunofluorescent reagents.

About 10 ml of blood were collected in a CellSave Blood draw Tubes (Immunicon, Huntingdon Valley, PA), which contained a slow fixing preservative. In this way, Leukocytes and CECs were stabilized, and the samples could have been processed up to 96 h after blood draw. Samples were maintained at room temperature, shipped via overnight courier to a central laboratory, and processed within 72 h of blood collection (Figure 9).

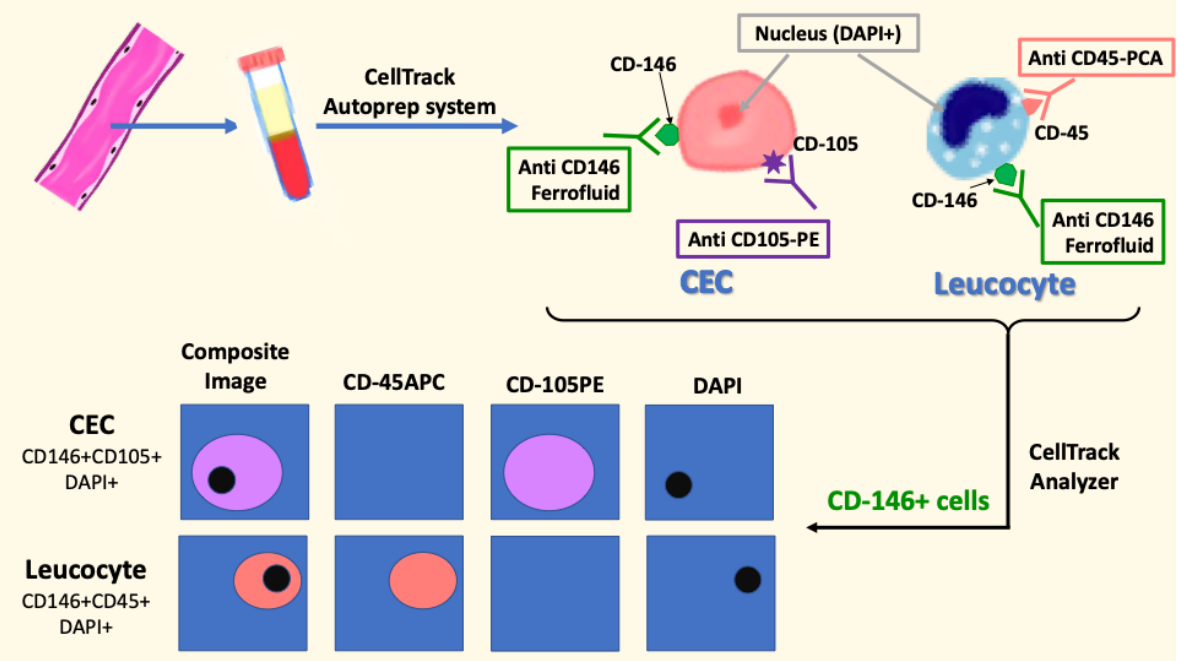


Figure 9: Circulating Endothelial cell identification by CellSearch. Tubes containing blood are centrifuged to separate blood into plasma, buffy coat and red blood cell layer. The blood tube is then placed into the CellTrack Autoprep system where blood cells are incubated with antibodies against CD146, CD105, CD45 and are stained with DAPI. In this step, CD146-positive CECs are labeled with anti-CD105-PE antibodies while leukocytes are labeled with anti-CD45-APC antibodies. The labeled cells are then analyzed and enumerated in CellTracks Analyzer. CECs are identified as CD105-positive/DAPI-positive/CD45-negative cells while leukocytes are identified as CD45-positive/DAPI-positive/CD105-negative cells. From Farina et al., Cells 2021[256].

The first step of the process is an immunomagnetic enrichment, using the CellTracks AutoPrep and CellTracks Analyzer II System (Immunicon Corp, Huntington Valley, PA, USA). As for manual immunomagnetic isolation, cells were isolated by CD146-coupled ferrofluids, but the CellTracks System is fully automated and, therefore, it's not operator dependent. Specifically, 7.5 ml of the fixed blood from the CellSave tube is pipetted into a specific CellSearch conical tube and

5.5 ml of CellSearch dilution buffer is added to the blood. Then, the diluited fixed blood is centrifuged at 800xg for 10 min without brake. Thereafter, the tube is carefully loaded into the AutoPrep system, and the diluted plasma will be removed until 1 cm above the red blood cell layer. Then, anti-CD146 ferrofluid and dilution buffer are added to the tubes and mixed by pipetting. The ferro-fluid reagent consists of nanoparticles with a magnetic core surrounded by a polymer layer coated with antibody directed towards the CD146 antigen for the selection of the CECs. CD146, also known as the melanoma cell adhesion molecule (MCAM), is a cell adhesion molecule currently used as a marker for endothelial cell lineage. The magnets were moved back and forward towards the tube to enhance the collisions between cells and ferrofluids. After an incubation period, the magnets remained against the tube, anti-CD146-ferrofluids, and the cells that have bound ferrofluid were pulled to the magnets, while the rest of the cells were removed in a single pipetting step.

After isolation, the suspension of CD146+ cells was stained with (i) 4',6-diamidino-2-phenylindole (DAPI) to identify nucleated cells, (ii) CD105-PE, fairly unique on endothelial cells, and (iii) CD45, to exclude CD146-expressing T cells. Therefore, staining reagents (<0.0006% mouse monoclonal antibodies specific to CD105 conjugated to phycoerythrin; <0.0013% mouse antiCD45 monoclonal antibodies conjugated to allophycocyanin in phosphate-buffered saline containing 0.5% BSA and 0.1% sodium azide) were added in conjunction with a permeabilization buffer to label the cells fluorescently. After incubation, magnetic separation was repeated to remove the excess staining reagent. After the final processing step, the cells were re-suspended in 300 uL of buffer and transferred to a chamber placed between two magnets that orientate the immunomagnetically labelled cells in a monolayer for analyses. The cells were then examined with a four-colour semi-automatedfluorescent microscope, the CellSpotter Analyzer II. A grey-scale charge-coupled device camera was used to scan the entire chamber surface, and each captured frame was then evaluated for potential CEC candidates by image analysis software (Figure 10). In summary, CECs were defined as $CD146^{+}CD105^{+}CD45^{-}DAPI^{+}$ cells. The endothelial feature of the cells meeting these phenotypic criteria was further demonstrated by global gene expression profiling, which clearly demonstrated the presence of endothelial markers[190].

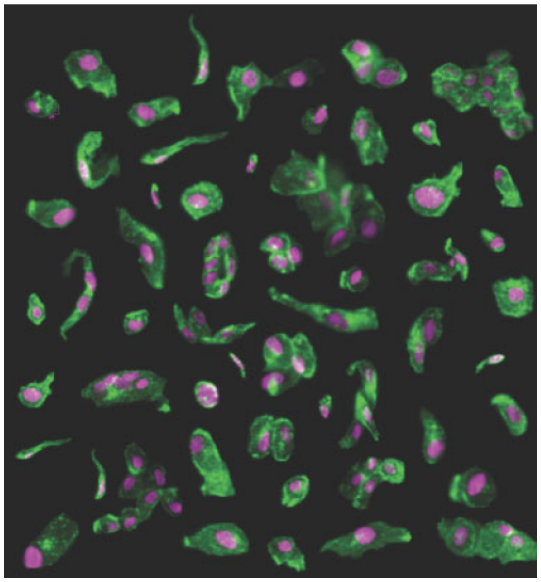


Figure 10: Gallery of morphological appearances of endothelial cells circulating in peripheral blood. In Purple, the nuclear stain DAPI, while in green, the CD105 staining. From Rowand et al.[187]

4.2.2 CECs collection by DEPArray System

The DEPArray system (Di-Electro-Phoretic Array system; by Silicon Biosystems, Bologna, Italy) is a semi-automated system that allows to isolate rare cells from mixed-cell populations at the single-cell level[257]. This platform utilizes high quality, image-based selection to identify and isolate the cells of interest. Fluorescently labeled cells can be visualized and isolated by means of a chip, consisting of various microelectrodes, which create electric cages in which individual cells are trapped. Alternatively activating and deactivating the microelectrodes on the chip results in moving the caged cells to a position in the chip that allows the recovery of these cells in a medium suitable for downstream analysis.

In detail, the DEPArray NxT System is composed of three elements: a benchtop instrument, a disposable microfluidic cartridge, and a proprietary software, the CellBrowser. The working principle of the DEPArray is the Dielectrophoresis (DEP), an electrokinetic principle based on the ability of a non-uniform electric field to exert forces on neutral, polarizable particles, such as cells, which are suspended in a liquid. The core of the technology is the microsystem cartridge, which is a single-use device integrating a microelectronic silicon chip, microfluidic chambers and valves. The silicon substrate in the cartridge integrates an array of over 300,000 micro-electrodes, each electrode can be programmed and energized with Alternating Current in-phase or counter-phase voltages with respect to the glass lid, which is conductive and transparent. By applying an appropriate pattern of phases, the array can generate up to 30,000 “DEP cages” in the Main Chamber, each one able to capture a cell in stable levitation, avoiding contacts between the cells and surfaces during the sorting process. DEP cages are able to trap and move cells of different type and size ranging from small sperm cells to large epithelial cells[258–260]. This electronic structure is integrated within an innovative microfluidic architecture that includes three micro-chambers in fluidic connection (Figure 11): The Main Chamber (where the sample is loaded), the Parking Chamber (where the target cells are collected before the recovery), and the Recovery Chamber.

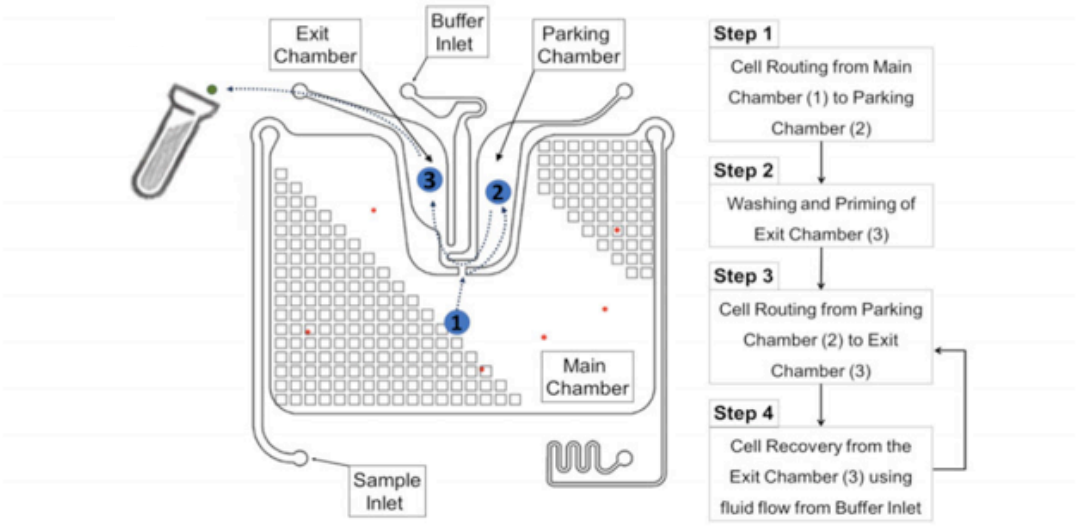


Figure 11: The DEPArray NxT system. After the phases of sample and buffer loading, the cells are randomly distributed into the Main chamber (1). Then, the selected cells are simultaneously moved toward the parking chamber (2). After a washing and priming phase of exit chamber (3), the cells are moved from the parking chamber (2) to exit chamber (3) for the cell recovery (4). *From Di Trapani et al.* [261]

Briefly, to allow loading of samples from CellSearch cartridges in a DEPArray cartridge, CellSearch CEC samples were aspirated from their CellSearch cartridge using a 200 ml gel loading tip pre-rinsed in a 2% BSA in PBS solution. The whole suspension was centrifuged for 10 min at 300g, cells were washed once in 1ml of SB115 buffer (a proprietary low-conductivity buffer for sorting fixed cells in the DEPArray cartridge) and finally re-suspended in 14 ml of SB115 buffer. Thereafter, DEPArray cartridges were manually loaded with 14 ml of sample and 800 ml of the buffer solution in which purified or single cells had to be recovered. After loading the cartridge into the DEPArray system, 9.26 ml of sample was automatically injected by the system into a microchamber of the cartridge, where the cells were spontaneously organized into a preprogrammed electric field consisting of 16 000 electrical cages, in which individual cells were trapped. Image frames covering the entire surface area of the microchamber for each of three fluorescent filter cubes (PE, APC and DAPI/Hoechst) and bright field images were captured. Cells were automatically detected by the system based on a DAPI/Hoechst fluorescence threshold and were assigned a unique cell ID. Captured images were digitally processed and presented in a software module that enables selection of cells of interest by the operator. Next, selected cells were moved simultaneously to a parking area adjacent to the main microchamber in the cartridge. Individual cells or groups of cells were, subsequently, moved to a recovery area, where a last visual confirmation of the targeted cell could have been performed. To recover a cell or group of cells, the content of the recovery area was flushed with two drops of buffer (ca. 30–40 ml) into a 200 ml PCR tube. The entire cell routing process was monitored under bright field imaging.

The proprietary CellBrowser software enables an automatic or operator-assisted identification of the desired cells through the elaboration of high-resolution images, minimizing the possibility to select inappropriate events, such as debris and doublets. The different cell populations are selected by using a manual or semi-automatic gating. Once identified, each target cell can be isolated from the bulk population, automatically, in the following way: the instrument moves the selected DEP cages (containing the target cells) by changing the electric field pattern step by step, deterministically, concurrently, and independently along trajectories calculated by the software, moving each selected cell from the original location into the Parking chamber. Afterwards, cells can be displaced, as single-cells or in pools of up to 507 cells. Typically, the process takes about 16 min for system calibration and Main Chamber loading, 20 min for image acquisition and analysis, 10 min for cell selection, 12 min for cell routing from Main Chamber to Parking Chamber, and 6 min for the priming and washing of the exit channel, resulting in a total time of 64 min (except for cell selection, all the steps are executed automatically by the system). The subsequent recoveries take 107 s for a single cell or 192 s for a pool of 20 cells. At the end of the process, the target cells can be eluted from the device directly into various types of supports, through an accurate microfluidic control, by flowing clean buffer loaded in the cartridge prior to use. The recovery procedure can be repeated to obtain from the same sample multiple separate recoveries of individual target cells (up to 96) and/or groups of cells[261]. Unlike other traditional bulk sorting, DEPArray technology isolates single and pure cell populations. The high-quality and accuracy of DEPArray technology has been thoroughly validated by using immunofluorescence and molecular-based approaches, with both spike in and real biological samples[258].

4.3 HSPCs detection: MILTENYI columns

CD34+ cells were purified by immunomagnetic positive selection (magnetic-activated cell sorting [MACS] CD34 MicroBead Kit by Miltenyi biotech, Bergisch Gladbach, Germany) from the mononuclear cell layer obtained after Ficoll centrifugation. The patented MACS Column Technology is based on the use of MACS MicroBeads, MACS Columns, and MACS Separators. Briefly, 10 mL of peripheral blood was collected in EDTA (Ethylene-diaminetetraacetic acid) tubes and examined within 6 h. The mononuclear cells layer obtained after Ficoll centrifugation (Lymphosepar I; IBL, Gunma, Japan) were magnetically labeled with CD34 MicroBeads [32]. Then, the cell suspension was loaded onto a MACS Column which was placed in the magnetic field of a MACS Separator. The unlabeled cells run through, while the magnetically labeled cells were retained on the MACS Column. The retained material was then washed with buffer to remove unlabeled material. After removing the column from the magnetic field, the magnetically retained CD34+ cells could be eluted as the positively selected cell fraction and counted using the Bürker-Turk chamber [33].

