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Mutations and epigenetica in Myeloproliferative Neoplasms

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"…I suggest that you keep your eyes open for things you are not supposed to know about. You may have something to contribute. At a meeting, don't gravitate to the poster that looks the most like something you are working on. Do a clean sweep around the room. Check out all the posters and find the one you know the least about and maybe think you have the least interest in. Now is the time to find out about it. Read it, and then ask the person standing there beside it what it's about and why he or she is interested in it. They will be happy to talk. Don't forget to check out the posters in your own field too - you have to make sure you don't miss out on some new development, but a poster session is a perfect opportunity to find out something brand new…"

Kary Banks Mullis

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1.INTRODUCTION

1.1 MYELOPROLIFERATIVE NEOPLASMS

In 1951, William Dameshek introduced the term ”myeloproliferative disorders (MPD)” to encompass polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myelogenous leukemia (CML).[1] Demeshek, was the first to suggest that these conditions might be related, recognising both the phenotypic overlap and tendency for phenotypic shift in these diseases.[1-2] The association of the Philadelphia (Ph)-chromosome with CML in 1960, distinguished the other three disorders as “classic” Ph-negative MPD. The first systematic attempt to classify MPD and MPD-like clinicopathologic entities was undertaken by the World Health Organization (WHO) committee for the classification of hematologic malignancies. According to the 2001 WHO classification system, CML, PV, ET, and PMF were included under the category of “chronic myeloproliferative diseases” (CMPD). The CMPD category also included other “nonclassic” MPD-like disorders such as chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia/hypereosinophilic syndrome (CEL/HES), and “unclassified CMPD.”[3] The identification of *BCR-ABL* as a CML-specific genetic event, in the context of CMPD, has facilitated accurate molecular diagnosis and effective targeted therapy. The lack of knowledge, until few years ago, on specific genetic defects in the other *BCR-ABL*-negative classic CMPDs necessitated that diagnosis rest on a combination of bone marrow histology and a few clinical and laboratory findings to distinguish clonal from reactive myeloproliferation and one CMPD from another.

The last 8 years have witnessed fundamental advances in understanding the molecular pathogenesis of classic *BCR-ABL*-negative CMPD, however the defining moment was the identification of an activating mutation in the *JAK2* tyrosine kinase. As a result, WHO diagnostic criteria have been revised, and the term “CMPD” has been changed to “myeloproliferative neoplasms (MPN).” [4-5]

Apart from the *BCR*/*ABL* rearrangement in CML, originated by a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34; q11),[6] or the chimeric *FIP1L1-PDGFRA* mRNA in some forms of eosinophilia, and *kit* mutations in cases with systemic mastocytosis, information concerning molecular abnormalities of MPN has been scanty until 2005, when four international groups described the same acquired point mutation in the *JAK2* gene in most patients with PV and around half those with ET or MF.[7-10] Diverse approaches were used to identify this mutation, comprising dissection of signalling pathways in PV, high throughput sequencing of kinase genes and sequencing of candidate genes within a region of chromosome 9 known to undergo loss of heterozygosity in PV patients. *JAK2*, one of four JAK family cytoplasmic tyrosine kinases (comprising JAK1, JAK2, JAK3 and TYK2), is essential for signalling by the erythropoietin receptor (EpoR) and thrombopoietin receptor (MPL) , and is also involved in signalling through the granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colonystimulating factor and interferon-γ receptors.[7-12] Studies of EpoR indicate that ligand binding results in a

conformation change in the receptor, with consequent phosphorylation of JAK2 and the receptor itself resulting in activation of downstream signalling pathways.[13] JAK2 also plays a vital role in EpoR trafficking, with absence of JAK2 resulting in retention of EpoR within the endoplasmic reticulum.[13] The central role of JAK2 in haematopoiesis is highlighted by a JAK2 knock-out mouse, which dies at embryonic day 12.5 due to a complete absence of definitive erythropoiesis. The JAK2 V617F substitution, resulting from a single base change, affects the pseudokinase (JH2) domain of the protein. This domain is required for both JAK2 activation and inhibition of basal kinase activity. The JAK2 V617F mutation results in substitution of a phenylalanine at a highly conserved residue, and is thought to impair autoinhibition of JAK2, leading to constitutive activation of tyrosine kinase activity. Expression of JAK2 V617F leads to cytokine independent growth of various cytokine dependent cell lines, with constitutive activation of pathways implicated in the control of proliferation, differentiation and cell survival such as STAT5, PI3K/AKT and MAPK.[19-22]

In the following 2 years, additional mutations in *JAK2* and *MPL* were reported (Table 1). These different mutant alleles all result in a gain of function due to the constitutive activation of tyrosine kinase-dependent cellular signaling pathways, particularly of the JAK-STAT pathway.[23-24]

Genetic Abnormality	**Disease**	**Frequency**
BCR-ABL	Chronic myelogenous leukemia	≅99%
*JAK2*V617F	Polycythemia vera	>95%
	Essential thrombocythemia	≅60%
	Primary myelofibrosis	≅60%
	MPN, unclassifiable	≅20%
	Refractory anemia with sideroblasts and thrombocytosis (RARS-T)	≅50%
JAK2 exon 12	Polycythemia vera	≅2%
*MPL*W515L/K	Primary myelofibrosis	≅8%
	Essential thrombocythemia	≅8%[†]
Involving *PDGFRA*	Myeloid neoplasms with eosinophilia	Unknown
	Mast cell disease	Unknown
Involving *PDGFRB*	Myeloid neoplasms with eosinophilia	Unknown
Involving *FGRF1*	Myeloid neoplasms with eosinophilia	Unknown
Involving *KIT* (D816V as the most frequent)	Mast cell disease	Unknown

Table 1. Recurrent Molecular Abnormalities Associated with Myeloproliferative Neoplasms

Transplantation of *JAK2*V617F mutated cells induced a PV-like phenotype in recipient mice, accompanied by leukocytosis of a different extent and eventually followed by changes suggestive of myelofibrotic transformation. More recently, by manipulating expression levels of the V617F allele, mice with an ET-like phenotype were also generated in the presence of low levels of mutated JAK2. Overall, these models indicated that

the *JAK2*V617F mutation is sufficient to induce a MPN-like phenotype in mice and suggested that the level of mutated allele may influence disease phenotype.[14-18]

Mutational frequency of *JAK2*V617F is estimated to be more than 95% in PV, 60% in ET or PMF, 40% to 50% in refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T), whereas it is very rare in AML or MDS. In most patients with PV or PMF, as opposed to a minority of those with ET, the mutation is harbored in a homozygous state, which is accomplished by mitotic recombination. In general, the highest V617F allele burden, that is the level of mutated allele relative to normal allele in a cell suspension such as granulocytes, is found in patients with PV followed by PMF and ET;[19-22] however, such variability in the allele burden does not represent a sufficient criterion for distinguishing among different clinical entities, nor does it satisfactorily help to explain the apparent paradox of "one mutant allele-different clinical phenotypes."[26,27] In fact, how a single V617F mutation can be the basis of different clinical disorders, as in the classic MPN, is still unclear. Interestingly, single nucleotide polymorphisms (SNPs) in *JAK2* have been associated preferentially with the diagnosis of PV, supporting the contribution of inherited host genetic characteristics to MPN phenotypic variability. Regardless, there is evidence to suggest that *JAK2*V617F may not be the initial clonogenic event in MPN and that a "pre-*JAK2*" mutated cell may exist.[26,27] In support of this is also a finding that leukemic blasts in patients who evolve to AML from a pre-existing *JAK2*V617F-positive MPN are often negative for the *JAK2*V617F mutation. Conversely, *JAK2*V617F, or other *JAK2* mutations, are likely a necessary component of the PV phenotype because they are detected in virtually all patients with the disease and are sufficient to reproduce the phenotype in mice. In summary, *JAK2*V617F mutation is integral to the classic MPN, but its exact hierarchical position in pathogenesis and its role in phenotypic variability remain to be clarified.[26,27]

In patients with a clinical picture suggestive of PV and who were found to be negative for the *JAK2*V617F mutation, several genetic abnormalities (ie, mutations, deletions, insertions) have been detected in a short region of *JAK2* exon 12.[23] These mutations, which probably account for less than 2% of patients with PV, affect autonomous cell proliferation and differentiation in a fashion similar to that of the V617F allele. Another recurrent molecular abnormality of MPN is represented by somatic mutations at codon 515 of *MPL*, which, as is the case with *JAK2*V617F, involve early myeloid and lymphoid progenitors. MPL (named after myeloproliferative leukemia virus oncogene homolog) is the receptor for the cytokine thrombopoietin (Tpo) and is highly expressed in early hematopoietic progenitors and in cells of the megakaryocytic lineage. *MPL*W515L induced both cytokine-independent growth and Tpo hypersensitivity in cell lines, resulting in constitutively activated JAK-STAT/ERK/Akt signaling pathways, and caused a PMF-like disease in mice.[24,25]

The gene encoding for the receptor of platelet-derived growth factor A (PDGFRA) is involved in at least four different genetic abnormalities associated with eosinophilia. The most frequent and best characterized abnormality is due to a karyotypically occult microdeletion at chromosome 4q12, where *PDGFRA* is located, resulting in a

chimeric *FIP1L1-PDGFRA* fusion gene. The latter encodes for an aberrantly activated tyrosine kinase as the consequence of disruption of the autoinhibitory activity encoded by *PDGFRA* exon 12, where the breakpoint is located; this constitutively active tyrosine kinase drives autonomous eosinophil progenitor proliferation, possesses transforming properties in vitro, and induces a myeloproliferative disorder with extensive eosinophil proliferation when expressed in transplanted mice. The fusion gene has been demonstrated at the level of hematopoietic stem cell compartment. Also the Beta type of PDGFR has been reported as being involved in rearrangements associated with imatinib-responsive eosinophilia. The *PDGFRB*is located at chromosome 5q31-32 and may fuse with different partners. One of the most common is the *ETV6*/*TEL* gene on chromosome 12p13, which encodes for a transcription factor with nonredundant roles in normal hematopoiesis. The fusion protein constitutively activates the cellular pathways normally associated with PDGFRB signaling and has transforming properties when expressed in cell lines. [29,30,32,33]A D816V mutation located in the catalytic domain of the tyrosine kinase receptor c-Kit occurs in systemic mastocytosis. c-Kit is the receptor for stem cell factor, a key cytokine involved in the generation and differentiation of mast cells from primitive hematopoietic progenitors; it is encoded by *kit*, located at chromosome 4q12. The D816V and other homologous mutations induce growth factor independent growth and cell differentiation in mast cell lines through activation of STAT5/PI3K/AKT signaling pathways.[36]

The main modification in the 2008 WHO[4,5] classification has been the substitution of the attribute “neoplasm” for “disease”. In fact, notwithstanding the analysis of the X chromosome inactivation pattern in informative females and other cytogenetic and/or molecular findings that established both “classic” and “nonclassic” myeloproliferative disorders as being clonal stem cell disorders, and the finding that evolution to AML is part of their natural history, the neoplastic nature of these conditions has been mostly dismissed until recently. The 2008 WHO classification for myeloid neoplasms, which incorporates novel information derived from molecular discoveries in *BCR-ABL* negative “classic” myeloproliferative states and clonal eosinophilic disorders, includes five major entities (Table 2) as follows: the Acute Myeloid Leukemia (AML) and the Myelodysplastic Syndromes (MDS) with their different subtypes, whose listing is outside the scope of this review; the Myeloproliferative Neoplasms (MPN); the category of overlapping Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN); and the Myeloid Neoplasms associated with eosinophilia and specific molecular abnormalities. AML is defined by the presence of either ≥20% blast cells in the bone marrow and/or peripheral blood or certain characteristic cytogenetic abnormalities. The MDSs are recognized and distinguished from MPN primarily on the basis of the presence of trilineage dyshematopoiesis in the absence of monocytosis in both bone marrow and peripheral blood.[4,5]