4.4 DNA Amplification

DNA extracted from isolated CEC and HSPC was amplified in order to obtain a quantity suitable for NGS analysis. The Whole Genome Amplification (WGA) was performed by Reply-g DNA library kit (Qiagen) following "Amplification of Genomic DNA from Single Cells" procedure. Our approach was based on the gene target capture sequencing. Specific probes (NimbleGen by Roche) have been used in order to hybridize all exons of the above-mentioned genes (141 kb). Briefly, up to 1000 cells were resuspended in PBS and treated by denaturating solution, which allow the membrane degradation and the DNA denaturation. This phase was followed by WGA obtained using Phi29 TaqPolymerase[262]. The WGA usually take 3 hours and may be concluded with Tagmentation, End-repair and A-tailing procedures in order to produce NGS library or stopped. Amplified genomic DNA is stable and NGS analysis can be subsequently performed.

4.5 Gene Panel NGS Analysis

DNA was first analyzed by MiSeq Illumina NGS platform, specific and sensitive to study multiple target genes when low amount of DNA is available. Firstly, 300ng of amplified genomic DNA from CECs or HSPCs was screened for mutations in 53 genes known to be associated to Myelofibrosis[14,38,50,53,252,253] (Table 7).

DNA was tagmented by enzimatic reaction. The fragmentation was immediately followed by end-repair reaction and the index and adaptors ligation. Index and adaptors are small sequences of DNA that need to be associated to the amplicon samples in order to uniquely identify each sample during the sequencing and the data analysis and to be recognized by the software as "true read". The DNA was then incubated with NimbleGen probes. The incubation was followed by the enrichment of the captured fragments, purifications by Ampure Beads and a final amplification. The captured sequences of CEC and HSC DNA from 4 subjects were thus pooled (8 samples per pool)[263] and sequenced following manufacturer's instructions by MiSeq Illumina NGS platform using 2x150 sequencing (V2 kit, TruSeq). One sequencing run was required in order to sequence 8 samples with a coverage about 3200x[264]. The .vcf files were analyzed using the free bioinformatics tool

wAnnovar (Wang Genomics Lab 2010-2020) [13,14]. The cutoffs to confirm the presence of the muta-tions were identification of mutant alleles in 30 and 50 reads both in forward and reverse, for HSPCs and CECs, respectively.

ABL1	CEBPA	HRAS	MYD88	SF3B1
ASXL1	CSF3R	**IDH1**	NOTCH1	SMC1A
ATRX	CUX1	**IDH2**	NPM1	SMC3
BCOR	**DNMT3A**	IKZF1	NRAS	**SRSF2**
BCORL1	ETV6/TEL	**JAK2**	PDGRFA	STAG2
BRAF	**EZH2**	JAK3	PHF6	**TET2**
CALR	FBXW7	KDM6A	PTEN	TP53
CBL	FLT3	KIT	PTPN11	**U2AF1**
CBLB	GATA1	KRAS	RAD21	WT1
CBLC	GATA2	MLL	RUNX1	ZRSR2
CDKN2A	GNAS	**MPL**	SETBP1	

Table 7: The 54 MF-related genes custom panel; in bold those that are more closely related to myelofibrosis[14,38,50,53,252,253]

4.6 Whole Exome Sequencing (WES)

WES approach would have been accomplished by an Illumina NGS platform, in order to obtain information about the genetic background and to identify new markers of Myelofibrosis in the cases resulted negative for the presence of mutations by Gene Panel NGS Analysis.

Whole Exome Sequencing protocol (Illumina Nextera Rapid Capture Exome) starts with the tagmentation of 50 ng of DNA in order to obtain sequences of oligonucleotides with a range of length between 200 and 500 bp. The performance of tagmentation is checked with Bioanalyzer using High Sensitivity chip. A PCR is performed on oligonucleotides, acquiring the contemporary amplification of the library and the creation of double indexes, produced by the binding of DNA sequences to their primers. The subsequent purification allows the size selection of the different fragments in order to pick out the sequences with a range of length between 350 and 550 bp. The library is checked with Bioanalyzer using DNA1000 chip. The protocol Illumina Nextera Rapid Capture Exome provides also the exome capture. Each library may be pooled with other libraries, 12 at the most. The exome capture involves two subsequently hybridizations of 19.5 hours with specific probes for human exome sequences. Hybridization will be followed by washes in order to remove the non-coding regions of the genome. Finally, a PCR will amplify the exome captured. NextSeq by Illumina will then sequence the exome library[265].

4.7 Statistical Analysis

Standard descriptive statistics were used to summarize the patient samples. Continuous data were expressed as median (range). Categorical variables were compared using the chi-square or Fisher's exact test. Mann-Whitney U test was used in univariate analysis for comparison of continuous variables. The clinical and laboratory parameters, as well as comorbid conditions and PMF treatment, were analyzed as possible factors related to the presence of molecular mutations on CECs and HSPCs and to the detection of shared mutations between the two subpopulations.

Overall survival was calculated from the date of sample collections to the last follow up or death, using the Kaplan-Meier method; the log-rank test was used to evaluate differences among subgroups. The cumulative incidence of acute myeloid leukemia (AML) progression in patients who shared somatic mutations and those who did not was performed with mortality as competing risk. Comparisons between cumulative incidences were performed using the Gray test. All reported P values are two-sided, and P values of less than 0.05 were considered to indicate statistical significance. Statistical analyses were performed with EZR software (v1.40) [42].

5. RESULTS

5.1 Patients and Healthy Controls Characteristics

Between July 2019 and October 2022, we prospectively evaluated 17 patients with primary myelofibrosis followed at the Hematology and Bone Marrow Transplantation Units at ASST Spedali Civili di Brescia, along with 5 healthy subjects, as controls.

The characteristics of patients and healthy controls are reported in Table 8 and 9. All the 17 patients were diagnosed with Primary Myelofibrosis according to WHO classification[1]. The patients' median age was 66 years (54-85), and the male sex was pre-dominant (70%; 12 out of 17 patients). The median time from diagnosis to sample collection was 26 months (1-216) (Table 10). Overall, 13 of the 17 patients (76%) presented splenomegaly at the time of samples collection, while four patients (24%) had constitutional symptoms. Three patients experimented thrombosis before being diagnosed with myelofibrosis: one had portal vein thrombosis, another one a central retinal artery occlusion, and the last one had deep vein thrombosis. Overall, 11 patients presented also other comorbidities: atrial fibrillation (n=2), low left ventricular function (n=1), hypertension (n=8), Chronic obstructive pulmonary disease (n=1), type II diabetes mellitus (n=1), metabolic syndrome (n=2), and Basedow disease (n=1).

Regarding the MPN driver mutations (Figure 12), 11 of the 17 (64%) patients were *JAK2 V617F* mutated, while 2 were CALR mutated, and 2 were *MPL W515L* positive. Conversely, two patients did not harbor any MPN driver mutations (triple-negative). Most of the patients (n=9) presented with an Intermediate-1 DIPSS score, 5 with intermediate-2, while 2 patients had a high-risk and 1 the low-risk DIPSS score class. Overall, the white blood cells (WBC) and platelets (PLT) count were in line with the values described for the general population, with a median value of 7.6 x 10^9 WBC/L and 201 x 10^9 PLT/L, respectively. Conversely, the hemoglobin levels were in median (10.9 g/dl) lower than the physiological range (Table 9). Most of patients had low or intermediate grade of bone marrow fibrosis. Indeed, 14 patients (82%) presented a grade 1 or 2 of bone marrow fibrosis, according to the World Health Organization classification[1]. More than 60% of patients didn't receive any treatment at the time of samples collection, while six patients were receiving hydroxyurea, as cytoreductive treatment. In general, patients were under treatment for a median of 12 months (6-24), and the therapy was well tolerated with no side effects.

The 5 healthy controls had no known illness. In details, they didn't have a previous history of cancers or vascular events, as well as they didn't present any cardiovascular risk. Their median age was 65 years (35-84), and 4 of 5 healthy controls were female. Their clinical features and peripheral blood counts are reported in Table 8 and 9.

The median follow-up from samples collection was 26 months (6–38).

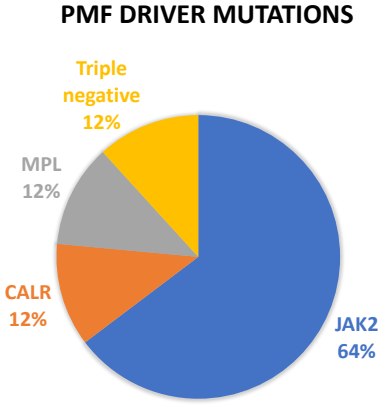


Figure 12: PMF Driver mutations distribution: *JAK2 V617F* was detected in 9 patients (64%; in blue), CALR mutations in 2 patients (12%; in orange), while 2 patients harbored the MPL mutations (12%; in grey). 2 patients didn't harbor any MPN driver mutations and, therefore, they were defined as triple negative (12%; in yellow).

	MYCEC_01	MYCEC_02	MYCEC_03	MYCEC_04	MYCEC_05	MYCEC_06	MYCEC_07	MYCEC_08	MYCEC_09	MYCEC_10	MYCEC_11	MYCEC_12	MYCEC_13	MYCEC_14	MYCEC_15	MYCEC_16	MYCEC_17	MYCEC_18	MYCEC_19	MYCEC_20	MYCEC_21	MYCEC_22
Disease	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	PMF	HC	HC	HC	HC	HC
Months from diagnosis	1	2	4	4	15	28	28	31	211	146	15	28	35	1	26	1	36	NA	NA	NA	NA	NA
Driver Mutation	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	JAK2	CALR	CALR	MPL	MPL	Triple neg	Triple neg	NA	NA	NA	NA	NA
JAK2 allelic ratio	27,3	55%	33%	77%	63%	35%	22%	76%	30%	92	41	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Sex	F	M	M	F	F	M	M	M	M	M	M	F	M	M	F	M	M	F	M	F	F	F
Age (years)	81	78	72	64	85	57	74	78	72	55	63	61	66	54	71	64	54	35	46	65	77	84
DIPSS risk class	Intm-2	Intm-2	Intm-2	Intm-1	Intm-1	High	Intm-1	Intm-1	Intm-1	Intm-1	Intm-1	Intm-1	Intm-2	Intm-2	Intm-1	High	Low	NA	NA	NA	NA	NA
Splenomegaly	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No
Spleen (cm below LCM)	3 cm	5 cm	9 cm	6 cm	5 cm	0 cm	16 cm	3 cm	3 cm	7 cm	6 cm	10 cm	9 cm	10 cm	0 cm	0 cm	0 cm	0 cm	0 cm	0 cm	0 cm	0 cm
Constitutional symptoms	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	Yes	No	Yes	No	NA	NA	NA	NA	NA
Previous thrombosis	No	No	Yes	No	No	No	No	No	Yes	Yes	No	No	No	No	No	No	No	No	No	No	No	No
Treatment	None	HU	None	None	HU	HU	None	None	HU	HU	None	None	None	None	None	None	None	None	None	None	None	None
BM fibrosis	1	1	2	2	2	1	2	1	1	1	3	2	3	1	1	2	1	NA	NA	NA	NA	NA
WBC (x10^9/L)	5,6	19,5	3,8	6,27	11	10,9	4,7	7,5	7,3	11.5	11.35	5,9	6,8	60	7,6	117,1	4.81	5,4	5,6	6,6	3,9	9.1
Hb (g/dL)	8,1	12,3	8,5	12,9	13	9,1	10,7	10,9	14,8	10.4	9.7	10,6	8	9,8	12,4	8,9	13	13	14,5	13,6	14,4	12
PLT (x10^9/L)	210	144	266	394	855	101	201	234	183	132	34	624	163	440	707	50	171	370	225	257	179	412

Table 8: Patients and healthy controls characteristics at the time of samples collection; PMF=Primary Myelofibrosis; M=male, F=Female; Intm=intermediate; HU=Hydrossiurea; NA=Not applicable; LCM=Left Costal Margin; BM=bone marrow; WBC=White blood count; Hb=Hemoglobin; PLT=Platelets

Features	PMF patients N or Median *(% or range)*	Healthy controls N or Median *(% or range)*	*p value*
Total	17	5	
Age (years)	66 *(54-85)*	65 *(35-84)*	*0.37*
Male	12/17 *(70%)*	1/5 *(20%)*	*0.12*
PMF	17/ 17	0/5	*NA*
Months from Diagnosis	26 *(1-211)*	*NA*	*NA*
WBC (x10^9/L)	7,6 *(3,8-117)*	5,6 *(3,9-9.1)*	*0.07*
Hb (g/dl)	10,9 *(8-14,8)*	13,0 *(12-14,5)*	*0.11*
PLT (x10^9/L)	201 *(34-885)*	232 *(179-412)*	*0.67*
Constitutional Symptoms	4 *(24%)*	*NA*	*NA*
Altered karyotypes	5 (*29%*)	*NA*	*NA*
Previous Thrombosis	3 *(18%)*	*0 (0%)*	*>0.99*
Splenomegaly			*NA*
N° patients	13 *(76%)*	0 *(0%)*	
cm below LMC	5 *(0-16)*	0	
Treatment			*NA*
Hydrossiurea	6 *(36%)*	0 *(0%)*	
None	11 *(64%)*	5 *(100%)*	
BM fibrosis			*NA*
WHO grade 1	7 *(41%)*	*NA*	
WHO grade 2	7 *(41%)*	*NA*	
WHO grade 3	3 *(18%)*	*NA*	
DIPSS (at samples collection)			*NA*
Low	1 *(6%)*	*NA*	
Intermediate 1	9 *(53%)*	*NA*	
Intermediate 2	5 *(29%)*	*NA*	
High	2 *(12%)*	*NA*	
Driver Mutations			*NA*
JAK2	*11 (64%)*	*NA*	
CALR	2 *(12%)*	*NA*	
MPL	2 *(12%)*	*NA*	
Triple negative	2 *(12%)*	*NA*	

Table 9: Patients and healthy controls characteristics; PMF=Primary Myelofibrosis; BM=bone marrow; WBC=White blood count; Hb=Hemoglobin; PLT=Platelets; NA=not applicable

5.2 CECs enumeration and collection

The CECs were detected from the peripheral blood of both PMF and healthy control using the CellSearch system, as previously described. All the $CD146^{+}CD105^{+}CD45^{-}$ and DAPI positive cells were considered as CECs. Overall, CECs were successful detected in all samples (17 patients and 5 controls; Table 10).

PMF patients presented a median of 18.5 CECs/ml (74CECs/4ml of peripheral blood, range: 15-1448), which was significatively higher than the median of 4.25 CECs/ml (17 CECs/4ml of peripheral blood, range: 11-19) detected in the healthy control (p: 0.002; Figure 13).

The number of CECs detected was not related with any of the clinical or laboratory variable analyzed (age, gender, grade of fibrosis, MPN driver gene mutations, DIPSS score, previous vascular

events, splenomegaly, constitutional symptoms, time to diagnosis, cytoreductive treatment, white blood count) (Table 11).

Patient	Disease	Driver Mutation	Sex	Age (years)	DIPSS risk class	BM fibrosis	Spleen cm below LCM	Constitutional symptoms	WBC (x10^9/L)	Treatment	Previous thrombosis	CECs detected (n/4ml)	CECs recovered (n/4ml)	CD34+ HSPCs (x10^4/ml)
MYCEC_01	PMF	JAK2	F	81	Intm-2	1	3 cm	No	5,6	None	No	21	0	67
MYCEC_02	PMF	JAK2	M	78	Intm-2	1	5 cm	Yes	19,5	HU	No	67	29	60
MYCEC_03	PMF	JAK2	M	72	Intm-2	2	9 cm	Yes	3,8	None	Yes	399	118	72
MYCEC_04	PMF	JAK2	F	64	Intm-1	2	6 cm	No	6,27	None	No	123	25	82
MYCEC_05	PMF	JAK2	F	85	Intm-1	2	5 cm	No	11	HU	No	54	8	127
MYCEC_06	PMF	JAK2	M	57	High	1	0 cm	No	10,9	HU	No	102	5	7
MYCEC_07	PMF	JAK2	M	74	Intm-1	2	16 cm	No	4,7	None	No	15	4	98
MYCEC_08	PMF	JAK2	M	78	Intm-1	1	3 cm	No	7,48	None	No	31	0	9
MYCEC_09	PMF	JAK2	M	72	Intm-1	1	3 cm	No	7,34	HU	Yes	145	27	57
MYCEC_10	PMF	JAK2	M	55	Intm-1	1	7 cm	No	11,5	HU	Yes	27	12	41
MYCEC_11	PMF	JAK2	M	63	Intm-1	3	6 cm	No	11,35	None	No	74	0	61
MYCEC_12	PMF	CALR	F	61	Intm-1	2	10 cm	No	5,86	None	No	116	30	72
MYCEC_13	PMF	CALR	M	66	Intm-2	3	9 cm	No	6,78	None	No	1448	122	63
MYCEC_14	PMF	MPL	M	54	Intm-2	1	10 cm	Yes	60	None	No	120	0	16
MYCEC_15	PMF	MPL	F	71	Intm-1	1	0 cm	No	7,6	None	No	290	32	15
MYCEC_16	PMF	Triple neg	M	64	High	2	0 cm	Yes	117,06	None	No	22	1	31
MYCEC_17	PMF	Triple neg	M	54	Low	1	0 cm	No	4,81	None	No	15	5	62
MYCEC_18	Normal control	NA	F	35	NA	NA	NA	NA	5,4	None	No	19	2	0,3
MYCEC_19	Normal control	NA	M	46	NA	NA	NA	NA	5,6	None	No	17	11	2,6
MYCEC_20	Normal control	NA	F	65	NA	NA	NA	NA	6,6	None	No	19	9	3,4
MYCEC_21	Normal control	NA	F	77	NA	NA	NA	NA	3,9	None	No	13	4	4,3
MYCEC_22	Normal control	NA	F	84	NA	NA	NA	NA	9,1	None	No	11	8	3,2

Table 10: Patients and healthy controls characteristics, CECs number detection and collection, and HSPCs collected.

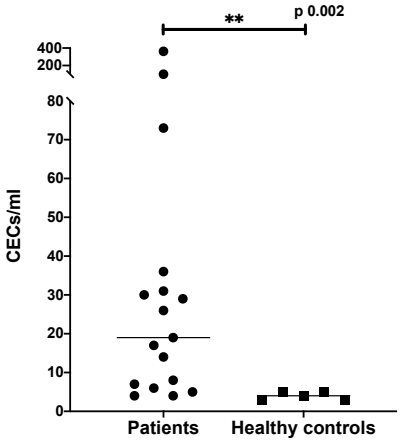


Figure 13: Scatter dot plot for the CECs detected in 1ml of peripheral blood for patients (black dots) and healthy controls (black squares). Line at median levels. The analysis was performed using the Mann-Whitney test. ** *p < o.o1*

Features	PMF patients		Healthy controls		*p value*
	CEC median (*range*); n pts	*p value*	CEC median (*range*); n pts	*p value*	
CECs detected	74 (15-1448); n=17		17 (11-19); n=5		***0.002***
Sex		*0.70*		*NA*	
Male	71 (15-1448); n=12		17; n=1		*NA*
Female	116 (54-290); n=5		16 (11-19); n=4		***0.02***
Age		*0.56*		0.2	
≥ 65 years	67 (15-1448); n=9		12 (11-13); n=2		***0.04***
< 65 years	88 (15-123); n=8		19 (17-19); n=3		*0.08*
Time from diagnosis		*0.79*		*NA*	
< 2 years	71 (21-399); n=8		*NA*		
> 2 years	116 (15-1448); n=9		*NA*		
White blood count		*0.46*		*NA*	
> 10 x 10^9/L	67 (22-120); n=7		0		*NA*
≤ 10 x 10^9/l	120 (15-1448); n=10		17 (11-19); n=5		***0.02***
Constitutional symptomns		*0.69*		*NA*	
Yes	94 (22-399); n=4		*NA*		
No	74 (15-1448); n=13		*NA*		
History of thrombosis		*0.43*		*NA*	*NA*
Yes	145 (21-399); n=5		0		
No	71 (15-1448); n=12		17 (11-19); n=5		
Splenomegaly		*0.56*		*NA*	*NA*
Yes	74 (15-1448); n=13		0		
No	62 (22-290); n=4		17 (11-19); n=5		
Treatment		*0.64*		*NA*	*NA*
Hydrossiurea	85 (27-290); n=6		0		
No treatment	74 (15-1448); n=11		17 (11-19); n=5		
DIPSS		*0.52*		*NA*	*NA*
Low-Interm1	64 (15-290); n=10		*NA*		
Interm2-High	102 (21-1448); n=7		*NA*		
Grade of BM fibrosis		*0.56*		*NA*	*NA*
1	67 (15-290); n=9		*NA*		
2-3	95 (15-1448); n=8		*NA*		
Driver mutations		*0.54*		*NA*	*NA*
JAK2	67 (15-399); n=11		*NA*		
Non JAK2 mutations	118 (15-1448); n=6		*NA*		

Table 11: Impact of patients and healthy controls' characteristics on CECs detection. Median and range of CECs detected in 4 ml of peripheral blood; n=number; pts=patients; Interm=intermediate; BM=bone marrow. Mann-Whitney test analysis. In bold the p value < 0.05.

Three patients (MyCEC10, 13 and 17) subsequently underwent alloSCT. The median of CECs detected before alloSCT was 6.75 CECs/ml (28 CECs/4ml of peripheral blood, range: 15-74), while one week after hematopoietic recovery post-alloTMO the patients presented a median of 34 CECs/ml (136 CECs/4ml of peripheral blood, range: 60-153) (p 0.2). The three patients didn't have any vascular complications related with alloSCT during the follow up.

Subsequently, CEC were collected for 13 of the 17 patients (76%) and in all the 5 healthy controls by DEEPArray system (global successful rate for CECs collection: 82%).

9 of the 22 samples (41%) showed the presence of unspecified PE-positive debris (Figure 14), which made the CECs recovery with DEPArray very challenging. It was not clear what that debris were, maybe they could be apoptotic cells of cells' fragments. Despite the presence of PE-debris, target single cells were selected as positive for CD105-PE mean intensity and negative for CD45-APC mean intensity (Figure 14-B). At the end, CECs recoveries were performed successfully for all samples except for 4 patients (MYCEC01, MYCEC08 and MYCEC11 and MyCEC14).

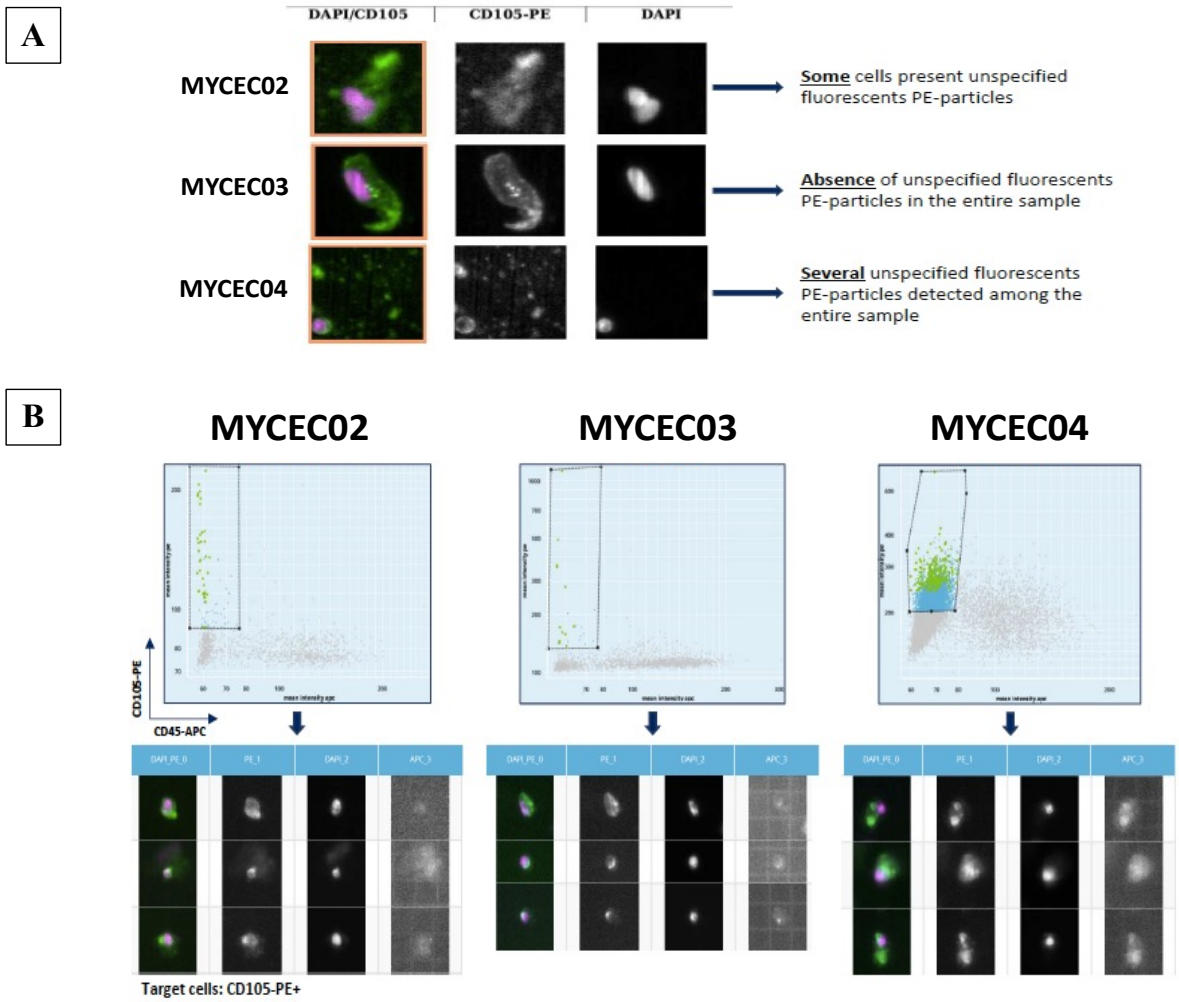


Figure 14: (A) CellSearch imagines of patients MYCEC02, MYCEC03 and MYCEC04. Some patients (MYCEC02 and 04) presented unspecified PE-positive debris. (B) Sample MYCEC02, MYCEC03 and MYCEC04 DEPArray imagines comparison. At the top the DEPArray scatter plot with the gate for CD105-PE positive (Y axis) and CD45-APC negative (X axis) cells. Down the original CellSearch imagines. Despite the presence of PE-debris (in light blue), target single cells were selected as positive for CD105-PE mean intensity and negative for CD45-APC mean intensity. Regarding the CellSearch analysis, in the first column the cells selected as CECs, which presented in purple the nuclear stain DAPI, while in green the CD105 staining. In the second column the selection of CD105-PE staining, while the third shown the DAPI staining. In the last column, the cells expressing CD45-APC stain were negative selected, because they were supposed to be leucocytes.

In PMF patients, a median of 25 CECs (range: 1-122) in 4 ml of peripheral blood was recovered (6,25 CECs/ml), while a median of 8 CECs (range: 2-11) in 4 ml of peripheral blood was collected in healthy controls (2 CECs/ml) (p 0.13; Figure 15-B). As expected, the median of CECs collected was inferior to what obtained during the CellSearch detection (Figure 15-A). No

significative differences were found between patients from whom we isolated CECs and those who did not successfully recover CECs.

In two (MyCEC10 and MyCEC17) of the three patients who subsequently underwent alloSCT, CEC were successfully collected also before (median: 10 CECs/4ml peripheral blood; range: 6-14) and after alloSCT (median: 8.5 CECs/4 ml; range: 5-12).

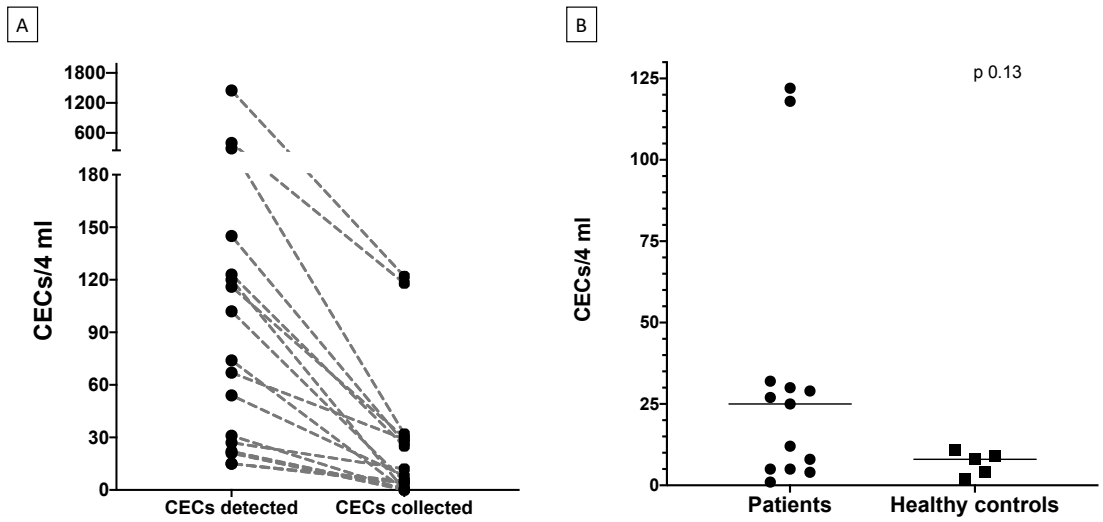


Figure 15: (A) Scatter dot plot for the CECs detected and collected in 4 ml of peripheral blood. (B) Scatter dot plot for the CECs detected in 4ml of peripheral blood for patients (black dots) and healthy controls (black squares). Line at median levels. The analysis was performed using the Mann-Whitney test.

5.3 HSPCs collection

A median of $6.15x10^5$ HSPCs/mL were collected in PMF patients (0.7–12.7) using the immunomagnetic CD34 positive selection (Table 12). Conversely, a median of $3.2x10^4$ HSPCs/mL (1.5-25) were collected in healthy controls (p = 0.0001; Figure 16; Table 12).

The number of HSPCs detected was not related with any of the clinical or laboratory variable analyzed, except for having or not PMF and for the grade of bone marrow fibrosis in PMF patients (p 0.005) (Table 15).

Finally, a median of $8.15x10^5$ HSPCs/mL (range: 5.6-10.7x10^5 HSPCs/mL) were collected after alloSCT in those patients who underwent allogenic transplantation.