1. Acute myeloid leukemia (AML) and related precursor neoplasms
2. Myelodysplastic syndromes (MDS)
3. Myeloproliferative neoplasms (MPN)
3.1. Chronic myelogenous leukemia (CML), *BCR-ABL1* positive
3.2. Polycythemia vera (PV)
3.3. Essential thrombocythemia (ET)
3.4. Primary myelofibrosis (PMF)
3.5. Chronic neutrophilic leukemia (CNL)
3.6. Chronic eosinophilic leukemia, not otherwise classified (CEL-NOS)
3.7. Mastocytosis
3.8. Myeloproliferative neoplasm, unclassifiable (MPN-u)
4. Myelodysplastic/Myeloproliferative neoplasms (MDS/MPN)
4.1. Chronic myelomonocytic leukemia (CMML)
4.2. Juvenile myelomonocytic leukemia (JMML)
4.3 Atypical chronic myeloid leukemia, *BCR-ABL1* negative
4.4. Myelodysplastic/myeloproliferative neoplasm, unclassifiable
4.5. Refractory anemia with ring sideroblasts associated with marked thrombocytosis
5. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*
5.1. Myeloid and lymphoid neoplasms associated with *PDGFRA* rearrangement
5.2. Myeloid neoplasms with *PDGFRA* rearrangement
5.3. Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

Table 2. The 2008 World Health Organization Classification for Myeloid Neoplasms

The four "classic" MPNs (ie, CML, PV, ET, and PMF) have been distinguished from the other "nonclassic" MPNs, which include chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), systemic mastocytosis (SM), and unclassifiable forms of MPN. Classic MPNs have been further functionally classified based on the presence or absence of the t(9;22) chromosomal translocation in the Philadelphia (Ph) chromosome resulting in the BCR-ABL 1 fusion protein (hallmark feature of Chronic Myeloid Leukemia, CML). Thus, it's now well established that the three main classical Ph-negative MPNs are PV, ET and PMF.[4,5,6]

1.2 THE "CLASSIC" Ph-negative MYELOPROLIFERATIVE NEOPLASMS

Classic Ph-negative MPNs are among the most frequent hematologic neoplasms, usually affecting the adult elderly population; however, they can also be found in children, and in this instance, they raise specific diagnostic and management issues. Epidemiological studies on chronic myeloproliferative neoplasms conducted during the last five decades have reported variable annual incidence rates. In PV, annual incidence rate per 100.000 inhabitants varies in the range of 1.9-2.6. As regards ET and IMF the same variability is observed with annual incidence rates ranging from 0.6-2.5 and 0.3-1.5 per 100.000 inhabitants, respectively. The median age at diagnosis is 69-74 years in PV , 67-72 years in ET and 67-76 years in PMF. There is a male predominance in PV and IMF , whereas ET is most common in females.[35-39] Familial clustering of these disorders is known, and even before the discovery of *JAK2*V617F mutation, this observation led to a suggestion of predisposition allele(s). This concept was further strenghtened when a clear molecular distinction of true familial MPN from other familial sindrome such as familial

erythrocytosis and hereditary thrombocythemia has become possible using clonality markers, cellular studies and JAK2 mutation analysis. A clear example of germline genetic factors influencing MPN pathogenesis was the discovery of the GGCC (also known as 46/1) haplotype of the JAK2 gene. Somatic mutations of JAK2 in MPN do not distribute equally between the two most common JAK2 gene haplotypes in Caucasian populations. The GGCC haplotype acquires over 80% of all V617F mutations as well as exon 12 mutations. The GGCC haplotype predisposes carries for JAK2 mutation positive MPN, and thus its major role is in the disease initiation. However, the hypothesis that GGCC haplotype might account for familial clustering of MPN ha recently been disproved in a study showing equal haplotype frequency in sporadic and familial MPN cases. The reason why the GGCC haplotype has negligible role in familial MPN is its weak ability to initiate the disease phenotype.[40-43]

Among classic MPNs, PV and ET are relatively indolent disorders, resulting in a modest reduction of lifespan compared with a control population; however, most patients ultimately suffer from one or more severe and potentially fatal complications directly attributable to the disease. Conversely, PMF has a severe course in most cases, and survival is significantly affected. The three clinical entities share several common features, such as their origin in a multipotent hematopoietic stem cell, a relatively normal cellular maturation, a striking overlap in clinical presentation (apart from PMF, which has its own peculiar manifestations), and in cases of PV and ET, the propensity to evolve into post-polycythemic or post-thrombocythemic myelofibrosis (or less frequently each into the other), and the possibility to transform into AML.[35-39]

Diagnosis. Because of similarities with reactive forms characterized by an increased count of mature peripheral blood cells on one side, and the significant phenotypic overlapping among them on the other, diagnosis of different MPNs has traditionally been challenging; the availability of the new molecular markers is expected to facilitate diagnosis (Table 3). As a matter of fact, molecular genotyping is integral to the 2008 WHO[4,5] diagnostic criteria, and tests for *JAK2* or *MPL* mutation already have become a standard tool in the diagnostic work up of MPN. In fact, detection of one of these mutations unequivocally establishes by itself the presence of a clonal MPN and rules out the possibility of reactive erythrocytosis, thrombocytosis, or myelofibrosis.[44-47] Unfortunately, they are of no help in distinguishing among the different forms of MPNs, although *JAK2* exon12[48] mutations have not yet been reported outside PV, and no patient with PV has been found to harbor an *MPL* mutation.[49,50]

Criteria	Polycythemia Vera	Essential Thrombocythemia	Primary Myelofibrosis
Major criteria	**1.** Hgb >18.5 g/dL (men) or >16.5 g/dL (women) **or** Hgb or Hct > 99th percentile of reference range for age, sex, or altitude of residence **or** Hgb >17 g/dL (men) or >15 g/dL (women) if associated with a documented and sustained increase of ≥2 g/dL from baseline that cannot be attributed to correction of iron deficiency **or** elevated red cell mass >25% above mean normal predicted value **2.** Presence of *JAK2*V617F or similar mutation	**1.** Sustained platelet count ≥450 x 10^9/L **2.** BM showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes. No significant increase or left-shift of neutrophil granulopoiesis or erythropoiesis **3.** Not meeting the WHO criteria for PV, PMF, CML, or MDS or other myeloid neoplasm **4.** Demonstration of *JAK2*V617F or other clonal marker **or** no evidence of reactive thrombocytosis	**1.** Megakaryocyte proliferation and atypia* accompanied by either reticulin and/or collagen fibrosis **or** In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (ie, pre-fibrotic cellular-phase disease) **2.** Does not meet WHO criteria for CML, PV, MDS, or other myeloid neoplasm **3.** Demonstration of *JAK2*V617F or other clonal marker **or** no evidence of reactive marrow fibrosis
Minor criteria	**1.** BM showing hypercellularlity for age and trilineage growth (panmyelosis)	—	**1.** Leukoerythroblastosis
	2. Subnormal serum Epo level		**2**. Increased serum LDH
	3. EEC growth		**3.** Anemia
	—		**4.** Palpable splenomegaly
Diagnostic combinations	Both major criteria + 1 minor criterion **or** first major criterion + 2 minor criteria	All 4 criteria must be met	All 3 major criteria + 2 minor criteria

Table 3. 2008 WHO Diagnostic Criteria for "classic" MPN

*Small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei and dense clustering

In patients with evidence of increased red cell mass, according to WHO criteria, demonstration of *JAK2*V617F mutation allows a diagnosis in greater than 95% of cases, as less than 2% of PV patients harbor *JAK2* exon 12 abnormalities. The compelling criterion for a diagnosis of ET is a sustained platelet count of greater than 450 × 10^9/L. Notably, this value is lower than the one originally used by the 2001 WHO[3] classification system (600 × 10^9/L), because the latter might have led to inadvertently overlooking classic ET cases with a lower platelet count. This assumption is supported by the discovery of the *JAK2*V617F mutation in some subjects who have a platelet count lower than 600 × 10^9/L. Diagnosis of ET requires exclusion of reactive thrombocytosis,[2] as well as of other

MPNs that present with thrombocytosis. In particular, exclusion of CML with FISH or PCR analysis for *BCR-ABL*rearrangement is mandatory. Positivity for *JAK2*V617F or *MPL* mutation cumulatively account for 60% to 70% of ET cases.[51-62,64,65,66] Therefore, the assessment of bone marrow morphology remains key to the diagnosis of ET[63]; bone marrow cellularity is normal or slightly increased, with abundance of large, mature-appearing megakaryocytes devoid of morphological abnormalities and generally dispersed throughout the biopsy. This appearance is distinct from both the panmyelosis typical of PV or the predominant granulocytic hyperplasia with highly bizarre megakaryocytes, often found in abnormally tight clusters, with aberrant nuclear to cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei that are found in PMF, even in initial stages without overt fibrosis.[2,63] (Figure 1a-b).

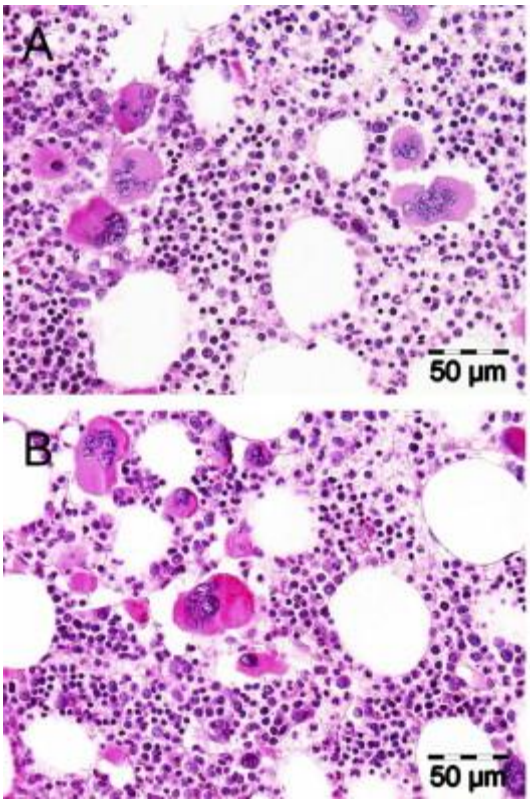

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Figure1. (A) Essential thrombocythemia (ET) or thrombocythemia vera (true ET). Increase and loose clustering of enlarged mature megakaryocytes with hyperlobulated nuclei and there is a slight increased cellularity. (B) Polycythemia vera (PV). Increase and clustering of small to enlarged or giant (pleomorphic) megakaryocytes with mature cytoplasm and hyperlobulated nuclei and increased cellularity due to increased erythropoiesis and granulopoiesis.

Bone marrow histology is required for the diagnosis of PMF. Although advanced reticulin or collagenic fibrosis is typically associated with classic stages of PMF (Figure 2), some degree of reticulin fibrosis can be found as well as in PV, or more occasionally in ET.