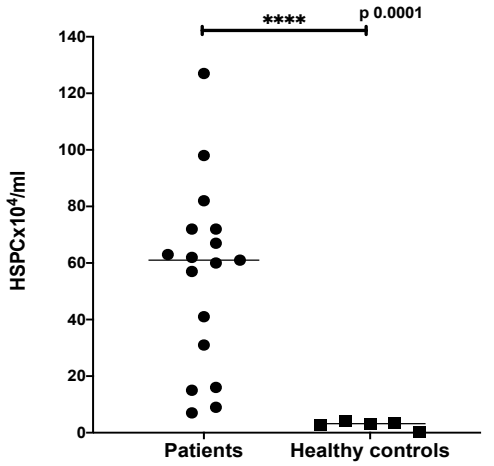


Figure 16: Scatter dot plot for the HSPCs collection in 1ml of peripheral blood for patients (black dots) and healthy controls (black squares). Line at median levels. The analysis was performed using the Mann-Whitney test. **** *p < 0.001*

Features	PMF patients		Healthy controls		*p value*
	HSPCs x10^4/ml; n pts	*p value*	HSPCs x10^4/ml; n pts	*p value*	
HSPCs collected	61,5 (7-127); n=17		3,2 (1,5-25); n=5		***0.0001***
Sex		*0.11*		*NA*	
Male	59 (7-98); n=12		2,6 ; n=1		*NA*
Female	72 (15-127); n=5		3,3 (0,3-4,3); n=4		***0.02***
Age		*0.44*		0,4	
≥ 65 years	63 (9-127); n=9		3,8 (3,2-4,3); n=2		***0.04***
< 65 years	51 (7-82); n=8		2,6 (0,3-3,4); n=3		***0.01***
Time from diagnosis		*0.84*		*NA*	
< 2 year	62 (7-127); n=8		*NA*		
> 2 year	60,5 (9-98); n=9		*NA*		
White blood count		*0.27*		*NA*	
> 10 x 10^9/L	50,5 (7-127); n=7		0		*NA*
≤ 10 x 10^9/l	67 (9-98); n=10		3,2 (0,3-4,3); n=5		***0.001***
Constitutional symptomns		*0.56*		*NA*	
Yes	45,5 (16-60); n=4		*NA*		
No	62 (7-127); n=13		*NA*		
History of thrombosis		*0.74*		*NA*	*NA*
Yes	57 (15-72); n=5		0		
No	61,5 (7-127); n=12		3,2 (0,3-4,3); n=5		***0.0003***
Splenomegaly		*0.06*		*NA*	*NA*
Yes	63 (9-127); n=13		0		
No	23 (7-62); n=4		3,2 (0,3-4,3); n=5		***0.02***
Treatment		*0.25*		*NA*	*NA*
Hydrossiurea	49 (7-127); n=6		0		
No treatment	63 (9-98); n=11		3,2 (0,3-4,3); n=5		***0.0005***
DIPSS		*0.43*		*NA*	*NA*
Low-Interm1	61,5 (9-127); n=10		*NA*		
Interm2-High	60 (7-72); n=7		*NA*		
Grade of BM fibrosis		***0.005***		*NA*	*NA*
1	41 (7-67); n=9		*NA*		
2-3	72 (31-127); n=8		*NA*		
Driver mutations		*0.48*		*NA*	*NA*
JAK2	61 (7-127); n=11		*NA*		
Non JAK2 mutations	46,5 (15-72); n=6		*NA*		

Table 12: Impact of patients and healthy controls' characteristics on HSPCs collection. Median and range of HSPCs collected in 1 ml of peripheral blood; n=number; pts=patients; Interm=intermediate; BM=bone marrow. Mann-Whitney test analysis.

5.4 Molecular alterations

From both CECs and CD34+HSPCs, the DNA was extracted and amplified, in order to obtain a quantity suitable for NGS analysis. Thereafter, the samples were analyzed using a 54 PMF-related gene custom panel.

Since the CECs collection was not possible for 4 patients due to technical issues, the molecular analysis was performed on 13 PMF patients and all the 5 healthy controls (Table 13). 3 of the 4 patients excluded by the molecular analysis were *JAK2 V617F* mutated, while one patient was *MPL* mutated. Therefore, the molecular profile of the 13 PMF patients analyzed was as follow: 8 patients harbored *JAK2 V617F* mutation, 2 patients were CALR mutated, 1 was MPL mutated, while 2 patients were "triple negative" for MPN driver gene mutations.

Of note, no mutations were found in HSPCs and CECs from healthy controls, in whom known polymorphisms in both the cells subpopulations were only observed. On the contrary, several somatic mutations in both HSPCs and CECs were assessed in PMF patients. Among the molecular alterations registered, only the mutations know to be pathogenic were considered (Table 13). Overall, most mutations found were non-synonymous mutations, while some were frameshift mutations (TET2, KMT2A, BCORL, SF3B1, SFRSF2, ASXL1, CUX1, CSF3R).

Patient	**Disease**	**Driver Gene**	**Sex**	**Age** (years)	**DIPSS risk class**	**WBC** (x10^9/L)	**Prev. thrombosis**	**HSPCs** (x10^4/ml)	**CECs** (n/4ml)	**Mutation on HSPC** *name (mutation; VAF%)*	**Mutation on CEC** *name (mutation; VAF%)*
MYCEC_01	PMF	JAK2	F	81	Intm-2	5,6	No	67	0	---	---
MYCEC_02	PMF	JAK2	M	78	Intm-2	19,5	No	60	29	***MPN driver mutations shared*:** **JAK2 (c.G1849T:p.; 61%)** ***NON MPN driver mutations shared*:** **IDH1(c.A593G:p.; 46.4%)** **ABL1 (c.T2035G:p.; 45.9%)** **TET2 (c.C3781T:p.; 38.2%)** *Somatic mutations*: TET2 (c.822delC:p.; 39.9%) ASXL1(c.1927dupG:p.; 34%) SRSF2 (c.C283G:p.; 38.2%)	***MPN driver mutations shared*:** **JAK2 (c.G1849T:p.; 16%)** ***NON MPN driver mutations shared*:** **IDH1(c.A593G:p.; 19%)** **ABL1(c.T2035G:p.; 5%)** **TET2 (c.C3781T:p.; 32%)** *Somatic mutations*: CSF3R(c.1404dupC:p.;11%) CBLB (c.G1613A:p.; 17%) KMT2A (c.2312dupC:p.; 13%) SRSF2 (c.287dupC:p.; 17%) SETBP1 (c.645dupC:p.;20%)
MYCEC_03	PMF	JAK2	M	72	Intm-2	3,8	Yes	72	118	***MPN driver mutations shared*:** **JAK2 (c.G1849T:p.; 28%)** ***NON MPN driver mutations shared*:** **ASXL1 (c.C1249T:p.; 26%)** **KMT2A (c.C89G:p.; 26%).** *Somatic mutations*: NOTCH1 (c.G6396C:p.; 52%) CEBPA(c.232_233insACCCGC:p.; 45%) ATXR (c.T4997A:p.; 18%)	***MPN driver mutations shared*:** **JAK2 (c.G1849T:p.; 97%)** ***NON MPN driver mutations shared*:** **ASXL1 (c.C1249T:p.; 47%)** **KMTA2 (c.C89G:p.; 20%)** *Somatic mutations*: JAK3(c.G3285T:p.; 62%; c.C2677G:p.; 21%; c.G1611A:p.; 25%) TET2 (c.5254delA:p.; 11%) CUX1 (c.1316dupC:p.; 12%) KMTA2 (c.2312dupC:p.; 30%) FLT3 (c.G1484A:p.; 35%) STAG2 (c.C1802T:p.; 46%)
MYCEC_04	PMF	JAK2	F	64	Intm-1	6,27	No	82	25	*MPN driver mutation*: None ***NON MPN-driver mutations shared*:** **WT1 (c.G362A:p.; 48%)** *Somatic mutations*: PDGRFA (c.C2778T:p.; 55%) NOTCH1 (c.C1023G:p.; 21%) CBLB (c.G1865C:p.; 52%)	*MPN driver mutation*: None ***NON MPN-driver mutations shared*:** **WT1 (c.G362A:p.; 14%)** *Somatic mutations*: U2AF1 (c.G461A:p.; 23%) KDM6A (c.G2056A:p.; 81%) SMC1A (c.G2820A:p.; 25%) ATRX (c.C3817A:p.; 19%)
MYCEC_05	PMF	JAK2	F	85	Intm-1	11	No	127	8	*MPN driver mutation*: JAK2 (c.G1849T:p.; 40%) ***NON MPN-driver mutations shared*:** **KIT (c.T2805A:p.; 45%)** *Somatic mutations*: TET2 (c.C3100T:p.; 41%) SF3B1 (c.T1155G:p.; 55%) ASXL1 (c.1927dupG:p.; 39%) BCORL (c.A1111C:p.28%)	*MPN driver mutation*: None ***NON MPN-driver mutations shared*:** **KIT (c.T2805A:p.; 75%)** *Somatic mutations*: ATXR (c.3031dupA:p.; 33%)
MYCEC_06	PMF	JAK2	M	57	High	10,9	No	7	5	*MPN driver mutation NOT shared*: JAK2 (c.G1849T:p.; 28%) ***NON MPN driver mutations shared*:** **SRSF2 (c.C283G:p.; 37.5%)** *Somatic mutations*: NOTCH1 (c.A311G:p.; 42%) ASXL1 (c.A2957G:p.; 53%) RUNX1 (c.C1193T:p.; 26%)	*MPN driver mutation*: None ***NON MPN driver mutations shared*:** **SRSF2 (c.C283G:p.; 21%)** *Somatic mutations*: JAK2 (c.1498dupC:p.; 20%) RAD21 (c.G1749C:p.; 21%) TP53 (c.G218A:p.; 59.5%)
MYCEC_07	PMF	JAK2	M	74	Intm-1	4,7	No	98	4	*MPN driver mutation*: JAK2 (c.G1849T:p.; 17%) ***NON MPN-driver mutations shared*:** **TP53 (c.G131A:p.; 12%)**	*MPN driver mutation*: None ***NON MPN-driver mutations shared*:** **TP53 (c.G131A:p. 28%)** *Somatic mutations*: ASXL1 (c.C369A:p.; 42%). KDM6A (c.G3409A:p.; 83%)
MYCEC_08	PMF	JAK2	M	78	Intm-1	7,48	No	9	0	---	---
MYCEC_09	PMF	JAK2	M	72	Intm-1	7,34	Yes	57	27	*MPN driver mutation*: JAK2 (c.G1849T:p.; 30.7%) *Somatic mutations*: ATAD3A(c.C1238T:p.; 5%) PDGRFA (c.G1731A:p.; 48%) FLT3 (c.T11A:p.; 27%)	*MPN driver mutation*: None *Somatic mutations*: MPL (c.C812A:p.; 30%) SF3B1 (c.3066dupC:p.; 28.8%; c.2422dupA:p.; 37.6%)

MYCEC_10	PMF	JAK2	M	55	Intm-1	11,5	Yes	41	12	*MPN driver mutation*: JAK2 (c.G1849T:p.; 89%) ***NON MPN-driver mutations shared:*** **SETBP1 (c.C668T:p.; 13%)** *Somatic mutations*: ASXL1 (c.1934dupG:p; 28%; c.A2957G:p.; 47.5%) CSF3R (c.1404dupC:p.; 11%) DNMT3A (c.176dup:p.; 6%) IDH2 (c.435dupG:p.; 9%) U2AF1 (c.A476G:p., 4%)	*MPN driver mutation*: None ***NON MPN-driver mutations shared:*** **SETBP1 (c.C668T:p.; 35%)**
MYCEC_11	PMF	JAK2	M	63	Intm-1	11,35	No	61	0	---	---
MYCEC_12	PMF	CALR	F	61	Intm-1	5,86	No	72	30	*MPN driver mutation*: None ***NON MPN-driver mutations shared***: **NOTCH1 (c.C4197T:p; 47%)**	*MPN driver mutation*: None ***NON MPN-driver mutations shared***: **NOTCH1 (c.C4197T:p; 60%)** *Somatic mutations*: IDH1 (c.G541A:p; 31%) PTEN (c.C1076T:p; 7%) PTPN11 (c.G214A:p; 17,5%) SETBP1 (c.C3860T:p, 14%) ASXL1 (c.1927dupG:p; 49%) STAG2 (c.G2968A:p.; 8%) JAK2 (c.T2267C:p.; 12%)
MYCEC_13	PMF	CALR	M	66	Intm-2	6,78	No	63	122	*MPN driver mutation*: None *Somatic mutations*: FBXW7(c.A221C:p; 47%) GNAS (c.C1268A:p; 33%)	*MPN driver mutation*: None *Somatic mutations*: CSF3R (c.G2485T:p; 9%) TET2 (c.1660dupC:p.; 24.4%; c.1842_1843insA:p.; 14.3%) KMT2A (c.2312dupC:p.;16%) TP53 (c.A182G:p.; 34%)
MYCEC_14	PMF	MPL	M	54	Intm-2	60	No	16	0	---	---
MYCEC_15	PMF	MPL	F	71	Intm-1	7,6	No	15	32	*MPN driver mutation*: MPL (c.G1544T:p.; 14%) *Somatic mutations*: CSF3R (c.T2087C:p.; 49%) ABL1^(c.C2429T:p.; 60%) SRFS2 (c.C283G:p.; 19.5%) ASXL1(c.1927dupG:p.;27.5%)	*MPN driver mutation*: None *Somatic mutations*: TET2 (c.C5167T:p.; 33%) KMT2A (c.C9673G:p.; 14%) ASXL1 (c.C369A:p.; 17%) ATRX (c.4630dupA:p.; 54%) STAG2 (c.T231A:p.; 26%; c.G1126A:p., 79%)
MYCEC_16	PMF	Triple neg	M	64	High	117,06	No	31	1	*MPN driver mutation*: None ***NON MPN-driver mutations shared:*** **TET2 (:c.T2599C:p. 49%;** **c.C5167T:p. 55%)** **NOTCH1 (c.C4930T:p 53%)** *Somatic mutations*: CALR (c.G566C:p; 53%)	*MPN driver mutation:* None ***NON MPN-driver mutations shared:*** **TET2 (:c.T2599C:p. 96%;** **c.C5167T:p. 97%**). **NOTCH1 (c.C4930T:p; 96%)**
MYCEC_17	PMF	Triple neg	M	54	Low	4,81	No	62	5	*MPN driver mutation*: None ***NON MPN-driver mutations shared:*** **SETBP1 (c.A1603G:p.; 6.1%)** *Somatic mutations*: ASXL1 (c.1934dupG:p; 3%) DNMT3A (c. 176dup:p.; 4%) SRSF2 (c.287dupC:p.; 24%) RUNX1 (c.C1193T:p.; 10%)	*MPN driver mutation:* None ***NON MPN-driver mutations shared:*** **SETBP1 (c.A1603G:p.; 42%)**
MYCEC_18	HC	NA	F	35	NA	NA	No	0,3	2	*None*	*None*
MYCEC_19	HC	NA	M	46	NA	NA	No	2,6	11	*None*	*None*
MYCEC_20	HC	NA	F	65	NA	NA	No	3,4	9	*None*	*None*
MYCEC_21	HC	NA	F	77	NA	NA	No	4,3	4	*None*	*None*
MYCEC_22	HC	NA	F	84	NA	NA	No	3,2	8	*None*	*None*

Table 13: **Mutations detected on CECs and HSPCs.** Patients' characteristics and mutations detected on CECs and HSPCs with their respective Variant allele frequency (VAF). The mutations shared by CECs and HSCs are in bold. WBC=white blood cout; M=male; F=female; PMF=Primary myelofibrosis; HC=Healthy control; NA=not applicable; Intm=Intermediate; MPN=Myeloproliferative neoplasms; CEC=Circulating endothelial cells; HSPC=Hematopoietic stem and precursors cells

In PMF patients, the previously identified MPN driver mutations were confirmed by NGS on HSPCs in all cases, except for one JAK2-mutated patients and for the two CALR-mutated patients, who presented CALR mutation under the detection limit.

In HSPCs, 27 of the 54 genes analyzed were mutated, with a median of 4 mutations (1–7) per cell and a variant allele frequency (VAF) of 4%, at least (Figure 17). Overall, seven patients harbored high molecular risk mutations (ASXL1, IDH1/2, SFRSF2, EZH2) on HSPCs. The most frequent mutated gene were *JAK2* and *ASXL1* (7 patients each), followed by *NOTCH1* (5 patients), *SRSF2* (4 patients)

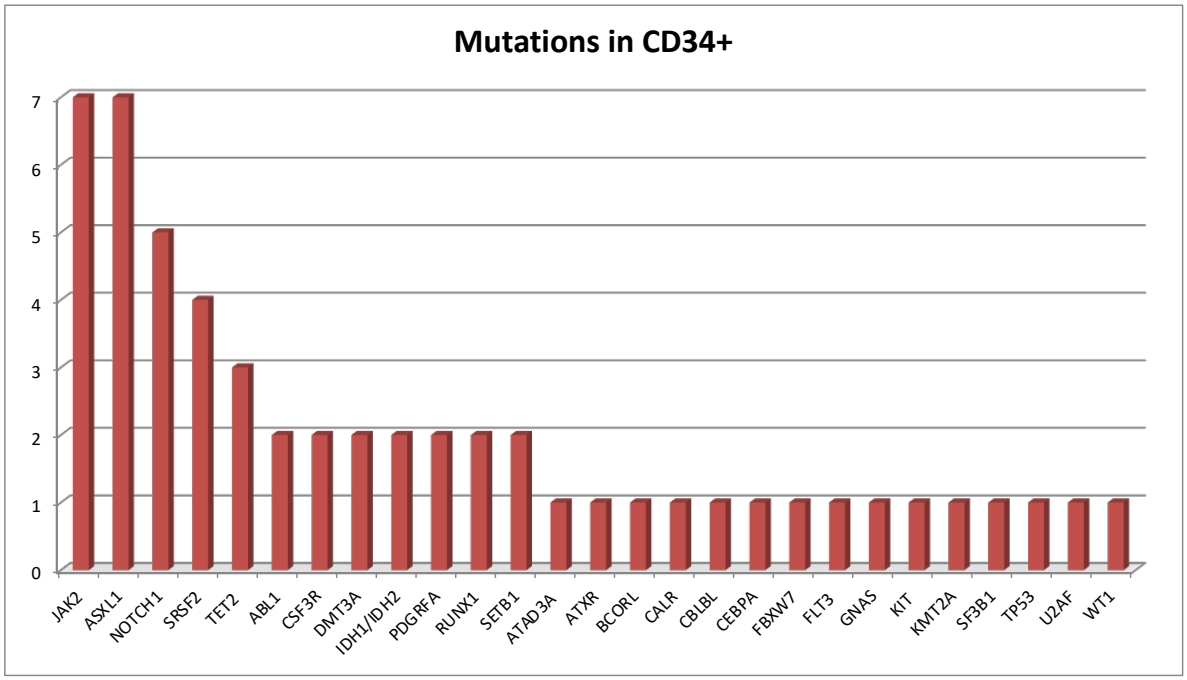
and *TET2* (3 patients). Interestingly, among the most frequent genes mutated, *ASXL1* and *SRFS2* belong to the high risk mutations described by Vannucchi and collegues[14]. In addition, *ASXL1* mutation is frequently associated with *JAK2* mutation (5/7).

Surprisingly, all the CECs from PMF patients hold at least 1 mutation related with myeloid malignancies, with a median of 4 mutations/patient (range: 1-9) (Figure 18). Overall, 28 different genes were mutated in CECs, with a VAF of 5%, at least. The *JAK2 V617F* PMF driver mutation was found in 2 of the 8 *JAK2+* patients (25%), while neither CALR nor MPL driver mutations were found in CECs. *TET2*, *KMT2A*, *ASXL1*, *TP53*, *STAG2* and *SETBP1* were the most frequently mutated genes in CECs (Figure 18). In particular, *TET2* was mutated in 5 patients, while *ASXL1* and *KMT2A* were altered in 4 patients, as well as *TP53*, *STAG2* and SETBP1 in 3 patients. Interestingly, *TET2* and *ASXL1* are also known to be high frequently mutated in Primary Myelofibrosis[14]. Overall, no relationships were found between the clinical characteristics and the number or type of genes mutated in the CECs (Table 14). The presence of *JAK2 V617F* on CECs was not related with previous thrombotic events, as well as it was not associated to any clinical or laboratory characteristics. The two patients with JAK2+CECs presented with constitutional symptoms and splenomegaly. Considering the blood count, one patient presented with leukocitosis, while the other one had anemia. Considering the impact of harboring "high molecular risk" genes mutations on CECs, no significative clinical or laboratory features seem to be related to the presence of these mutations, maybe also because of the small number of patients. Moreover, a longer follow up will be necessary to see the clinical impact of harboring these mutations on CECs and in general of harboring mutations on CECs.

Features	Mutations >4		HR molecular CECs	
		p value		*p value*
Number	7		6	
Sex (Male)	4 (57%)	*0.56*	4 (67%)	*>0.99*
Age (≥ 65 years)	4 (57%)	*>0.99*	4 (67%)	*0.59*
Time from diagnosis (> 2 year)	4 (57%)	*>0.99*	3 (50%)	*>0.99*
White blood count (> 10 x 10^9/L)	2 (29%)	*0.59*	2 (33%)	*>0.99*
History of thrombosis	1 (14%)	*0.56*	1 (16%)	*>0.99*
Splenomegaly	5 (71%)	*>0.99*	4 (67%)	*>0.99*
Treatment (Hydrossiurea)	3 (43%)	*>0.99*	3 (50%)	*>0.99*
DIPSS (Interm2-High)	4 (57%)	*0.27*	3 (50%)	*0.59*
Driver mutations (JAK2+)	4 (57%)	*>0.99*	4 (67%)	*>0.99*

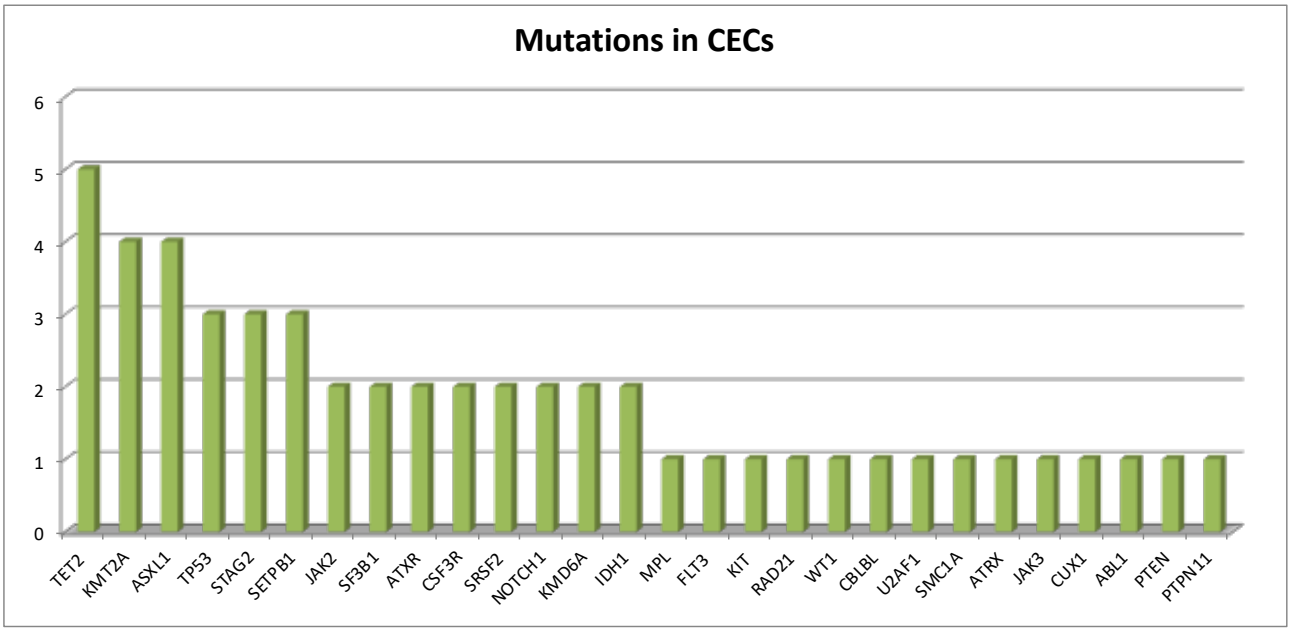
Table 14: **Relationship between patients' characteristics and number and type of mutations on CECs.** The presence of more than 4 mutations/patients on CECs, as well as of molecular high-risk mutations on CECs was not related with any of the clinical/laboratories patients' characteristics analyzed. The cut-off of 4 mutations/patients was based on the median number of mutations on CECs per patient. Interm=Intermediate; CEC=Circulating endothelial cells; HR=high-risk

When comparing mutational profiles of HSPCs and CECs in PMF patients, 10 of 13 patients (77%) shared at least one mutation between the two cells subpopulations (Figure 19). Two of the eight JAK2+ patients shared the MPN driver mutation between HSPCs and CECs, and they also shared the highest number of genes mutations between the two subpopulations: *ABL1*, *IDH1* and *TET2* in one case, and *ASXL1* and *KMT2A* in the other case. No other MPN driver mutations were shared between CECs and HSPCs. 8 patients shared only NON MPN-driver somatic mutations between the two cells subpopulations. In details, *TET2* and *NOTCH1* in one case, and individual paired mutations in *TP53*, *KIT*, *SRSF2*, *SETBP1* (twice), *NOTCH1* and *WT1*, in the other 7 patients.



	MyCEC_02	MyCEC_03	MyCEC_04	MyCEC_05	MyCEC_06	MyCEC_07	MyCEC_09	MyCEC_10	MyCEC_12	MyCEC_13	MyCEC_15	MyCEC_16	MyCEC_17
Molecular Marker	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	CALR	CALR	MPLW515L	Triple neg	Triple neg
JAK2	■	■		■	■	■	■	■					
CALR													
MPLW515L											■		
CALR c.G566C:p												■	
MPL c.C812A:p													
ATAD3A							■						
PDGRFA			■				■						
FLT3							■						
KIT				■									
TET2	■			■								■	
SF3B1				■									
ASXL1 c.1927.dupG:p	■	■		■							■		
ASXL1 c.C1249T:p													
ASXL1 c.A2957G:p					■			■					
ASXL1 c.1934dupG:p					■			■					■
BCORL				■									
ATXR		■											
FBXW7										■			
GNAS										■			
CSF3R								■			■		
KMT2A		■											
TP53						■							
JAK2 c.1498dupC:p													
RAD21													
SRSF2 c.C283G:p	■				■						■		
SRSF2 c.287dupC:p													■
NOTCH1		■	■		■				■			■	
RUNX1					■								■
WT1			■										
CBLBL			■										
U2AF1								■					
KMD6A													
SMC1A													
CEBPA		■											
JAK3													
CUX1													
STAG2													
IDH1	■												
ABL1	■										■		
PTEN													
PTPN11													
SETBP1								■					■
IDH2								■					
DNMT3A								■					■

Figure 17: Molecular alterations discovered on HSPCs. On the top the frequency of mutated genes on HSPCs. At bottom, the molecular alteration detected for each PMF patient.



	MyCEC_02	MyCEC_03	MyCEC_04	MyCEC_05	MyCEC_06	MyCEC_07	MyCEC_09	MyCEC_10	MyCEC_12	MyCEC_13	MyCEC_15	MyCEC_16	MyCEC_17
Molecular Marker	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	JAK2V617F	CALR	CALR	MPLW515L	Triple neg	Triple neg
JAK2	■	■											
CALR													
MPLW515L													
CALR c.G566C:p													
MPL c.C812A:p							■						
ATAD3A													
PDGRFA													
FLT3		■											
KIT				■									
TET2	■	■								■	■	■	
SF3B1	■						■						
ASXL1 c.1927.dupG:p		■							■				
ASXL1 c.C3860T:p						■					■		
ASXL1 c.A2957G:p													
ASXL1 c.1934dupG:p													
BCORL													
ATXR				■							■		
FBXW7													
GNAS													
CSF3R	■									■			
KMT2A	■	■				■				■	■		
TP53					■					■			
JAK2 c.1498dupC:p					■								
RAD21					■								
SRSF2 c.C283G:p					■								
SRSF2 c.287dupC:p	■												
NOTCH1									■			■	
RUNX1													
WT1			■										
CBLBL	■												
U2AF1			■										
KMD6A			■			■							
SMC1A			■										
ATRX			■										
CEBPA													
JAK3		■											
CUX1		■											
STAG2		■							■				
IDH1	■								■				
ABL1	■												
PTEN									■				
PTPN11									■				
SETBP1								■	■				■

Figure 18: Molecular alterations discovered on CECs. On the top the frequency of mutated genes on CECs. At bottom, the molecular alteration detected on CECs for each PMF patient.

The most frequently mutated genes shared between CECs and HSPCs were *JAK2*, *ASXL1*, *TET2*, *NOTCH1*, *SETBP1* and *SRSF2* (Figure 20). All these genes were shared twice. *JAK2*, *ASXL1*, *TET2* and *SRSF2* are also known to be the most frequently mutated genes in Myelofibrosis[14]. We also identified individual paired HSPC/CEC with shared mutations in *TP53*, *KIT*, *KMT2A*, *IDH1*, *WT1* and ABL1. One patient shared 4 mutations between HSPC and CEC, while 4 and 2 patients shared 1 and 2 mutations, respectively. Four of the seven molecular high-risk patients shared mutations both on CECs and HSCs. The shared gene mutations were: *JAK2, ASXL1, KIT, SRSF2, IDH1, TET2,* and *ABL1*. In one case the shared genes mutations were 4. In addition, some patients presented different mutations in the same "high molecular risk" gene (g.e. ASXL1 and SRSF2 in patients MyCEC-15 and MyCEC-02, respectively).

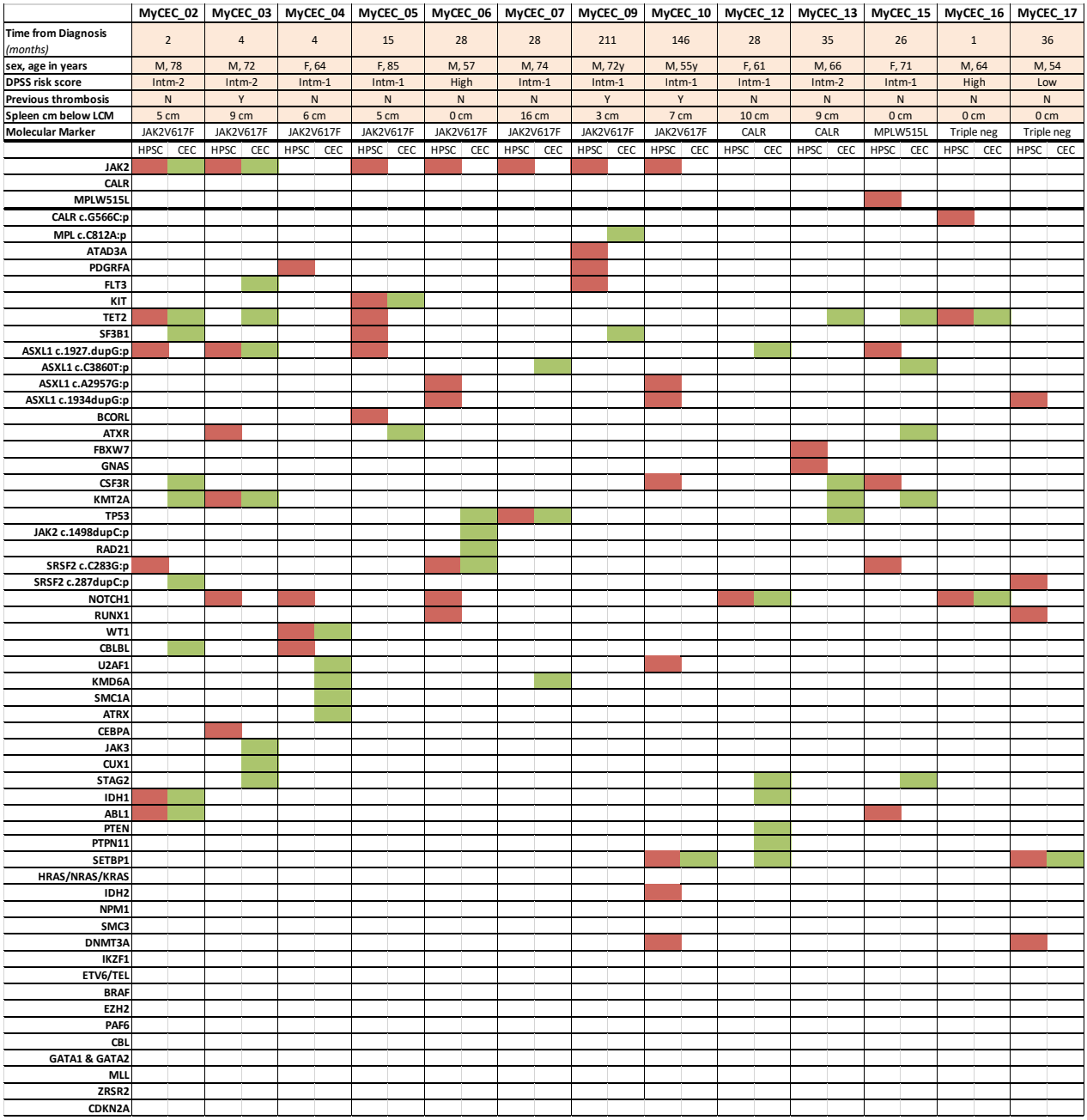


Figure 19: **Molecular profiles of both CEC and HSPCs in patients with PMF.** The molecular lesions found in the HSPCs are in Red, while in Green the ones discovered in the CECs. At the top of the table the clinical characteristics of patients, who successfully recovered CECs.