Therefore, fibrosis by itself is not synonym for PMF, and diagnosis of PMF can be made even in the absence of overt fibrosis.[2,63]

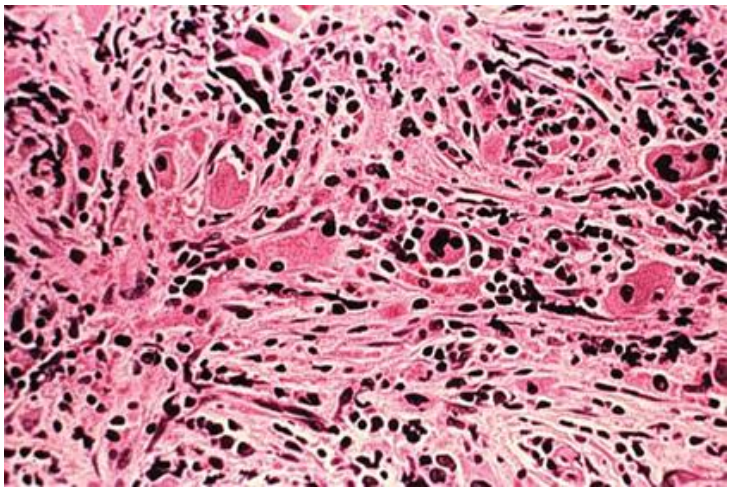

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Figure 2. Bone marrow morphology of PMF.

Also the leukoerythroblastic features of blood smears, with immature myeloid precursors, nucleated red cells, and abnormally shaped erythrocytes (tear-drop cells), is very characteristic, but not diagnostic, of PMF. Some cytogenetic abnormalities, such as del(13)(q12;q22), are frequently encountered and may be diagnostically specific in this context. Acquired cytogenetic abnormalities are, in fact, present in up to 50% in those with PMF, suggesting a greater degree of genetic instability within myelofibrosis clone. Several studies have indicated a role for mutant JAK2 in driving genetic instability, with increases in homologous recombination, point mutations and small deletions observed in cell lines expressing mutant compared to wildtype JAK2. In fact, other factors have been demonstrated to be implicated in the development of myelofibrosis including reduced GATA-1 expression, increased thrombopoietin and transforming growth factor β signalling and altered expression of molecules involved in stem cell trafficking such as CXCR4. More recently, the use of high-resolution SNP (single-nucleotide polymorphism) analysis and high throughput sequencing methods led to the identifications of additional genetic mutations with potential pathogenetic implications in PMF development. Genes affected included *TET2*, *IDH1/2 (*isocitrate dehydrogenase 1/2 enzyme)*, ASXL1* (additional sex combslike1), *EZH2 (*an histone Methyltransferase)*, DNMT3a* (DNA-methyltransferase), *SRSF2,LNK* and *c-CBL* (casitas B-lineage lymphoma proto-oncogene) which have been reported to be mutated in a small subset of patients with PMF.[1,27,67-72] (Figure 3)

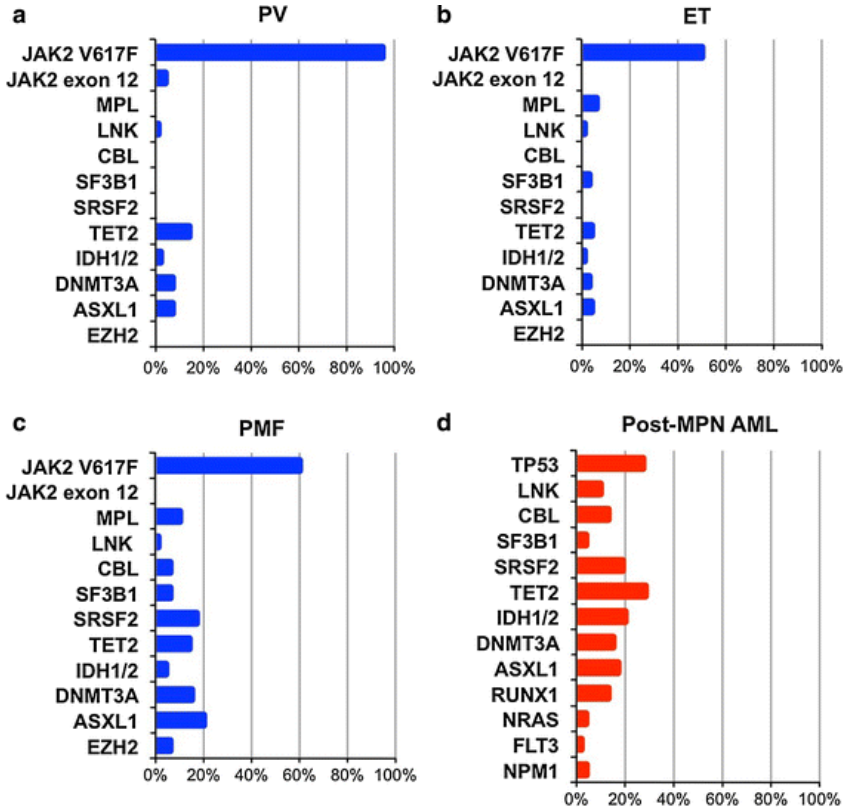

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Figure 3. Frequencies of mutations in commonly affected genes in patients during chronic myeloproliferative phase (a–c) and acute leukemic phase (d) of the disease.

However, the specific role of these new gene alterations as possible new diagnostic molecular markers and/or as new prognostic factors is still inconclusive.In fact, considering that most of these mutations are shared by other myeloid malignances, in particular myelodysplastic syndromes and chronic myelomonocytic leukemia, it is unclear whether such changes are directly involved in the pathogenesis of PMF. Furthermore, it remains to be established whether they antedate or follow the acquisition of *JAK2* V617F point mutation. [1,27]

Anemia, palpable splenomegaly, and raised lactate dehydrogenase levels are additional diagnostic criteria.

Clinical Course and Risk Stratification. Thrombosis, hemorrhage, evolution to post-polycythemic or post-thrombocythemic myelofibrosis, and AML transformation represent the most clinically relevant issues in the course of classic MPN. Most thrombotic events occur at or in the two years before diagnosis. However, epidemiologic studies suggested that the cumulative rate of thrombosis during the disease course ranged from 2.5% to 5.0% and from 1.9% to 3% per patient-year in PV and ET, respectively, depending on whether the patient was in a low-risk or high-risk category. Arterial thrombosis accounts for 60% to 70% of all cardiovascular events and includes acute myocardial infarction, ischemic stroke, and peripheral arterial occlusion. Events involving the venous system,

more common among PV patients, are represented by lower extremity deep venous thrombosis, pulmonary embolism, and splanchnic vein thromboses (SVT).[73-97] Recent data indicate that at least 40% of patients with SVT not attributable to other causes actually harbor the*JAK2*V617F mutation[98,111-116]; therefore, *JAK2*V617F genotyping represents a first-line test for these conditions. Occasional SVT patients harboring *MPL* mutation have also been reported. Conversely, involvement of the microcirculatory system is more typically associated with ET and manifests as erythromelalgia (a rare disorder characterized by burning pain, warmth, and redness of the extremities due to arteriolar fibrosis and occlusion with platelet thrombi, typically aspirin-sensitive), transient ischemic attacks, visual or hearing transitory defects, recurrent headache, and peripheral paresthesia; however, because of the lack of objective diagnostic criteria, true incidence of microvessel disturbances is difficult to assess. Pathogenesis of thrombosis in classic MPNs is multifactorial; rheologic abnormalities due to increased red cell mass in PV, abnormal function of platelets and their enhanced interaction with leukocytes and endothelial cells, are all possible contributing factors; however, neither thrombocytosis nor increased hematocrit (at least until 52%) are clearly associated with occurrence of thrombosis.

Mortality rate is age-dependently increased in PV, being 1.6-fold and 3.3-fold higher than in the reference population in patients younger or older than 50 years, respectively. Conversely, survival of ET patients is reduced by about 2-fold compared with the general population starting from the first decade after diagnosis. Major causes of shortened survival in PV or ET are represented by thrombotic events and transformation to myelofibrosis or AML. Therefore, because of the finding that thrombosis represents the most common event that complicates the courses of PV and ET, and eventually is the leading cause of death, it was considered appropriate to stratifying patients according to their risk. Older age (greater than 60 years) and a previous history of thrombosis are standard risk factors for thrombosis in both PV and ET [73-119](Table 4).

Risk Category	Age >60 Years or History of Thrombosis	Generic Cardiovascular Risk Factors
Low	No	No
Intermediate	No	Yes
High	Yes	Irrelevant

Table 4. Risk-Stratification of Patients with Polycythemia Vera or Essential Thrombocythemia

Finally, several studies have domonstrated that *JAK2*V617F mutated status in ET, and a high V617F allelic burden in both ET and PV are associated with increased risk of thrombosis. Therefore, both leukocytosis and *JAK2*V617F mutated status could represent novel, powerful, disease-associated, risk factors; however, they need validation in prospective studies.[111-116]

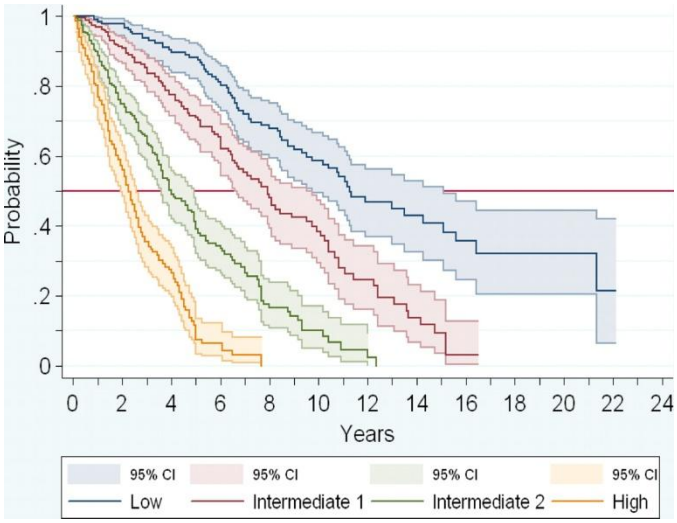
Life expectancy in PMF is 31% lower than in an age-matched and sex-matched population, with a median survival of 5 years, although younger patients may experience longer survival. Major causes of death are represented by portal hypertension or hepatic-splenoportal thrombosis, thromboses in various anatomic sites, heart failure due to splenic pooling, infections, pulmonary hypertension, bleeding caused by thrombocytopenia or hemostatic defects, and transformation to AML. Prognostic staging systems for PMF have been developed that allow separation of patients with low-risk and high-risk disease associated with significantly different survival times[119-130] (Table 5).

Prognostic Scoring System	**Prognostic Factors**	**No. of Prognostic Factors by Risk Category**			**No. of Months of Survival by Risk Category**		
		Low	**Intermediate**	**High**	**Low**	**Intermediate**	**High**
All patients							
Lille	Hb <10 g/dL WBC <4 or >30x10^9/L	0	1	2	93	26	13
Cervantes	Hb <10 g/dL PB Blasts ≥1% Constitutional symptoms	0-1	—	2-3	99	—	21
Mayo	Hb <10 g/dL WBC <4 or >30x10^9/L Plt <100x10^9/L Monocytes >1x10^9/L	0	1	≥2	173	61	26
Younger patients							
Cervantes, aged ≤55 y	Hb <10 g/dL PB Blasts >1% Constitut. symptoms	0-1	—	2-3	176	—	33
Dingli, aged <60 y	Hb <10 g/dL WBC <4 or >30x10^9/L Plt <100x10^9/L	0	1	2-3	155	69	24

Table 5. Prognostic Scoring Systems Used for Risk Assessment in Patients with PMF

A stratification according to the risk is particularly relevant for younger patients who can potentially exploit the curative effect of allogeneic hematopoietic stem cell transplantation; in fact, at present, only allogeneic hematopoietic stem cell transplantation (ASCT) is considered potentially curative in MF. In this regard, both myeloablative and reduced intensity conditioning (RIC) strategies have been employed.[129,135-138] The most consolidated and powerful risk-stratification system, the IPSS, was developed by the IWG-MRT using a multi-institutional series of 1001 patients with PMF. This system employs 5 variables, estimated at diagnosis, for prediction of survival: (1) age > 65 years, (2) hemoglobin level < 100 g/L, (3) leukocyte count > 25 × 10^9/L, (4) ≥ 1% blasts in the peripheral blood, and (5) the presence of constitutional symptoms (eg, night sweats, loss of > 10% body weight in the last 6 months, noninfectious fever) (Table 4). Projected survival ranged from 135 months (95% CI = 117-

181 months) in the low-risk category (no risk factors) to 95 months (95% CI = 79-114 months) in the intermediate-1 category (one risk factor), 48 months (95% CI = 43-59 months) in the intermediate-2 category (2 risk factors), and 27 months (95% CI = 23-31 months) in the high-risk category (3 or more risk factors).[131-133] (Figure 4)



Cervantes F et al. Blood 2009

Figure 4. Survival curves of the 4 risk groups of patients according to the new PMF prognostic system.

Considering that modification of the disease over time, with acquirement of additional risk factors not present at diagnosis, could profoundly affect survival, the IWG-MRT subsequently developed the DIPSS, relying on the data obtained in a cohort of 525 PMF patients with extensive follow-up information. The DIPSS includes the same 5 variable as the IPSS, but the acquisition of anemia was assigned a score of 2 because this event affected survival with an hazard ratio roughly double the other parameters. In this series, 63% of the patients became anemic at 15 years from diagnosis, 22% acquired the risk variable "leukocytosis," 39% acquired "blasts ≥ 1%," and 22% developed constitutional symptoms. Therefore, the advantage of the DIPSS score is that it permits a tailored framework for clinical decision-making at any time during the disease course.

Additional factors not included in the IPSS that affect survival are represented by red cell transfusion need, thrombocytopenia, and "unfavorable" karyotype. Regarding the latter, patients with unfavorable karyotype, which includes a complex karyotype or sole or 2 abnormalities such as +8, −7/7q−, i(17q), inv(3), −5/5q−, 12p−, or 11q23, had a median survival of 2 years compared with 5.2 years for those with a "favorable" karyotype, defined as no abnormality or any other apart from those included in the above category, the 5-year survival rates were 8% and 51%, respectively. The newly devised DIPSS Plus score incorporates these additional 3 variables for improved prognostic categorization[132,133] (Table 6).

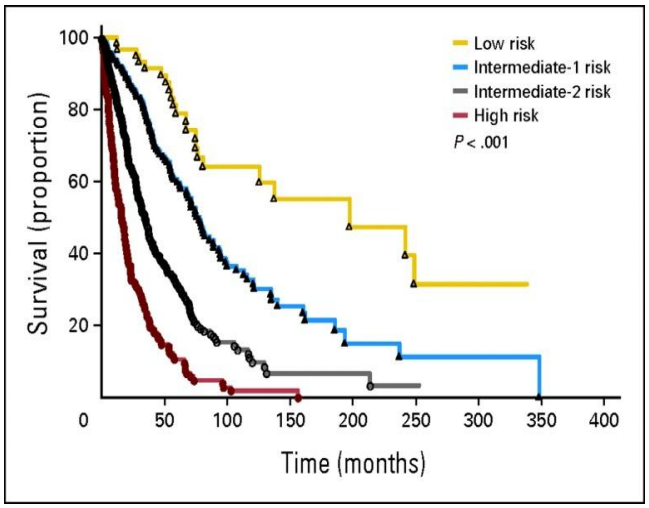
Variable	IPSS[9]	DIPSS[10]	DIPSS Plus[11]
Age > 65 y	√	√	√
Constitutional symptoms	√	√	√
Hb < 100 g/L	√	√	√
Leukocyte count > 25 × 10^9/L	√	√	√
Circulating blasts ≥ 1%	√	√	√
Platelet count < 100 × 10^9/L			√
RBC transfusion need			√
Unfavorable karyotype*			√
	1 point each	1 point each but Hb = 2	1 point each

Hb: hemoglobin.

*Unfavorable karyotype indicates any of the following: +8, −7/7q−, i(17q), inv(3), −5/5q, 12p−, or 11q23 rearrangements

Table 6. Prognostic Score System for patients with PMF

In a series of 793 patients, median survival times were 185, 78, 35, and 16 months for the low, intermediate-1, intermediate-2, and high-risk categories, respectively. (Figure 5)



Gangat N et al. Journal of Clinical Oncology 2011

Figure 5. Survival data of 793 patients with primary myelofibrosis evaluated at time of their first Mayo Clinic referral and stratified by their Dynamic International Prognostic Scoring System (DIPSS) + karyotype + platelet count + transfusion status prognostic scores.

Management of Classic Ph-negative MPN. Because thrombotic complications are the leading cause of morbidity and mortality in patients with ET or PV, reducing the frequency of thrombosis is the main aim of therapy in these patients. In fact, at present, drugs should not be used in ET or PV with the intent to either prolong survival or prevent disease transformation into AML or MF. However, controlled studies have shown significant reductions in the incidence of thrombotic complications in patients with PV treated with low-dose aspirin and in high-risk patients with ET treated with hydroxyurea (HU). Also, there is compelling, although not controlled, evidence to support the use of phlebotomy in all patients with PV and hydroxyurea in those with high-risk disease. Taken together, current recommendations for treatment in PV include phlebotomy and low-

dose aspirin in all patients and the addition of hydroxyurea in high-risk disease treatment strategy, with the exception of phlebotomy, also applies to ET (low-dose aspirin in all patients and the addition of hydroxyurea for high-risk disease). In PV patients undergoing phlebotomy, it is generally recommended but not mandated to keep the hematocrit level below 45% in men and 42% in women.[139-178]

Regarding PMF, the only approach that has resulted in a prolongation of survival time in PMF and has the potential to be curative is allogeneic HSCT. [180,183] At present, it should be reserved for patients with high-risk disease after careful clinical evaluation and thorough patient counseling, particularly considering the option of inclusion in trials with innovative drugs. Both myeloablative and reduced-intensity conditioning regimens have been used, with similar efficacy in terms of survival (3-year event-free survival in the range of 50% to 60%) but lower mortality rate with the use of the latter in older patients.[183,184] Therefore, a myeloablative strategy may be considered as the most appropriate for younger patients, whereas the reduced-intensity regimen would be the best for older patients. Furthermore, in patients who relapse after HSCT, a graft-versus-myelofibrosis effect could be demonstrated after donor-lymphocyte infusion with a remarkable reduction of bone marrow fibrosis.[178,179.181] Given that a conventional drug therapy does not significantly modify disease course and is largely ineffective, it is reserved for patients who present either with symptomatic anemia or splenomegaly. Low-dose thalidomide[186] in combination with prednisone improves anemia or thrombocytopenia in 30% to 50% of cases. Lenalidomide,[187] a thalidomide analog, has produced excellent and durable responses in the relatively infrequent PMF patients who have the del(5q) abnormality, and it can be recommended as first-line therapy in this patient subset.[188,189] When there is the need to control excessive myeloproliferation, ie, leukocytosis, thrombocytosis, or progressive splenomegaly, HU is the current drug of choice.[193,194] Splenectomy has a role for alleviating mechanical symptoms due to extreme splenomegaly and can also ameliorate anemia in approximately 25% of transfusion-dependent patients. However, splenectomy in PMF bears an approximately 10% procedure-related mortality, and it should be performed by experienced surgeons.[190] Furthermore, up to 25% of patients present with accelerated hepatomegaly and extreme thrombocytosis after splenectomy, and these patients require further cytoreduction.[195-196]

The involvement of JAK-STAT pathways in most patients who have classic MPN and harbor mutations in *JAK2* or *MPL* and the experimental evidence that suggests that the same signaling abnormalities may be at the basis of mutation-negative patients are behind active efforts to develop anti-JAK2 drugs. Many molecules have undergone preclinical testing, in vitro and also in vivo, and some have already been introduced into clinical trials. Concerning selective JAK2 inhibitors, we have listed only those that are already in clinical trials or whose activity has been demonstrated in *JAK2*V617F-mutated murine models (TG101348). (Table 7) Among these, INCB018424, XL019, CEP-701, and

TG101348 are currently undergoing clinical trials in patients with advanced stages of PMF, post-PV/ET myelofibrosis, PV, and *JAK2*V617F-positive ET.[191,197-202]

Drug	α-JAK2 vs JAK1	α-JAK2 vs JAK3	Other targets	Phase	N
Ruxolitinib (INCB018424)	×1.0	×153	None known	1/2 3	153 628*
TG101348	×35	×332	FLT3, Ret	1	59
CYT387	×0.6	×8.6	JNK1, CDK2	1/2	36
CEP-701	NA	×3.0	FLT3, TrkA	2	22
AZD1480	×5.0	×15	Aurora A, TrkA FGFr1	1/2	NA
SB1518	×58	×24	FLT3	1/2	31
LY2784544	NA	NA	None known	1/2	NA

Table 7. JAK2 inhibitors under clinical development in patients with MF

Preliminary results have been encouraging in terms of activity against splenomegaly and constitutional symptoms, with minimal toxicity. Although the number of patients treated until now is limited with any single drug and, thus, prevents us from making any definitive comment, the hope that this molecularly targeted approach may finally result in improving quality of life and possibly the chance of cure for patients with classic MPN is enormous.

2. THE MOLECULAR PATHOGENESIS OF MPN

2.1 MUTATIONS AFFECTING JAK-STAT SIGNALING PATHWAY

Myeloproliferative neoplasms are characterized, as any other malignancy, by genetic defects that culminate in the neoplastic phenotype. Initially, MPNs were considered as simple, single-hit diseases that lead to an increase in mature blood cells. In 2005, in fact, the discovery of a recurrent molecular abnormality characterized by a gain of function in the gene encoding Janus kinase 2 (JAK2)[7-11] paved the way for greater knowledge of the pathophysiology of myeloproliferative neoplasms. JAK2V617F mutation was the first important breakthrough in the understanding MPN: it demonstrated the role of the pathologic JAK/STAT signaling pathway. This pathway is essential for normal hematopoiesis and is "turned on" after activation of cell receptors by its ligands, establishing the link between extracellular stimuli and the cellular effects of numerous growth factors, cytokines, interferon, etc. Upon binding of the ligand, conformational changes in the receptor lead to JAK and STAT phosphorylation. Once phosphorylated, STATs form homodimers and translocate to the nucleus where they will promote transcription of specific genes (Figure 6).