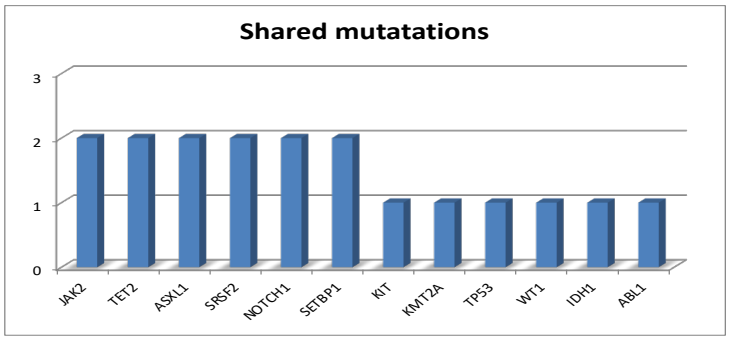


Figure 20: Genes mutated shared between HSPCs and CECs

Notably, the two patients who subsequently underwent alloSCT presented both old and new mutations on HSPCs after the hematopoietic stem cell transplantation (Figure 21). Interestingly, some of the new mutations on HSPCs were also shared with CECs (two in one patient [*IDH2* and *U2AF1*], and one in the other patient [DMT3A]). In addition, CECs analyzed from patients after alloSCT presented also their own gene mutations, not shared with HSPCs (*ETV6* and *NRAS* on one case, *SETBP1* in the other case).

	PRE alloSCT MyCEC_10		*POST alloSCT* MyCEC_10		*PRE alloSCT* MyCEC_17		*POST alloSCT* MyCEC_17	
Sex, Age in years	M, 55y		M, 55y		M, 54		M, 54	
DPSS risk score	Intm-1		Intm-1		Low (--> Intm-2)		Low (--> Intm-2)	
Previous thrombosis	Y		Y		N		N	
Spleen cm below LCM	7 cm		7 cm		0 cm		0 cm	
Molecular Marker	JAK2V617F		JAK2V617F		Triple neg		Triple neg	
	HPSC	CEC	HPSC	CEC	HPSC	CEC	HPSC	CEC
JAK2								
CALR								
MPLW515L								
CALR c.G566C:p								
MPL c.C812A:p								
ATAD3A								
PDGRFA								
FLT3								
KIT								
TET2								
SF3B1								
ASXL1 c.1927.dupG:p								
ASXL1 c.C3860T:p								
ASXL1 c.A2957G:p								
ASXL1 c.1934dupG:p								
BCORL								
ATXR								
FBXW7								
GNAS								
CSF3R								
KMT2A								
TP53								
JAK2 c.1498dupC:p								
RAD21								
SRSF2								
NOTCH1								
RUNX1								
WT1								
CBLBL								
U2AF1								
KMD6A								
SMC1A								
ATRX								
CEBPA								
JAK3								
CUX1								
STAG2								
IDH1								
ABL1								
PTEN								
PTPN11								
SETBP1 c.A1603G:p								
SETBP1 c.G1193T:p								
HRAS/NRAS/KRAS								
IDH2								
NPM1								
SMC3								
DNMT3A c.176dup:p								
DNMT3A c.327duoG:p								
IKZF1								
ETV6/TEL								
BRAF								
EZH2								
PAF6								
CBL								
GATA1 & GATA2								
MLL								
ZRSR2								
CDKN2A								

Figure 21: Molecular profiles of both CEC and HSC in patients with PMF who underwent alloSCT. The molecular lesions found in the HSPCs are in Red, while in Green the ones discovered in the CECs. At the top of the table some of the patients' clinical characteristics (patient MyCEC_17 had DIPSS Low at the enrollment and then Intm2 at the time of alloSCT), and the time according to alloSCT: before alloSCT in light blue, while after alloSCT in violet. Intm=Intermediate

Considering the polymorphic alleles, in the loci analyzed we didn't find loss of the heterozygosity (LOH) in HSPCs in any PMF patients, while the CECs from 3 out of 13 patients presented LOH in different loci (GATA2 C15G; P5P; PDGFRA C2472T; V824V; and JAK2 G2490A; L830L on MyCEC04, MyCEC09 and MyCEC06 patients, respectively).

At baseline, no clinical differences were found between patients who shared mutations in HSPCs and CECs and those who did not (Figure 22).

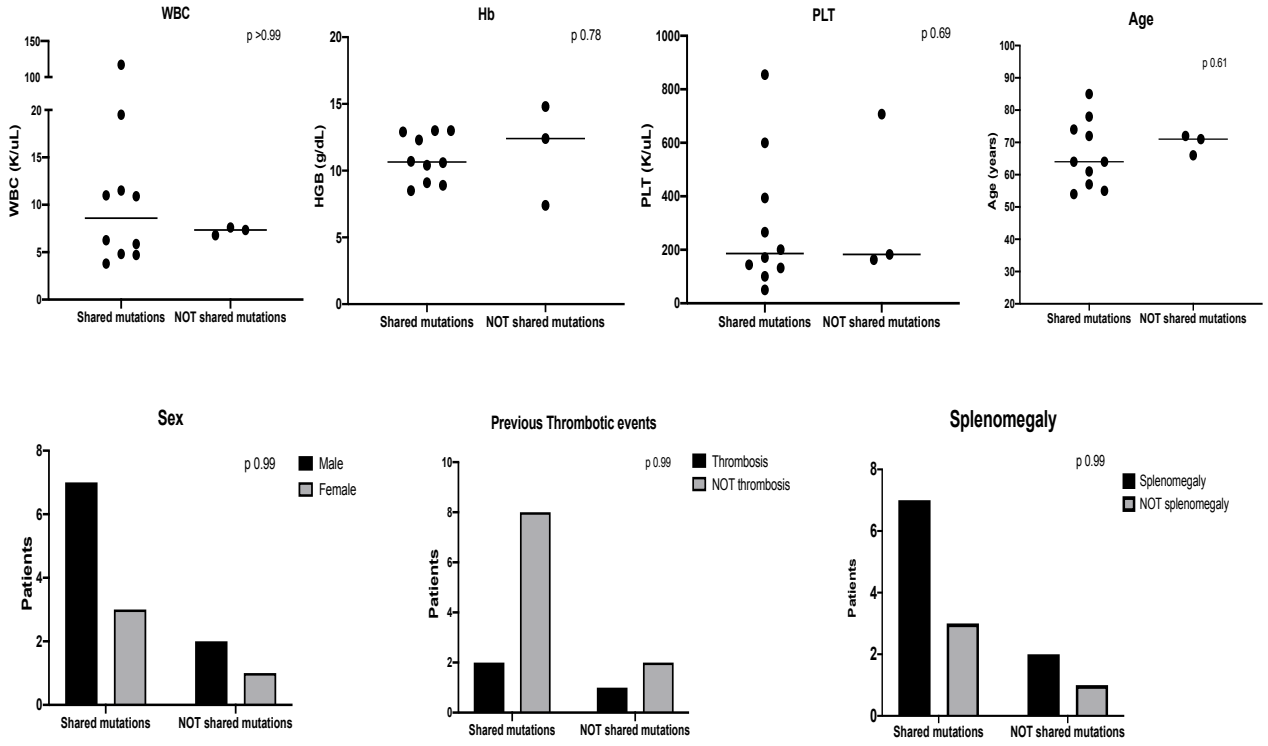


Figure 22: Patients' clinical characteristics according to harboring or not shared mutations between CECs and HSPCs. No significative difference in were found between patients who shared mutations both in HSCs and CECs and those who did not share molecular alterations.

Notably, patients with the samples collected within 1 year from PMF diagnosis presented a higher number of shared mutations (p = 0.03) (Figure 23-A). In particular, the patients who shared the highest number of mutated genes were studied within 4 months from diagnosis. Interestingly, also the patients with JAK2+ CECs shared a higher number of mutated genes between HSPCs and CECs (Figure 23-B).

Patients who shared somatic mutations between the two cells populations had similar follow up to those who did not share any mutations (p 0.09). The presence of shared mutations between CECs and HSPCs did not apparently impact on outcome, neither for the overall survival (p = 0.21) nor for the acute myeloid transformation cumulative incidence (p = 0.50) (Figure 24). No vascular events were observed in all patients during the follow up.

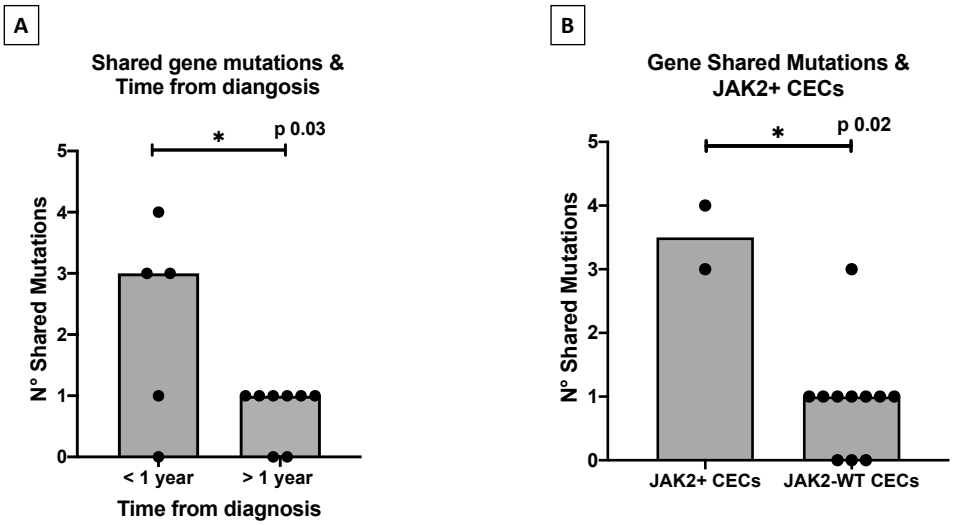


Figure 23: **Number of shared mutations between CECs and HSPCs, according to the time from diagnosis (A) and the presence of *JAK2 V617F* mutation on CECs (B).** Patients enrolled within 1 year from PMF diagnosis shared a higher number of mutations between the two subpopulations compared with patients enrolled after 1 year (p = 0.03), as well as those patients who harbored the *JAK2 V617F* mutation on CECs had a higher number of shared mutations between the two cells populations (p 0.02).

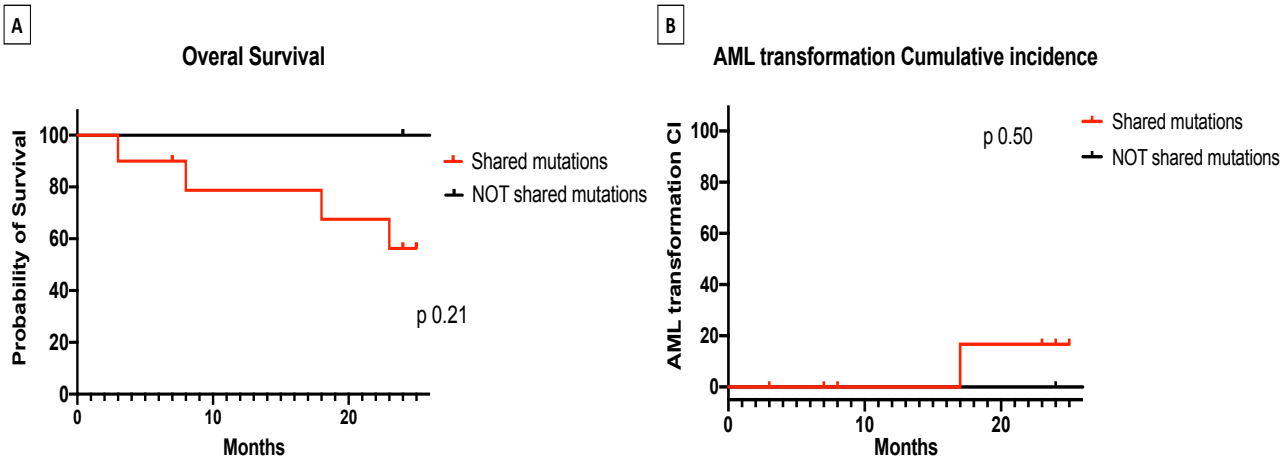


Figure 24: **Impact of harboring shared mutations between CECs and HSPCs on clinical outcome of the PMF patients.** The presence of shared gene mutations (red line) did not impact on overall survival (A), neither on Acute myeloid transformation cumulative incidence (B). Analysis was performed using Gray's Test.

6. DISCUSSION

Primary myelofibrosis is a Myeloproliferative neoplasm characterized by a high rate of vascular complications[109,110,266] and increased bone marrow and spleen vascularity[267]. Several studies have tried to understand the mechanisms underlying these events. However, even if significant advances have been made in understanding the pathogenesis of this disease, the causes of vascular complications in PMF remains uncertain and have been subjects of debate and speculation[112]. Recently, new insights into factors contributing to the development of thrombotic and hemorrhagic events in PMF patients have become available[268], including the role of endothelial cells that contain the MPN driver mutations. *JAK2 V617F* mutation has been detected in endothelial cells captured by laser microdissection[219,242] and in endothelial precursors cells [214,218,245] in patients diagnosed with myeloproliferative neoplasms, suggesting a link between the presence of *JAK2 V617F* in Endothelial cells and the high incidence of vascular events in these diseases. However, the concerns on defining the "true" circulating endothelial precursors on one hand, and the technical limitations of studying "in vivo" mature endothelial cells captured by laser microdissection on the other hand, don't allow to clearly validate the endothelium involvement in PMF pathogenesis.

In the MyCEC study, we wanted to investigate the role of endothelium in Primary Myelofibrosis, comparing the molecular profile of paired CD34+ hematopoietic stem and progenitors cells and circulating endothelial cells from PMF patients to trace a biological and possibly a pathogenetic link between these two cell populations in myelofibrosis.