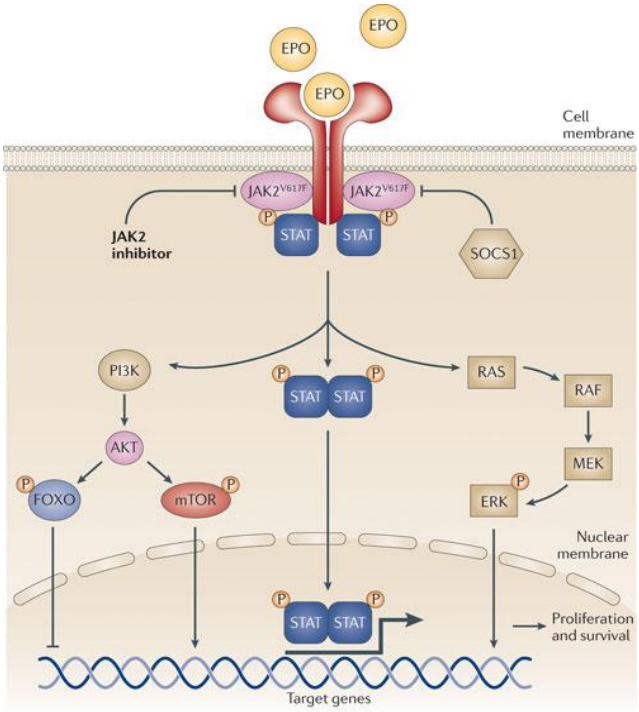


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Figure 6. JAK2^{V617F} signaling pathway in MPNs

The hypothesis that hypersensitivity of hematopoietic stem and progenitor cells to cytokines might largely account for the pathogenesis of myeloproliferative neoplasms has been corroborated by the discovery of mutations that affect other cytoplasmic proteins

involved in cytokine signaling, either resulting in a gain-of-function (MPL) or a loss-of-function (CBL and LNK). Dysregulation of tyrosine kinases is a recurrent theme in chronic myeloid neoplasms, as exemplified by the constitutive activation of ABL caused by oligomerization of the BCR-ABL fusion protein in chronic myelogenous leukemia, the gain-of-function mutation of the tyrosine kinase receptor c-KIT in mastocytosis, and the activation of platelet-derived growth factor receptor-α or -β and fibroblast growth factor receptor in hypereosinophilic disorders.[203]

2.1.1. *JAK2* MUTATIONS

JAK2 is a member of the Janus kinase family composed by four tyrosin-kinases (JAK1, 2, and 3 and TYK2) that attach to cytokine receptor cytosolic domains. JAK kinases possess two highly homologous domains at the carboxyl terminus: an active kinase domain (JAK homology, JH1) and a catalytically "inactive" pseudokinase domain (JH2) which negative regulates the JH1 kinase activity. At the N-terminus, the JH5-JH7 domains contain a FERM (Band-4.1, ezrin, radixin, and moesin)–like motif, which plays a role in the binding to the cytosolic domain of cognate cytokine receptors. JAK2 plays a central role in the signaling from "myeloid" cytokine receptors. In fact, it binds to the three homodimeric "myeloid" receptors (erythropoietin receptor [EPO-R], myeloproliferative leukemia [MPL; TPO-R], G-CSF receptor [G-CSF-R]), to the prolactin and growth hormone receptors, to heterodimeric receptors (GM-CSF-R, IL-3-R, and IL-5-R, which share the common β chain of IL-3-R and the gp130 family of receptors), and to IFN-γ R2. JAK2 is the only JAK capable of mediating the signaling of EPO-R and MPL. JAK2 also functions as a chaperone for trafficking of these 2 receptors to the cell surface and their stability. More recently, JAK2 was also shown to promote G-CSF-R cell-surface localization.Therefore, JAK2 and the 3 "myeloid receptors" form functional units and have been shown to be required for the promotion of JAK2V617F signaling. [204] (Figure 7)

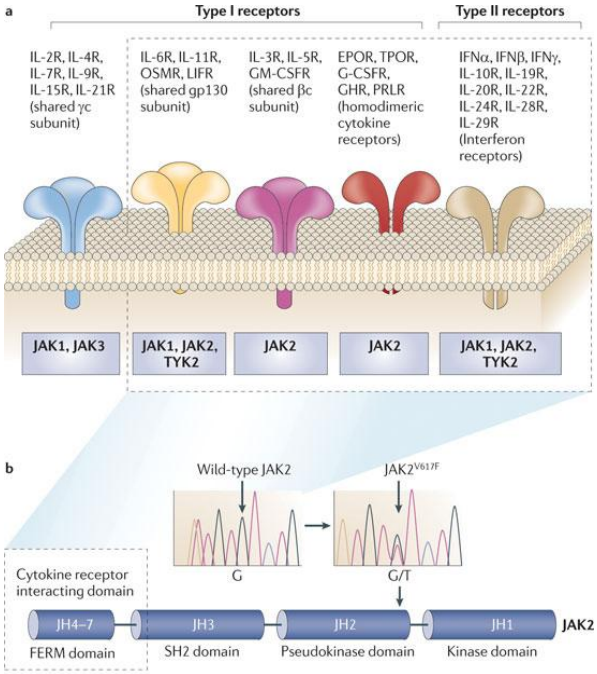

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Figure 7. Cytokine receptors and JAK2

The JAK2V617F point mutation at base pair 1849 (G→T), cause substitution of the normal valine to phenylalanine in codon 617 (V617F) of exon 14 of the gene. This valine is located at one of the predicted interfaces between JH1 and JH2 domains, and the change to a phenylalanine appears to relieve the inhibition of the JH2 domain on the JH1 kinase domain. JAK2V617F mutation was defined as "gain- of- function " because the expression of mutated allele in cytokine-dependent cell lines conferred cytokine independence and cytokine hypersensitivity through the constitutive activation of STAT5, Akt and ERK-dependent pathway. [7-11] The JAK2 gene is located on chromosome 9p24. Following the observation that acquired uniparental disomy (UPD) of chromosome 9p is present in 30% of patients with PV, the JAK2V617F mutation was discovered as the prominent genetic aberration in patients with BCR-ABL–negative MPNs (~ 95% of patients with PV, 50%-70% with ET, and 40%-50% with PMF), as well as in some cases of atypical MPN (30%-50% splanchnic vein thrombosis and sideroblastic anemia associated with a thrombocytosis).[205.206] The high prevalence of the V617F mutation in three clinically distinct MPNs begs the question of what additional factors contribute to phenotypic diversity between PV, ET, and PMF. First, mutant allele burden of V617F may modulate phenotype: the mutation can be found on one or both alleles (homozygosity) due to a mitotic recombination process that occurs in most patients with PV or PMF and a minority only of ET. Recently, in-vivo studies have corroborated these findings: murine retroviral transplant models resulting in high levels of V617F expression produced a PV-like phenotype with marked erythrocytosis.Conversely, transgenic models with more physiologic levels of V617F expression resulted in phenotypes resembling ET and PMF.

For ET patients, positivity for V617F tends to confer a PV-like phenotype, with higher hemoglobin and lower platelet counts than in V617F-negative ET patients. Differences in intracellular signaling arising from V617F may also explain the development of PV compared with ET: preferential activation of STAT1 constrains erythroid differentiation and promotes megakaryocytic development, leading to an ET phenotype.[207-210] In contrast, reduced STAT1 phosphorylation promotes erythroid development as observed in PV. Host genetic background may also influence disease presentation. In retroviral transplant models, disparate phenotypes were observed depending on the mouse strain. In C57Bl/6 mice, transplantation with JAK2 V617F transduced cells resulted in a PV-like disease predominantly characterized by erythrocytosis.[208] However, in Balb/C mice, similar experiments yielded mice with erythrocytosis, but also leukocytosis and the subsequent development of myelofibrosis.[211] However,most recently several lines of evidence support that the most compelling basis for MPN diversity comes from the additional molecular abnormalities that either precede or follow the acquisition of V617F. The aggregate data suggest that there is no strict temporal order of mutation occurrence that defines the development or natural history of specific MPNs and V617F may arise on a pre-existing abnormal clonal substrate: 1) in some patients, the V617F burden is relatively small compared with the proportion of cells with a coexistent clonal karyotypic abnormality; and 2) in AML arising from a V617F-positive MPN, the mutant V617F allele can frequently no longer be detected.[212] This suggests that the MPN and AML share a clonal origin that likely preceded the acquisition of V617F. Furthermore, in V617F-positive PV and ET patients, both JAK2 wild-type and V617F-positive EECs have been detected within the same patient, suggesting that a separate event may confer clonal, erythropoietin-independent growth, before V617F. One study in ET patients demonstrated that V617F was acquired on separate alleles as multiple, independent events.[213]

Additional complex mutations, deletions or insertions have been detected in exon 12 of *JAK2* and are usually associated with clinical features typical of a *JAK2*V617F-negative PV or idiopathic erythrocytosis. These gain-of-function mutations span the linker region between the SH2 and JH2 domains. Although not located in the pseudokinase domain, these mutations may modify the structure of the JH2 domain in a very similar fashion as V617F. Along these lines, residue F595, located in the helix C of the pseudokinase domain, was shown to be required for both V617F and K539L mutants but not for cytokine-induced JAK2 activation. However, in contrast to JAK2V617F, exon 12 mutations are not associated with ET and PMF, although JAK2 exon 12 PV may progress to a secondary myelofibrosis.[23,57] Beside its prominent role in the cytokine receptor signaling cascade, JAK2 can act as an epigenetic regulator mainly by influencing chromatin structure.[33] In hematopoietic cells, nuclear JAK2 phosphorylates histone H3Y41, thereby blocking recruitment of the repressor heterochromatin protein 1α and allowing increased expression of several genes, including the LMO2 oncogene.[214] (Figure 8 and 10). In the same line, JAK2V617F has been recently described to interact with and phosphorylate the

protein arginine methyltransferase PRMT5 with a much greater affinity than wild-type JAK2.

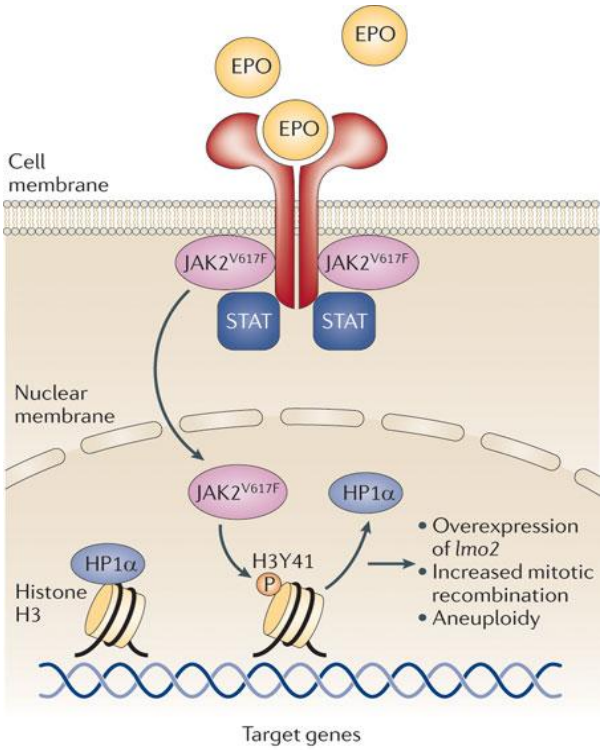

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Figure 8. Nuclear activity of JAK2 and JAK2 ^{V617F}

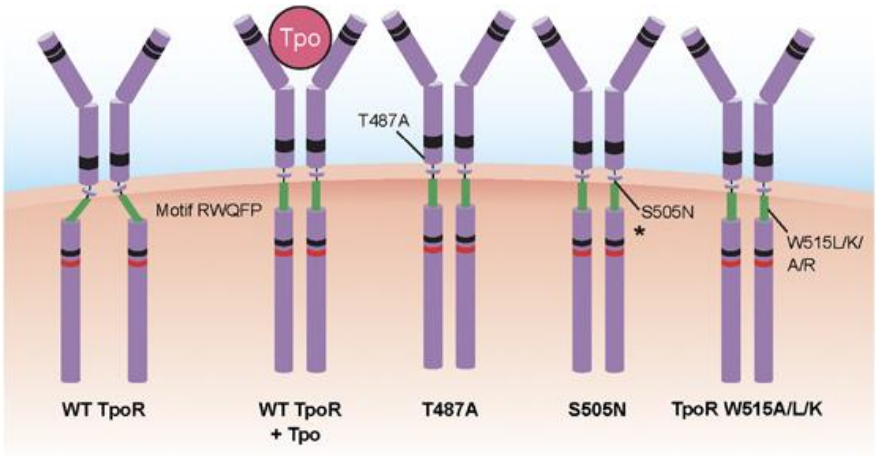
This property is specific (or enhanced) for the mutant protein and has been shown to disrupt the interaction between PRMT5 and its cofactor MEP50, leading to a decreased methyltransferase activity. The knockdown of PRMT5 increases colony formation and erythroid differentiation of primary cells.[215]

Due to its high prevalence, the detection of JAK2 mutations has become an essential step in the diagnosis of myeloproliferative disorders. Regarding the prognostic importance of JAK2 mutations, the findings are not conclusive yet; most studies have not found differences in survival or progression to AML between patients with and without JAK2 mutations.[216] Intriguingly, we and others demonstrated that a low JAK2V617F allele burden is associated with worse survival in PMF.[217,218]

2.1.2 *MPL* MUTATIONS

A third recurrent molecular abnormality is represented by mutations involving codon 515 of *MPL*, the gene encoding the receptor for Tpo (named after myeloproliferative leukemia virus oncogene homolog), which result in the ligand-independent activation of the receptor itself. These point mutations (which lead to a W to L, K or A transition) are located in a RWFQP motif in the transmembrane-juxtamembrane junction, a short region implicated in maintaining MPL in an inactive conformation in the absence of the

ligand; substitutions at W515 probably affect the inactive conformation of MPL and result in its ligand-independent activation. They are detected in ≅10% of patients with PMF or ≅8% of *JAK2*V617F-negative ET.[29,32,58,59,210] Furhtermore, activating MPL trans membrane S505N and extracelluar juxtamembrane T487A mutations have been identified in familial ET and in rare megakaryoblastic leukemia cell lines, respectively. (Figure 9). Interestingly, in some patients multiple *MPL* mutations or the coexistence with *JAK2*V617F allele have been reported.[66] As a matter of fact, while additional MPL mutations have been observed in patients with PMF, only those occurring at codon 515 have functional relevance by causing activation of the JAK-STAT pathway, promoting the G_1/S phase transition of the cell cycle.[61] *MPL*W515L introduction in cell lines resulted in a cytokine-independent growth and hypersensitivity to thrombopoietin. The *in vivo* expression of the W515L allele in mice through a retroviral transplant-based approach resulted in a very different phenotype compared with that induced by the JAK2 V617F mutation; in fact, these mice developed extreme thrombocytosis and leukocytosis, reticulin fibrosis in the bone marrow, extramedullary hematopoiesis in the spleen and liver, and their lifespan was significantly shortened to a few weeks. Therefore, the disorder produced in mice mimicked human PMF rather than PV or ET.[73]



W. Vainchenker and S. N. Constantinescu. Oncogene 2012

Figure 9. *MPL* mutations

2.1.3 *LNK* MUTATIONS

LNK, also called SH2B3, belongs to a family of adaptor proteins (e.g.,also SH2-B, APS) that acts as a negative regulator of cytokine signaling [219]LNK protein specifically interacts with JAK2 (wild type or mutant V617F), MPL (wild type or mutant W515L) inhibiting the downstream activation of STAT. This family of adaptor proteins share several structural motifs, including a proline-rich N-terminus, a pleckstrin homology (PH) domain, an SH2 domain, and a conserved tyrosineresidue near the C-terminus. . LNK binds to MPL via its SH2 domain and colocalizes to the plasma membrane via its PH domain. On cytokine stimulation with Tpo, LNK binds strongly to JAK2 and inhibits downstream STAT

activation, thereby providing critical negative feedback regulation.[220-222]In addition, LNK negatively regulates c-KIT and FMS signaling.LNK-deficient mice have an increased HSC pool with enhanced self-renewal properties and increased quiescence.This phenotype probably results from increased TPO/MPL signaling because TPO is required for maintaining HSC quiescence and the HSC reservoir. In addition,Lnk knockout mice develop an MPN phenotype, characterized by splenomegaly with marked fibrosis, due to accumulation of abnormal levels of erythrocytes, megakaryocytes and marked B-cell overproduction. As expected from its negative role in JAK2 signaling, LNK is also capable of attenuating the signaling induced by MPLW515L or JAK2V617F. As a conseguence, in murine models lacking LNK the of MPN induced JAK2V617 is accelerated. In JAK2V617F-positive patients, LNK expression is increased and modulates the myeloproliferative process. [220-224] Recently, 2 mutations have been identified in *LNK* exon 2, one in a patient with PMF and the other with ET; both MPNs were JAK2V617F negative.[225] The first mutation leads to a premature stop codon resulting in the absence of the PH and SH2 domains, whereas the second (E208Q) is a missense mutation in the PH domain. In the first mutation, the capacity to inhibit TPO signaling is lost, whereas in the second mutation, some inhibitory function is maintained. The frequency of mutations in *LNK* is low.However, other mutations of *LNK* have been found in leukemic transformation of MPN at a greater frequency (~13%).[226] The few mutations described affect predominantly a hot spot at exon 2, between codons 208 and 234. and are heterozygous. Interestingly, some of these mutations appear to be late events involved in disease progression because they were not found in the chronic phase.[226] In addition some LNK mutations were associated with JAK2V617F, although it is not known whether LNK mutants and JAK2V617F were present in the same cell. It has been also reported that *LNK* exon 2 mutations can be found in pure erythrocytosis.One mutation (A215V) had been previously described in PMF blast crisis, and another (E208X) leads to absence of the PH and SH2 domains as the mutant described in PMF. Primary samples from patients harboring LNK mutations presented increased STAT3/5 activation, suggesting that the loss of function caused by LNK is analogous to JAK2 or MPL gain of function mutations.

2.1.4 *CBL* MUTATIONS

The Casitas B-cell lymphoma (CBL) family includes 3 homologs: c-CBL, CBL-b, and CBL-c.[228]

c-CBL, the founding member, is the cellular counterpart of a murine viral oncogene involved in B-cell and myeloid malignancies. CBL proteins are multifunctional adapter proteins with ubiquitin ligase activity. The CBL protein can act either as a positive or negative regulator of tyrosine kinase signaling. It can bind to activated receptors, acting as an adaptor protein, recruiting downstream molecules, but it also has E3 ubiquitin ligase activity, 'marking' active kinases for degradation, such as FLT3, KIT and MPL.[228,229] *c-CBL* is located at 11q.23.3 and contains several domains: an N-terminal tyrosine kinase binding domain followed by a Ring domain, which is important for the transfer of

ubiquitin moieties. A linker separates these 2 domains. The C-terminus part contains a proline-rich domain involved in the binding of several SH3 proteins. Finally, a C-terminal UBA/LZ domain is implicated in CBL oligomerization and ubiquitin binding.[229] In the chronic phase of classic MPN, c-*CBL* mutations have been found in a low percentage of PMF patients (6%) but were not detected in a small series of PV and ET patients.[229] In one case, the c-CBL mutation occured after JAK2V617F. However, during progression of the disease, JAK2V617F was outcompeted by the *CBL* mutant, suggesting that the 2 mutations had occurred in 2 different cells. Similarly a c-CBL mutation has been detected in blasts from a JAK2V617F-positive MPN, which became JAK2V617F negative during transformation.Usually variants are missense mutations, which are homozygous because of an acquired uniparental disomy or, rarely, because of a deletion of the wild-type copy. For these reasons, *CBL* has been considered a tumor suppressor gene. Most mutated CBL forms behave as loss-of-function molecules having a dominant-negative effect not only on c-CBL but also on CBL-b, leading to an excessive sensitivity to a variety of growth factors.[33,229] Transfection of CBL mutants was sufficient to induce an oncogenic phenotype in fibroblast cell lines. Additionally, mice with the CBL null phenotype developed a disease characterized by splenomegaly and augmented hematopoietic progenitor pool, suggesting a role for this gene in the development of MPNs.[230] Additionally, mutated CBL inhibited E3 ubiquitin ligase activity of wild type CBL in transfected fibroblasts, revealed a dominant negative role for the mutated protein and not only a classical tumor suppressor effect.

2.2 EPIGENETICS AND MUTATIONS IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

Very recent studies have demostrated that mutations in signaling molecules are not sufficient for disease development in humans and that several cooperating genetic hits might be required to induce disease and allow progression. As a consequence of the development of whole genome assays and next-generation sequencing an increasing number of mutations have been observed in MPNs, and most of these mutations involve genes modifying epigenetic regulation. 'Epigenetics' describes the study of stable, reversible alterations affecting gene expression that are not stored within the primary DNA sequence. Since the identification of epigenetic regulation, there has been rapid progress in elucidating the nature and role of the mechanisms involved in this type of gene expression control. It has been shown that epigenetic factors play a vital role not only in development and differentiation, but also in pathogenesis and, in particular, in cancer development. There are four main molecular mechanisms of epigenetic regulation in normal and malignant development: DNA methylation, histone methylation, histone acetylation and regulation activity of small RNAs. Methylation and hydroxymethylation of cytosines in DNA, as well as methylation, acetylation, and other modifications of histones, are the most common mechanisms of achieving transcriptional repression. DNA methylation occurs when a cytosine residue at a "CpG" site, ie a cytosine that precedes a guanine, becomes covalenty bound with a methyl group at the 5-carbon position, a

reaction which is catalyzed by a family of DNA methyltransferases. There are CpG dinucleotide-rich regions in specific locations along the genome that are are called “CpG islands”, usually at the 5’ regulatory region of many genes; when methylation occurs at high frequency in this CpG-rich regions of promoter, the gene cannot be efficiently transcribed. Promoter methylation is a normal process for the orchestrated control of transcriptional activity during development and cell-lineage specification, but it also contributes susbstantially to tumorigenesis. Hypermethylation at specific gene loci, particularly of tumor suppressor genes, and global hypomethylation of genomic DNA, as well as inactivation of microRNA genes by DNA methylation, are all common findings in human cancer cells. The reduced global methylation found in cancer cells, that increases during cancer progression, is mainly due to hypomethylation of DNA repetitive sequences, coding regions and introns, particularly in areas of the genoma characterized by a low content of coding genes. Loss of methylation in normally silent regions of the genoma could also cause the inappropriate expression of genes normally silenced, including imprinted genes or genes of the inactive X chromosome. On the opposite, abnormal gain in DNA methylation with aberrant silencing of transcription may occur at specific gene promoter regions and represent a mechanism for inactivation of tumor-suppressor genes. As a matter of fact, differential chemical modifications of histone tail, ie acetylation and methylation, affect DNA replication and DNA repair, but may also affect gene transcription. Acetylation of histones is accomplished by competing activity of histone acetyl transferase (HAT) and histone deacetylases (HDAC). Acetylated histones have a reduced interaction with the negatively charged DNA, due to lowered positive charge of lysine residue in the presence of acetyl group, and on turn this facilitates the interaction of transcription factors to consensus target sequences on gene promoter. However, while drugs acting as HDAC inihibitors can facilitate the re-expression of genes whose promoter sequence is not hypermethylated, they are generally little or no effective in contrasting gene silencing when hypermethylation of gene regulatory sequences is present. A sequential treatment scheduling, that involves exposure to a demethylating agent followed by HDAC inhibitors, usually results in synergistic re-expression of silenced genes, at least in most culture conditions.