Physiologically, endothelial cells line the interior surface of blood vessels and they are responsible for maintaining vascular integrity and generating an anti-thrombotic surface[269]. Because of its anatomic characteristics, the study of the endothelial compartment is very difficult and laborious. Indeed, most of the published papers are "ex-vivo" studies on circulating endothelial precursors (EPC). However, the definition of EPC is controversial and, therefore, the significance of such findings have been questioned by some authors[214,217,219]. Historically, EPCs are mononuclear cells which may be distinguished into colony forming unit–endothelial cells (CFU-ECs) and endothelial colony-forming cells (ECFCs). The first studies have shown the *JAK2 V617F* mutation on the CFU-ECs[143,196,270]. However, more recently, Yoder et al[137] clearly settled that CFU-ECs, acknowledged by many authors as endothelial in origin[132], derive from the hematopoietic system, with no ability to form secondary endothelial colonies in vitro or new vessels in vivo. In contrast, ECFCs belonged to the endothelial lineage, showing a vessel-forming capacity in vivo and being able to form cells endothelial colonies[137]. Until now, it is a matter of debate if ECFCs can act as endothelial progenitor cells and harbor the *JAK2 V617F* mutation in PMF patients. Several studies have tried to investigate this hypothesis, but the results are in contrast. Piaggio et al. have repeatedly documented that circulating ECFCs lack the *JAK2 V617F* mutation[214], as well as Rosti and colleagues further demonstrate the absence of mutations in the ECFCs resident in the spleen[219]. More recently, Guy and colleague confirmed the absence of JAK2 mutations on ECFC from MPN patients[222]. Conversely, Teofili et al. have shown that ECFCs from patients with myeloproliferative disorders can carry the *JAK2 V617F* mutation, speculating that a mutated endothelium could derive from a mutated common progenitor cell[218]. Notably, mutated ECFCs were detected only in patients with a previous history of thrombotic events[218].

Trying to overcome the discussion on the EPCs, Sozer[271] and Rosti[219] have analyzed

mature endothelial cells captured by laser microdissection, and they have shown that they may harbor the *JAK2 V617F* mutation. However, ethical reasons prevent accessibility to extra-hematopoietic and extra-splenic tissues, thus limiting the possibility of searching for mutated endothelial cells in organs not involved in active neo-angiogenesis (e.g., the spleen of PMF patients) and further validate these results.

In the present study, we decide to investigate the relationship between endothelial cells and myelofibrosis studying the circulating endothelial cells, which are matured differentiated cells deriving from the turn-over of endothelial liar. In particular, we wanted to investigate the role of CECs in Myelofibrosis and whether CECs may harbor PMF-associated somatic mutations and eventually share them with paired hematopoietic precursors (HSPCs).

The MyCEC study is characterized by several novelties in comparison to the previously published works. We can summarize these innovations in two main aspects: (1) the use of a completely different methodology for studying the interaction between endothelium and myelofibrosis using mature circulating endothelial cells (i.e. through the use of CECs), and (2) the molecular study of CECs and paired HSPCs with a 54-gene panel. Indeed, for the first time, endothelial cells were investigated for other PMF-associated somatic mutations besides *JAK2 V617F*, and the somatic mutational profile of the CECs isolated from PMF patients have been compared with the same one of paired HSPCs.

Considering the first innovative aspect, i.e. the method of study, we decide to overcame the discussion on the "true" endothelial precursors analyzing mature circulating endothelial cells collected by CellSearch/DEPArray system. Thanks to the high sensitivity and efficacy of CellSearch system in detecting CECs (CECs were detected in all samples) and of DEPArray system in sorting them (collection successful rate: 82%), we were able to overcome also the limits and the ethical concerns of using laser microdissection for studying mature endothelial cells. The true endothelial origin of CECs detected with CellSearch system was previously confirmed by Smirnov and colleagues[190] analyzing the gene expression profile of CECs isolated with this methodology. Among the genes overexpressed in CECs from cancer patients, some of them were known to be associated with endothelial function: TIMP2 (tissue inhibitor of metalloproteinase, which regulates angiogenesis), THBD (thrombomodulin, an endothelial cell surface glycoprotein), ENG or CD105 (endoglin, a membrane glycoprotein primarily associated with human vascular endothelium), VEGF (vascular endothelial growth factor), and CD146, a gene that encodes endothelial cell adhesion protein that is also used to capture CECs from peripheral blood.

The CellSearch/DEPPArray technology combine the two traditional methods used to isolate CECs (i.e., anti CD146-immunomagnetic and immunofluorescent selection) and it's the only single cell detection method approved by Food and Drug Administration[272]. Being a semi-automated system, it guarantees standardization in CECs identification and high-level of reproducibility, specificity and sensitivity [27,34], overcoming the lack of standardization of the other CECs detection methodologies. Cells were identified as CECs when they presented the following surface immunophenotype: CD146+, CD105 +, DAPI+ and CD45-. Even if there is no consensus on markers that define the CECs, the phenotype that we have used is the most accepted and recognized by the different groups.

Comparing to the previous methods used for CECs isolation, CellSearch technology has some

advantages. Indeed, although CD146+ immunomagnetic separations [147] is the most widely used method to isolate CECs, it has some limitations. The first limit is that also EPC and mononuclear cells may express CD146 and, therefore, the specificity of this method is limited. Then, it is a manual-based isolation and thus largely dependent on researcher's experience. On the other hand, although immunofluorescent selection by flow cytometry allows to select the EPC and to distinguish mature CECs from other cells with overlapping expression of antigens (e.g., lymphocytes, hematopoietic progenitor cells), however, no consensus there is on which surface markers may better identify CECs. In addition, the lack of standardization between different laboratories remains a main limitation of immunofluorescent selection. In the past, all these features have led to a wide variation in the reported range of CECs in the literature (1–5,700 per mL), making the interpretation and comparison of existing studies quite difficult, if not impossible[180,273,274] and, more importantly, hampering future investigations. Combing the two methods with the CellSearch system, we were able to overcome some of the limitations of the two technologies commonly used for CECs selection, allowing to have a high sensitivity and specificity reproducible method, with a reduced operator-dependency.

The CellSearch has been confirmed as a reliable and effective method. Indeed, CECs were detected in all the subjects. PMF patients had higher levels of CECs compared with healthy controls (18.5 CECs/ml vs. 4.25 CECs/ml; p 0.002; Figure 13). This result was consistent with previous findings[165], suggesting an endothelium damage in PMF[275]. Conversely, CECs levels are generally very low in healthy controls, resulting from the physiological endothelial turnover. Our data in healthy controls were in line with previous findings in healthy individuals. CECs levels have been reported elevated in several disease characterized by a high endothelial turnover (myocardial infarction, infectious vasculitis, kidney transplant rejection, and cancer), reflecting vascular damage or neo-angiogenesis process[182,188]. Previous studies had shown that female may have a higher level of CECs [276], while some others not[165,277]. Considering the clinical characteristics, in our cohort the number of CECs detected was not related with any of the variable analyzed, including gender.

CECs collection was performed using the DEEPArray system, which is an image-based cell-sorting technology. It combines microelectronics and microfluidics, in an highly automated platform, enabling a simple and reliable way of isolating pure, single, viable rare cells from an heterogeneous sample, with unprecedent purity for molecular analysis[258,278,279]. In our cohort, the DEPArray technology allow to collect the CECs in almost all subjects (18 of 23 samples, 82%), despite the low number of CECs and the presence of unspecified PE-positive debris, which made the recovery very challenging. It is not clear what these debris were, but they could have been apoptotic cells or cell fragments. However, despite the presence of PE-debris, target single cells were selected as positive for CD105-PE mean intensity and negative for CD45-APC mean intensity. As expected, the median of CECs collected was inferior to the one obtained during the CellSearch isolation (Figure 15). No significative differences were found between patients from whom we were able to collect CECs and those we were not. Moreover, no significative correlations were found between the number of CECs collected and the clinical characteristics of the patients (Table 11).

As expected, PMF patients had also a significative higher number of CD34+ HSPCs compared with healthy control, confirming the hyperproliferation of hematopoietic precursors in Primary Myelofibrosis[280]. Neither clinical nor laboratory characteristics influenced the number of HSPCs

collected, except for bone marrow fibrosis in PMF patients (Table 12). This data was expected because the higher grade of bone marrow fibrosis favored the migration into peripheral blood of HSPCs, resulting in higher circulating CD34+ hematopoitic cells[281].

Since the CECs collection was not possible for 4 patients due to technical issues, the molecular analysis was performed on 13 PMF patients and all the 5 healthy controls. The first significant molecular result of our study was that only the CECs from PMF patients presented MPN-related genes mutations, while no genomic alterations were found in the CECs isolated from the healthy controls (Table 13). These findings strongly suggest that the acquisition of myeloid-associated genes mutations is strictly related to the PMF development.

Surprisingly, all PMF patients presented CECs with at least one mutation on genes known to be related with myeloid malignancies. It was the first time that cells from endothelium were investigated with a panel of MPN-related genes besides *JAK2 V617F*. Notably, 28 different genes of the 54 genes panel were found to be mutated in CECs from PMF patients. This result was completely unexpected and highlights the involvement of endothelium in PMF development. Interestingly, TET2 and ASXL1, which are known to be high frequently mutated in myelofibrosis[14], were also among the most frequently mutated genes in CECs. Moreover, some “high molecular risk” genes were found mutated in CECs, as ASXL1, SRSF2 and IDH1. Overall, no relationships were found between the clinical characteristics and the number, or the type of genes mutated in the CECs. However, the small sample size and the short follow-up don’t allow to make any speculations about the impact of these mutations on endothelial cells.

Considering the molecular profile of HSPCs, the previously identified MPN driver mutations were confirmed by NGS on HSPCs in all cases, except for one JAK2-mutated patients and for the two CALR-mutated patients. The absence of CALR on HSPCs analyzed may derive from the know technical difficulties on detecting this mutation with NGS[282,283]. No mutations were found on HSPCs in healthy controls. In PMF patients, 27 of the 54 genes analyzed in HSPCs were mutated, with a median of 4 mutations (1–7) per patients. The frequency distribution of gene mutated was in line with previous studies on PMF patients[14]. Interestingly, among the most frequent genes mutated, *ASXL1* and *SRFS2* belong to the high risk mutations described by Vannucchi and collegues[14]. More than half of patients (n=7) presented a high molecular risk mutation (ASXL1, IDH1-IDH2, SRSF2, EZH2) on HSPCs. Furthermore, the high frequency of mutations in *ASXL1*, *TET2* and *SRFS2*[14,284] and the *ASXL1* association with *JAK2* mutation, are also in line with what was previously described in PMF patients[14].

Interestingly, when we have compared the molecular profile of HSPCs with the paired CECs, several myeloid-associated mutations were shared between the two cells populations in PMF patients. Indeed, 77% of patients (10 out of 13) shared at least one mutation between the two cells subpopulations. To the best of our knowledge, this is the first time that the same somatic mutations besides *JAK2 V617F* were described both on endothelial cells and hematopoietic progenitor cells.

Considering the MPN driver mutations, 2 of the 7 JAK2+ HSPC patients (28.6%) shared the *JAK2 V617F* between HSPCs and CECs, while neither MPL nor CALR mutations were detected in the CECs. Notably, the patients with JAK2+ HSPCs/CECs shared also the higher number of gene mutations between the two cells subpopulations (4 and 3, respectively). The JAK2 mutation was previously described on mature endothelial cells captured by laser microdissection, while it is argued if

endothelial precursors may or not harbor it. Interestingly, also in previous published studies not all the patients analyzed presented the JAK2 mutations on endothelial cells. This data suggests that the endothelium of patients with myeloproliferative neoplasms may be composed by a mix of wild-type and JAK2 mutated Endothelial cells. CEC derive from the whole-body vessels, thus from both tissues involved and not by the disease. Therefore, the mutated CECs may represent a very low fraction of all CECs, making difficult to identify the mutations with NGS. All these aspects may explain why we did not observe the JAK2 driver mutation in the CECs of all patients. Overall, the presence of the *JAK2 V617F* on CECs was not related to any patient or disease characteristic, including a previous history of thrombosis. These findings are in line with the observations of Sozer[242] and Rosti[219], while differ from Teofili's study, in which the *JAK2* positive endothelial precursors (ECFCs) were described only in a subset of patients with thrombosis[218].

Considering the non-driver MPN somatic mutations in the CECs, ASXL1, TET2 and SRSF2 genes were among the most frequently shared mutations and are also known to be the most frequently mutated genes in Myelofibrosis[14]. Notably, the discovery that CECs and HSPCs shared molecular alterations other than MPN-driver JAK2 mutation is particularly relevant for those patients who harbored other MPN driver mutations or who did not ("triple negative"), highlighting the endothelium involvement in PMF pathogenesis also in these settings of patients, regardless of the MPN driver genes mutational status. Conversely, previous studies explored only the presence of *JAK2 V617F* mutations in endothelial cells.

Considering the clinical characteristics analyzed, no significative differences were found between patients who shared mutations between HSPCs and CECs and those who did not (Figure 22). In addition, the presence of shared mutations or not between the two cells subpopulations did not impact on disease progression or survival (Figure 24).

Interestingly, patients with samples collected within 1 year from PMF diagnosis presented a higher number of shared mutations ($p= 0.03$; Figure 23). These results may suggest that during the disease progression, the PMF clones and the EC clones might independently be lost or acquire growth advantages/disadvantages over time, showing a divergent evolution from a common progenitor cell. At the same time, it may also be possible that patients not sharing somatic mutations on CECs and HSPCs may have a more indolent course resulting in a longer survival, while patients harboring shared mutations between the two cells subpopulations may have an adverse outcome early in the disease course. Additional prospective, systematic and larger studies will be needed to better clarify this aspect.

For the first time, we also compared the molecular profile of paired HSPCs and CECS before and after alloSCT. Of note, the two patients who subsequently underwent alloSCT, presented on HSPC both previous and new mutations, while CECs presented only new mutations. The presence of new gene mutations in both HSPCs and CECs after alloSCT may be due to the presence of clonal hematopoiesis in the alloSCT donors, or, more unlikely, it may be related to the chemotherapy and treatments related to the alloSCT itself. Before alloSCT, the two patients presented both shared and unshared mutations between the two cells subpopulations. Notably, also in the post-alloSCT setting, HSPCs and paired CECs shared at least one mutation (1 in one patient and 2 in the other one). These results highlight, once again, the relationship between hematopoietic precursors cells and

endothelial cells. However, the low number of after-alloSCT analyzed patients didn't allow any other speculations.

Finally, the study of polymorphic alleles showed that LOH is a rare phenomenon in the studied setting of PMF patients and it affects only CECs. HSPCs did not present LOH. However, the low number of patients and the limits deriving from the study of only few loci did not allow any speculation on this data.

Altogether, the presence of myeloid-associated mutations in CECs only from PMF patients, the frequency distribution of mutated genes in CECs, superimposable to the ones described in HSPCs in PMF [14], and the high frequency of patients who shared at least one mutation between HSPCs and CECs, support a primary involvement of endothelial cells in PMF. However, how the endothelial cells may acquire myeloid-associated gene mutations remain an open question.

An intriguing hypothesis already proposed in previous studies is that HSPC and endothelial cells may originate from a common mesoderm-derived precursor cell, known as the "hemangioblast"[231]. The term ''hemangioblast'' was initially coined by Murray in 1932[231]. Subsequently, in the late 1990's, some authors described that single mesodermal cells isolated from in vitro differentiating mouse embryonic stem cells could give rise to both blood cells and endothelium[233,234], supporting this theory. On the contrary, several studies have suggested that endothelial and hematopoietic lineages are independently derived from mesodermal cells[285,286]. To date, there is still no conclusive evidence demonstrating that, in higher vertebrates, a hemangioblast does indeed give rise to both endothelium and blood cells in vivo, and, therefore, its existence is still a matter of debate [287,288]. The detection of *JAK2 V617F* in endothelial cells, CECs or EPCs from MPN patients may support this theory. Moreover, the recent evidence that JAK2 mutation was acquired in utero or childhood in MPN patients[243,244] may be at least chronologically consistent with involvement of "hemangioblast" by MPN driver mutations. In addition, the results of the MyCEC study may give new significant elements supporting the Murray's theory. Indeed, (1) the high frequency of patients who shared at least one mutation between CECs and HSPCs (77%), (2) the number of mutations shared per patients (up to 4/patient) besides *JAK2 V617F,* even after alloSCT, and the (3) presence of myeloid-associated mutations on CECs, strongly support the hypothesis of a common precursors between HSPCs and ECs, which might act also as the cell of origin of PMF.