Somatic mutation in epigenetic modifiers are shared among all myeloid malignancies and affect *TET2*,*IDH1/2*, *DNMT3A*, *SOCS*, *ASXL1* as well as members of the polycomb repressor complex 2 (PRC2). In MPN they are predominantly found in patients with PMF, or patients who show disease progression to AML; however, their specific role remains unclear, as these mutations are widely detected in different cancers.

2.2.1 *SOCS* MUTATIONS

Suppressor of cytokine signaling (SOCS1, SOCS2, and SOCS3) proteins are important negative regulators of the JAK–STAT signaling and are both induced by and act in a negative feedback loop to downregulate JAK/STAT signaling. Several studies have demonstrated that SOCS1 is involved in cytokine signaling [232] and in direct activation of p53-dependent senescence. SOCS1-inactivating mutations have been described in B-cell

lymphoma.[233] Some mutations in the different SOCS have been found in MPNs, but they seem rare. Epigenetic silencing of SOCS1/3 is an additional pathogenetic mechanism leading to cytokine signaling hypersensitivity. SOCS1 hypermethylation has been reported in a fraction of patients with Ph-negative MPNs and can be seen in both JAK2V617F-positive and JAK2 wild-type patients[234,235] However, the methylation pattern that was observed in these studies was noted in SOCS1 exon 2 (also seen in normal control blood cells) but not the gene promoter site and thus the relevance of this observation to MPN pathogenesis is not evident. Hypermethylation of SOCS3 has been detected in PMF but not PV/ET patients. A trend for lower SOCS3 expression in JAK2V617F-negative PMF patients was noted in one study.[236] SOCS methylation status was not correlated with any identifiable clinical variables or outcome.[237] *SOCS2* silencing by hypermethylation has also been shown in MPN-derived cell lines as well as primary MPN cells and can coexist in cells that carry the JAk2V617F mutation.[238]

2.2.2 *IDH1/2* mutations

Isocitrate dehydrogenase 1 and 2, *IDH1* and *IDH2*, located on chromosome 2q33.3 and 15q26.1, respectively, encode $NADP^+$-dependent enzymes that catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate.[32] Heterozygous mutations of *IDH1* and *IDH2* have been first identified in solid tumors and in AML. Mutated IDH1/2 are neomorphic enzymes that catalyze the reduction of αKG to (R)-2-hydroxyglutarate (Figure 11). A subsequent overproduction of (R)-2-hydroxyglutarate has been proposed to affect the function of αKG-dependent enzymes such as TET2, resulting in a decrease of 5hmc.[239,240,241] Both *IDH1/2* mutations and *TET2* mutations lead to similar hypermethylation signatures and patterns of impaired myeloid differentiation and increased expression of stem cell markers. An analysis of 1473 patients with MPN revealed a low incidence of IDH1/2 mutations in chronic phase ET, PV, and MF (0.8%, 1.9%, and 4.2%, respectively) contrasting with a 21.6% frequency in blast phase.[242] Thirty-eight *IDH* 1/2 mutations have been discovered in a large screening study of MPN patients and can coexist equally with mutations in *JAK2*, *MPL*, and *TET2*[32]. The types of IDH1/2 mutations seen in MPNs are distinctly different than the ones observed in solid tumors and overlapped with those documented in AML and include IDH1-R132, IDH2-R140, and IDH2-R172. Over 21% of patients with blast phase-related MPN carry an IDH1/2 mutation, and this was irrespective of JAK2V617F status.[242,243]This appears to indicate that IDH1/2 mutations can also influence the transformation of MPN to blast phase disease. Interestingly, leukemic blasts and progenitor cells can possess both mutated *IDH2* and *JAK2V617F*, and in other patients with MPN-transformed leukemia, the mutated IDH1/2 may be present in blasts with wild-type JAK2 and absent in the progenitor cells with JAK2V617F.[243] These data have shown the possibility of the presence of two subclones originating from a yet unidentified primary clone or two independent competing clones arising in the same individual.

2.2.3 *EZH2* MUTATIONS

EZH2, located on 7q36.1, encodes for the PcG enhancer of zeste homolog 2, the catalytic component of the polycomb repressive complex 2 (PRC2) that methylates histone H3 at lysine 27 (H3K27me3) (Figure 10).[244] PRC2 is involved in various cellular processes, including proliferation, differentiation, cell-identity maintenance, aging, and plasticity.[245,246] In addition to EZH2, PRC2 includes EED, RbAp46/48, SUZ12, AEBP2, JARID2, and PCL. It is thought that PRC2 contributes to chromatin structure regulation. The SET domain of EZH2 is specifically involved in the trimethylation of K27. H3K27me3 is a marker of inactive chromatin, as opposed to H3K4 trimethylation which is a marker of transcriptionally active status. EZH2 also associates with DNA-methyltransferases to direct DNA methylation. EZH2 overexpression is observed in numerous solid tumors such as breast and prostate cancers and may induce dedifferentiation.[247] It was found mutated in B-cell lymphomas and the recurrent mutation targeting Y641 results in a gain of function: the mutated enzyme preferentially generates trimethylated K27. In contrast,macro- and micro-deletions of the genomic region containing EZH2 have been found in about 10% of myelodysplastic syndromes, with a few subjects presenting loss-of-heterozygosity due to acquired uniparental disomy.[248,249] Mutations of *EZH2* have been reported in patients with primary myelofibrosis, myelodysplastic syndromes, and myelodysplastic syndromes/myeloproliferative neoplasms; they are scattered throughout the gene and include missense, nonsense and premature stop codons resulting in loss of function. Both monoallelic and biallelic mutations have been described. Additionally, it has been shown that EZH2 mutations in myeloid malignancies cause absence of histone 3 methylation at Lysine 27, revealing a loss-of-function phenotype compatible with a tumor suppressor function.[249] In one of the first studies, forty-nine *EZH2* mutations have been found in 42 individuals out of 614 patients with myeloid disorders. Thirteen percent of MF patients in this cohort harbored an EZH2 mutation. A total of ten *EZH2* mutations were identified in exons involving deletions, insertions, and missense mutations in patients with PMF, post-PV/ET MF, and MPN-associated acute myeloid leukemia.[249] Microarray and SNP analysis did not show association with copy number alterations or uniparental disomy. Additionally, no association was seen with *JAK2V617F* allele burden. Degree of splenomegaly and leukocytosis was clinical findings found to be statistically associated in MPN patients expressing *EZH2* mutations. Moreover, the presence of EZH2 mutations has recently been associated with decreased overall survival in patients with PMF. Three-deazaneplanocin A(DZNep) is a carbocyclic adenosine analog that inhibits *s*-adenosylhomocysteine hydrolase and results in the accumulation of *s*-adenosylhomocysteine, disrupting methylation of targets by EZH2.[250] Although the effects of DZNep are global and not specific to EZH2, this drug has been tested as a single agent in solid tumor cell lines and in combination with a HDACi in primary AML cells. The combination of this agent with the pan-HDACi, LBH589 (Panobinostat, Novartis), was shown in vitro to selectively induce apoptosis in AML primary cells and not normal $CD34^+$ cells. This effect was correlated with reduction in EZH2 protein level and induction in *p16*, *p21*, and *p27* gene expression. Combined therapy in a NOD/SCID mouse model

with HL-60 AML led to an improvement in survival when compared to each agent alone.[251] This compound is currently being investigated in early phase clinical trials. Furthermore, expression of miR-101-1 and 101-2, which negatively regulate EZH2, has been shown to be decreased in MPNs and displayed an inverse relationship with *EZH2* mRNA expression.[252] This may provide an additional mechanism for *EZH2* gene dysregulation and contribute to MPN disease progression and disease severity.

2.2.4 *ASXL1* MUTATIONS

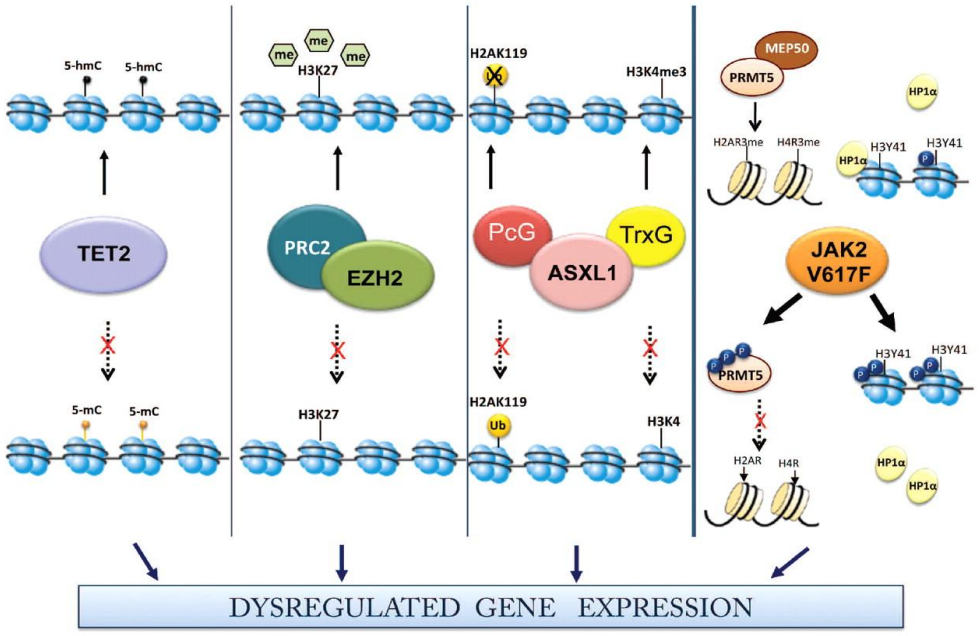
ASXL1 encodes the *A*dditional *SeX* combs–*L*ike protein-*1* which is one of the three mammalian homologs of *Drosophila* Additional Sex Comb (*Asx*) gene, named after the fact that *Asx* deletion caused homeotic transformation due to dysregulation of *Hox* genes, whose spatially and quantitatively appropriate expression is essential for the anterior-posterior specification of axial structures during mammalian development. Constitutional *de novo* nonsense mutations of *ASXL1* have recently been described in half of the subjects with Bohring-Opitz syndrome (MIM605039), a disorder characterized by severe intellectual disability, distinctive facial features and multiple congenital malformations.*ASXL1* maps to human chromosome 20q11.21, consists of 12 exons and encodes a protein composed of 1,541 amino acids. All mammalian ASXL proteins have conserved sequence features: the amino-terminal ASX homology region, which contains two of the three putative nuclear receptor box domains, and a carboxy-terminal plant homeo domain finger. It is a member of the enhancer of trithorax and polycomb (ETP) family that enlists proteins required for both the maintenance of activation and silencing of gene expression by modifying chromatin configuration. For example, ASXL1 can interact with retinoic acid receptor in the presence of retinoic acid and enhance the transcription of some genes while repressing that of others, depending on the cell context. The fine details of the mechanism of action of ASXL1 are not well defined yet, but the protein is involved in distinct multiprotein complexes that bind to and modify chromatin at target gene regions.[244] Scheuermann *et al.* demonstrated that ASXL1 exists in a complex, named polycomb repressive deubiquitinase, with BAP1, a ubiquitin carboxy-terminal hydrolase that removes monoubiquitin from histone 2A in nucleosomes (Figure 10).[244,253] ASXL1 also associates with the histone acetyltransferase SRC-1, the histone methyltransferase MLL and forms a ternary complex with heterochromatin protein-1 (HP1) and the histone demethylase LSD. Thus, ASXL1 has pleiotropic and context-dependent repressive or activating effects on transcription through chemical modification of histones. Frameshift mutations, nonsense mutations, and large 20q11 deletions of *ASXL1* have been described in 10–15% of myeloproliferative neoplasms and myelodysplastic syndromes, 40% of CMML (particularly in the myeloproliferative subset, 60%), in refractory anemia with ring sideroblasts and thrombocytosis, a few patients with chronic myelogenous leukemia and 15–20% of acute leukemias. Most *ASXL1* mutations are found in exon 12, spanning the region from Tyr591 to Cys1519, and disrupt the protein downstream of the ASX homology domain with loss of the plant homo domain. Germline targeted disruption of *Asxl1* in mice resulted in embryonic/perinatal death while

in the few mice who survived to birth only mild hematopoietic defects were detected with no evidence of myelodysplastic or myeloproliferative disorder.[17] From a genetic point of view, the mutations in *Asxl1* deleted mice differ from the mutations seen in patients, which usually result in the deletion of the plant homeo domain finger while sparing its N-terminal motifs; this would suggest that *ASXL1* mutations generate a dominant-negative protein that can inhibit its wild-type counterpart.[254] In some studies, *ASXL1* mutation was associated with an unfavorable outcome in acute myeloid leukemia, CMML and myelodysplastic syndromes, while there is not enough information on this aspect in classic myeloproliferative neoplasms because of the relatively small series of patients. In this issue of the Journal, Stein *et al.* report on 166 patients with myeloproliferative neoplasms who were analyzed for exon 12 *ASXL1* mutations.[18] Extending previous results, they detected *ASXL1* mutations very rarely in PV and ET, while the frequency reported in patients with myelofibrosis (included patients with primary and post-PV/post-ET myelofibrosis) was significantly higher (36%). Phenotypic correlations revealed a higher prevalence of anemia-directed therapy in *ASXL1*-mutated patients; however, the relatively low number of cases (n=77) examined hampered analysis of prognostic correlations.