Other mechanisms might explain the detection of myeloid associated mutations in endothelium. One of them refers to the ability of monocytes of generating cells that closely resemble ECs, the so called "endothelial like cells" (ELCs) or angiogenic monocytes[289]. However, in humans it is currently thought that ELCs influence angiogenesis by secreting pro-angiogenic factors, rather than directly participate in neovascularization[246]. Moreover, the high frequency of shared mutations in our cohort and the presence also of different mutations between the two cells subpopulations, make this hypothesis unlikely.

Finally, another possible mechanism might be the fusion of mutated hematopoietic cell with an endothelial cell or the phagocytosis of cell-free DNA or extracellular vesicles[290,291], but also this hypothesis seems very unlikely in our study because of the complexity and variability of the CECs molecular profile.

Regardless of the existence or not of a common precursor, the presence of somatic

mutations in endothelial cells may have important consequences in the insurgence of vascular complications in PMF patients and in the disease development. Indeed, mutated ECs in PMF may represent a "neoplastic" vascular niche in both the bone marrow and the spleen[220], which allow blood cells adhesion, vascular complications and the tumor cell growth, as demonstrated for JAK2-mutated ECs using in vitro and in vivo assays [220–222,224,225,227]. Primary myelofibrosis is also characterized by higher microvascular density both in bone marrow and spleen[292], highlighting the relevance of neo-angiogenesis in this disease, which could be influenced by molecular alterations of endothelial cells, too. Moreover, given the high incidence of thrombosis in the splanchnic area in patients with myelofibrosis[66], genetic abnormalities of endothelial cells might influence the coagulation mechanism locally. Recently, Guy et al[222] evaluate whether vascular EC expression of *JAK2 V617F* is sufficient to promote a pro-thrombotic state, using a "in vitro" model of human endothelial cells overexpressing *JAK2 V617F* and an "in vivo" model of mice with endothelial-specific *JAK2 V617F* expression. Interestingly, these mice displayed a higher propensity for thrombosis, due to a pro-adhesive phenotype deriving from an increased endothelial P-selectin exposure, secondary to degranulation of Weibel-Palade bodies. Therefore, they proposed that *JAK2 V617F*-expressing endothelial cells promote thrombosis through induction of endothelial P-selectin expression. Interestingly, Guadall et al[225] confirmed that *JAK2 V617F*-positive ECs display pro-thrombotic characteristic, using *JAK2 V617F* and JAK2wild-type-induced pluripotent stem (iPS) cells from an MPN patient and redirected these iPS cells towards the endothelial lineage. All these observations reinforce the theory proposed by Teofili of neoplastic vascular niche in patients with myeloproliferative neoplasms. According to Teofili et al[220] *JAK2 V617F* mutated endothelial cells, producing abnormally high levels of adhesion molecules such as ICAM-1, V-CAM, and E-selectin, might represent the "neoplastic" vascular niches where trapped hematopoietic stem cells are forced to proliferate (Figure 25).

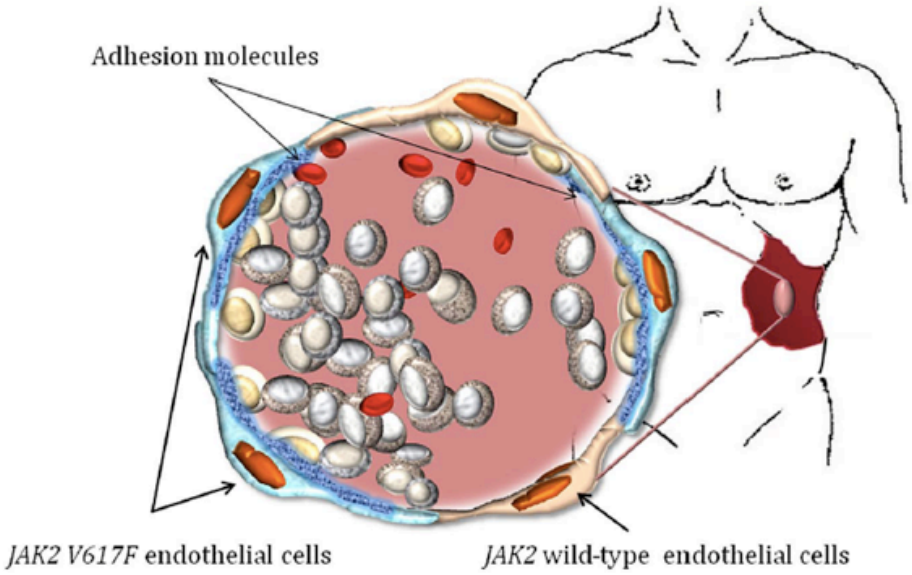


Figure 25: The "neoplastic" vascular niche. The figure illustrates how mutated endothelial cells, producing abnormally high levels of adhesion molecules such as ICAM-1, V-CAM, and E-selectin, might represent the "neoplastic" vascular niches where trapped hematopoietic stem cells are forced to proliferate. Indeed, in spleen microenvironment, neoplastic myeloproliferation and angiogenesis are tightly interrelated. From Teofili et al.[220]

More recently, the Teofili's theory on the "neoplastic" vascular niche was reinforced by some evidence by Zahn and colleagues. Using both in vitro[226] and in vivo[227] models, the authors shown that the *JAK2 V617F*-bearing vascular niche promotes *JAK2 V617F* hematopoietic clonal expansion, while it inhibits WT hematopoiesis. Moreover, these data support the previous reports that the marrow microenvironment of myeloid malignancies is altered to impair normal hematopoiesis, while favoring malignant stem cell expansion[228,229]. Recently, several observations have provided evidence that the vascular niche plays an important role in influencing hematopoietic stem cell dormancy, self-renewal, and proliferation[293,294]. In addition, Zhang has shown how the *JAK2 V617F*-mutant HSPC transplanted in a WT recipient mice are either insufficient to develop a MPN phenotype in the absence of additional disease-promoting mechanism, as for example a *JAK2 V617F*-mutated bearing vascular niche, or require a longer period of time to develop the disease phenotype in WT environment than in mutant environment[227]. However, there are evidence in mouse models indicating the presence of the JAK2 mutation in HSCs alone is sufficient to induce an MPN[283].

Our results support the theory that in MPN patients there could be a "neoplastic vascular niche", constituted by mutated endothelial cells and which may favor vascular complications on one hand, and allow MPN clone growth and expansion on the other hand. In our study, all the PMF patients harbored myeloid-associated somatic mutations besides *JAK2 V617F*, which could play an important role within the vascular niche. Unfortunately, how these mutations can interact with hematopoietic cells and the neoplastic clone is not yet known at present. Our results, however, certainly open new scenarios that will deserve to be investigated to better understand the constitution and role of the vascular niche.

The MyCEC study presented also some limitations. The small number of patients analyzed, as well as the small number of CECs collected in some patients, together with the low sensitivity of NGS are the main limitations to clearly say whether some mutations found in HSPCs and not in CECs, or vice versa, are the result of mutational heterogeneity. Probably, only some of the CECs collected derive from mutated EC involved with the disease and this factor could make further difficult to analyze the molecular profile of the CECs and compare it with the one of HSPCs.

However, on the other hand, we think that the discovery of shared and un-shared somatic mutations, despite the low number of CECs collected and the low NGS sensitivity, highlights the ECs involvement in PMF and reinforce the hypothesis of a common precursor between ECs and HSPCs, as well as put new lights on the theory of a "neoplastic" vascular niche in MPN patients. Increasing the number of patients analyzed, also in the post alloSCT setting, it cannot be excluded that this involvement may be even higher and that the mutations shared between CECs and HSPCs may be more and would help to better investigate the role of endothelium in PMF development. Thus, new and larger studies specifically aimed to evaluate the frequency of HSPCs and CECs shared mutations and its correlation with clinical characteristics of disease are needed.

7. CONCLUSIONS

The MyCEC study aims to investigate the role of endothelial cells in Primary Myelofibrosis. In doing so, we compared the molecular profile of circulating endothelial cells (CEC) with paired hematopoietic stem and progenitors cells (HSPC) in order to (1) evaluate the presence of myeloid-associated somatic mutations on endothelial cells, which may constitute a neoplastic vascular niche and possible explain the higher frequency of vascular complications and angiogenesis in Primary Myelofibrosis, and to (2) explore the theory of a common precursor between endothelial and hematopoietic cells, which may act as the cell of origin of PMF.
With this purpose, we isolated and amplified DNA from paired CECs and CD34+ HSPCs from 17 PMF patients and 5 healthy controls. The DNA from the two cell populations was then investigated with a 54 MF-related genes NGS custom panel. For two patients this analysis was performed also after they underwent allogenic hematopoietic stem cell transplantation (alloSCT).

Previous studies have tried to explore the relationship between endothelium and myeloproliferative neoplasms, investigating whether endothelial cells may harbor the *JAK2 V617F* MPN-driver mutation, using both ex vivo and in vitro studies, on human and mice models as well. Since the endothelial cells line the inner surface of all vessels, the study of the endothelial compartment is challenging, and several approaches have been proposed. Considering the anatomical difficulties in studying endothelium “ex vivo”, most of the published studied focused on the circulating endothelial precursors, but there is no consensus in which type of cells may be considered the “true” endothelial precursor. At the beginning, some authors studied the Colony forming unit-endothelial cells (CFU-EC), but they are now no longer considered true EPCs. Subsequently, several studies focused on the endothelial colony-forming cells (ECFCs), which are now considered potential endothelial precursor cells. Unfortunately, the results of the studies investigating if circulating endothelial precursors cells may harbor or not the PMF driver gene *JAK2 V617F* mutation were in contrast. More recently, some authors found the JAK2 mutation in mature endothelial cells captured by laser micro-dissection, but ethical and technical reasons limited the use of this procedure in clinical practice. Therefore, we decide to investigate the role of endothelium in myelofibrosis using an innovative method in order to overcome some difficulties emerged in the published studies. Indeed, we decide to study the circulating endothelial cells, which are mature endothelial cells deriving from the turn-over of endothelial liar and they are considered as markers of endothelium damage. In order to study the CECs, we used the CellSearch and DEPArray sistems, a non-invasive and standardizable procedure with high sensitivity and efficacy, and which is the only single cell detection method approved by Food and Drug Administration[272]. This technology combines the two historically methods used for CECs detection, i.e. anti CD146-immunomagnetic and immunofluorescent selection. Moreover, the true endothelial nature of the CECs obtained with the CellSearch technology was demonstrated through Gene expression profiles studies, allowing us to be confident in the real endothelial origin of the CECs collected with this methodology. CECs were defined as cells with the following immunophenotype: CD146+DAPI+CD105+CD45-.

In addition to the methodological approach chosen, the MyCEC study is also characterized by the following further innovations: (1) the investigation of the CECs molecular profile using a 54-PMF related genes custom panel; and (2) the comparison of the molecular profile of the CECs with the ones of paired HSPCs.

In the MyCEC study, CECs levels in PMF patients were higher than in healthy controls, corroborating the endothelial involvement in the pathogenesis of the disease and the diffuse vascular damage.

Notably, only PMF patients presented MPN-related genes mutations on the CECs, while no genomic alterations were found in the CECs collected from the healthy controls. These findings strongly suggest that the acquisition of myeloid-associated genes mutations was strictly related to the PMF development.

Surprisingly, all the PMF had mutated CECs with a median of 4 (1–9) mutations/patient. To the best of our knowledge, this is the first time that myeloid-associated mutations other than *JAK2 V7617F* have been detected in endothelial cells. Moreover, 77% of the patients shared at least one mutation between CECs and HSPCs, up to a maximum of 4 shared mutations per single patient. Interestingly, two patients shared the *JAK2 V617F* PMF-driver mutation between the two cell populations, and they were also the patients with the highest number of shared gene mutations between CECs and HSPCs (4 and 3, respectively). Time from PMF diagnosis was also related with the number of shared mutations. Indeed, samples collected within 1 year from PMF diagnosis presented a higher number of shared mutations between the two cells subpopulations. These results may suggest that during the disease progression, the PMF clones and the EC clones might independently be lost or acquire growth advantages/disadvantages over time, showing a divergent evolution from a common progenitor cell. Notably, also in the post-alloSCT setting, HSPCs and CECs shared at least one mutation (1 in one patient and 2 in the other one).

Altogether, the presence of myeloid-associated mutations only in the CECs from PMF patients, the frequency of mutated genes in CECs, and the high frequency of patients (77%) who shared at least one mutation between HSPCs and CECs, support a primary involvement of endothelial cells in PMF. How the endothelial cells may acquire myeloid-associated gene mutations remains an open question. An intriguing hypothesis already proposed in previous studies is that HSPC and ECs may originate from a common precursor cell, known as the “hemangioblast”[231]. The recent evidence that JAK2 mutation was acquired in utero or childhood in MPN patients [243,244] may be at least chronologically consistent with involvement of “hemangioblast” by MPN driver mutations. The results of MyCEC study give new elements supporting this theory. Indeed, (1) the high frequency of patients who shared at least one mutation between CECs and HSPCs (77%), (2) the number of mutations shared per patients (up to 4/patient) besides *JAK2 V617F,* even after alloSCT, and the (3) presence of myeloid-associated mutations on CECs, strongly support the hypothesis of a common precursors between HSPCs and ECs, which might act also as the cell of origin of PMF.

Irrespective of the existence or not of the “hemangioblast”, the use of a 54 genes panel allows us to investigate several PMF-related genes mutations, and not only the *JAK2 V617F* as in previous study, showing an involvement of the endothelium regardless the driver mutation status. The presence of somatic mutations in ECs may have important consequences in the disease development and the insurgence of vascular complications in PMF patients. Indeed, mutated ECs in PMF may represent a “neoplastic” vascular niche in both the bone marrow and the spleen[220], which allow blood cells adhesion, vascular complications and the tumor cell growth, as demonstrated for JAK2-mutated ECs using in vitro and in vivo assays [220–222,224,225,227]. A

longer follow up of our patients and new studies investigating the “neoplastic” vascular niche in humans are needed to validate this hypothesis.

The small number of CECs collected in some patients and the low sensitivity of NGS are the main limitations to clearly say whether some mutations found in HSPCs and not in CECs, or vice versa, are the result of mutational heterogeneity. Probably, only a part of the CECs collected derive from mutated EC involved with the disease and this factor could make difficult to analyze the molecular profile of the CECs and compare it with the one of HSPCs. However, on the other hand, we think that the discovery of shared and un-shared somatic mutations, despite the low number of CECs collected and the low NGS sensitivity, highlights the endothelial cells involvement in PMF and reinforce the hypothesis of a common precursor between ECs and HSPCs.

In conclusion, the MyCEC study through a new methodological approach describes for the first time the genomic mutational profile of both HSPCs and CECs in PMF patients and provides new knowledge on the cell of origin in myeloproliferative neoplasms and the potential role of ECs in the “neoplastic” vascular niche. These preliminary results have also a particular value because they open to further studies aiming to clarify the clinical relevance of the reported mutational status in the two cells populations and provide new insights into the mechanisms for the shared mutations. In doing so, it will be necessary to expand the cases and create an animal model for functional studies.

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