2.2.5 *TET2* MUTATIONS

TET2, which stands for ten-eleven-translocation-2, is member of a family that includes also *TET1* and *TET3. TET2* is located on 4q24 and contains 11 exons. The founder of the *TET* family, *TET1*, was originally identified as a fusion partner of *MLL* in acute myeloid leukemia with the t(10;11)(q21;q32) translocation. One known function of TET proteins is to accomplish 5-methylcytosine hydroxylation resulting in the generation of 5-hydroxymethylcytosine; the significance and role of this modified base is still largely unknown, but 5-hydroxymethylcytosine has been found enriched in actively transcribed genes and in the promoters of polycomb-repressed elements that are normally activated during development of mouse embryonic stem cells (Figure 10-11).[244] TET1 and 2 proteins appear to play a crucial role in stem cell biology as shown in the control of pluripotency of murine embryonic stem cells. Initially, it was suggested that TET1 was involved in the self-renewal of ES cells by controlling Nanog,[255] but recent evidence suggests that both TET1 and TET2 are necessary for pluripotency by controlling cell fate through the regulation of distinct sets of genes.[256] Thus, TET2 may have a similar role on HSC. Preliminary results show that in the mouse, in vitro knockdown of *TET*2 by shRNAs increases monocytic differentiation in the presence of GM-CSF, causing an excess of immature forms.[257] Targeting *Tet2* in mice caused a progressive expansion of hematopoietic stem and progenitor cells leading to a myeloproliferative phenotype with splenomegaly, extramedullary hematopoiesis and marked expansion of the monocytic compartment.[244] *TET2* mutations have been discovered in a wide range of myeloid malignancies,[6]including classic myeloproliferative neoplasms (approximately 14%), mastocytosis, myelodysplastic syndromes, chronic myelomonocytic leukemia (CMML; 50%) and in post-myeloproliferative neoplasm or *de-novo* acute myeloid leukemia.

Sequential analysis of the presence of *TET2* mutations during the progression of myeloproliferative neoplasms has shown that these mutations may precede or follow the *JAK2*V617F mutation[26] or occur at the time of disease transformation to acute myeloid leukemia.[258] Mutations are scattered over the gene and consist of small insertions, deletions and nonsense mutations, all expected to result in a loss-of-function of the protein, and missense mutations affecting conserved amino acids in catalytically active regions. Accordingly, patients with *TET2* mutations have lower global 5hmC content than wild type. Of note, the global level of 5hmC may not directly impact the mC level as evaluated on a gene-specific base because *TET2*-mutated patients have lower mC in MDS samples and greater mC in AML samples.[257] *TET2* alterations are most commonly heterozygous, suggesting that TET2 haploinsufficiency may be a mechanism sufficient for transformation, as indicated also by the phenotype of $Tet^{+/-}$ mice. Inhibition of TET2 catalytic activity is also driven by the neomorphic IDH1/2 mutant proteins. *TET2* mutations are found in approximately 14% of MPNs ranging from ET (11%) to PMF (19%).[259] In roughly 20% of the patients, 2 mutations are observed, suggesting that the inactivation of a single copy of TET2 is sufficient for the transformation process. TET2 mutations are not responsible for the familial MPNs identified to date, although a germline mutation has been described.In vitro studies have first shown that *TET2* mutations occur before JAK2 that the converse may happen and also that *TET2* mutations may occur when MPNs transform to AML.[260] Mutations have been shown to be present in the HSC/progenitor populations. In vivo analyses demonstrated that the *TET2*-mutated cells from MPN samples are able to engraft into immunodeficient mice. Clone-amplification studies have shown that *TET2* mutations and JAK2V617F act synergistically to induce a clonal dominance at the level of hematopoietic progenitors in PV patients.[26]



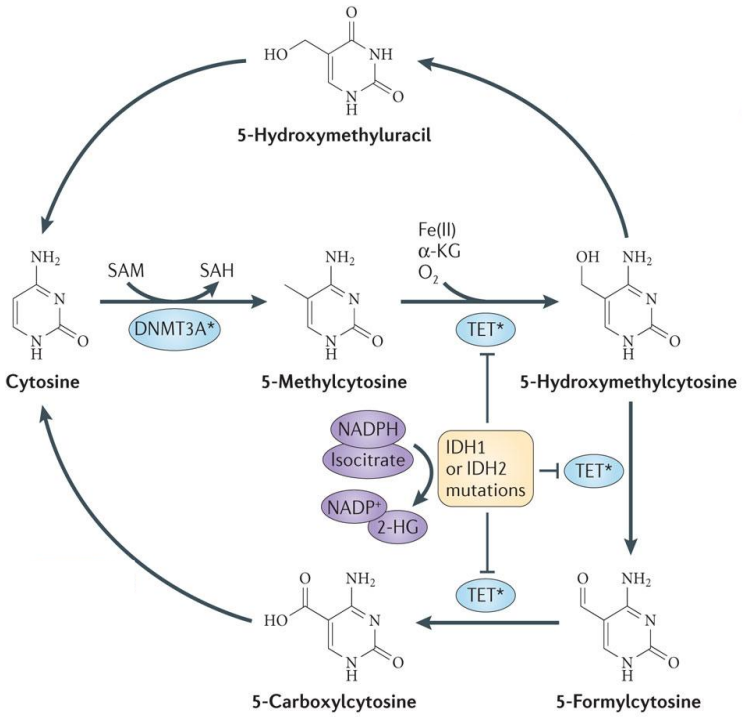
Vannucchi A M , Biamonte F Haematologica 2011

Figure 10. Known functions of EZH2, TET2, ASXL1 in epigenetic regulation of gene expression

2.2.6 *DNMT3A* MUTATIONS

DNMT3A is a member of the mammalian family of methyltransferases that enzymatically add a methyl group to cytosine in CpG dinucleotides in DNA (Figure 11). Initial studies of the role of DNMT3A in mouse haematopoiesis demonstrated no overt phenotype unless both *Dnmt3a* and *Dnmt3b* were lost. More recently, it was carried out a more extensive analysis *in vivo* by studying the effect of haematopoietic-specific conditional *Dnmt3a* deletion on self-renewal in serial transplantation assays. These studies revealed that deletion of *Dnmt3a* resulted in progressive expansion of the long-term haematopoietic stem cell (LT-HSC) pool without increasing HSC proliferation or altering haematopoietic differentiation. Careful analysis of the methylome in HSCs revealed that deletion of *Dnmt3a* resulted in both hypermethylation and hypomethylation of different loci; notably, they observed hypomethylation and increased expression of a core set of genes that are crucial for regulating HSC self-renewal and promoting malignancy. Whether the same set of genes is subject to altered epigenetic patterning in *DNMT3A*-mutant AML cells has not been investigated.[261,262] Ley and colleagues[263] first identified somatic mutation of *DNMT3A* in adults with AML after whole-genome sequencing of a human sample of AML with normal cytogenetics. They found that *DNMT3A* mutations were present in 22% of adult cases of AML and were associated with an increased risk of relapse in a single-institution patient cohort. Interestingly, these mutations seem to be associated with intermediate-risk AML, a finding also noted with TET2- and IDH-mutant disease. In contrast, no cases of favorable risk AML contained these mutations.[263] Unlike the exclusivity of TET2 and IDH mutations in recent studies of AML, DNMT3A mutations often cooccurred with IDH mutations, suggesting that these

latter two mutations may not have overlapping functions in leukemogenesis. Somatic mutations in DNMT3A have been reported as nonsense, frameshift, and missense mutations throughout the open-reading frame. However, the majority of somatic *DNMT3A* mutations result either in premature truncation of the protein product (nonsense or frameshift mutations), or occur at a single amino acid, R882. Mutation of R882 occurred in nearly 60% of the *DNMT3A*-mutant samples (37 of 62 mutations) and has been shown to decrease catalytic activity and DNA binding affinity.[264,265] Although one study reported a decrease in DNA methylation activity of >50% with the DNMT3A R882 mutant in an in vitro methyltransferase assay, AML patient samples with DNMT3a mutations were not found to have altered total 5-methylcytosine content or altered patterns of methylation . Equally important is the fact that the DNMT3A R882A mutation appears to occur exclusively as a heterozygous mutation suggesting a potential gain of function which may or may not require a wildtype copy of DNMT3a for altered function.[263,265] Immunoprecipitation of methylated DNA followed by hybridization to DNA microarray chips (MeDIP–chip) revealed that AMLs with *DNMT3A*^{R882} mutations had decreased methylation at 182 specific genomic loci compared with *DNMT3A*-wild-type AML. However, changes in methylation were not correlated with robust changes in gene expression. Mutations in *DNMT3A* have also been observed in patients with MDS, as well as less commonly in patients with classic MPNs. The clinical and prognostic implications of*DNMT3A* mutations in chronic myeloid malignancies are not yet clarified. Likewise, little is known about the timing of *DNMT3A* mutations during disease evolution. One study compared MDS and MPN with the corresponding secondary AMLs that had *DNMT3A*mutation and identified the same *DNMT3A* mutation in the antecedent disorder as in the secondary AML in all ten cases.[266] This suggests that *DNMT3A* mutations may be an early event of clonal evolution.[267]



Alan H. Shih, Omar Abdel-Wahab, Jay P. Patel & Ross L. Levine. Nature Reviews Cancer 2012

Figure 11. DNA methylation and demethylation pathway.

2.3 OTHER MUTATIONS

2.3.1 *TP53* MUTATIONS

TP53 gene encodes p53, a major tumor suppressor protein involved in various biologic activities, including the control of cell-cycle checkpoints and apoptosis. Germline loss-of-function mutations in *TP53* predispose patients to a multiplicity of cancers, and acquired mutations in p53 occur in approximately 10% of AML samples. Several studies have demonstrated that *TP53* mutations are not associated with the chronic phase of MPNs.[268] However, mutated *TP53* have been found with a 20% frequency in post–MPN-AML patients.[269] The picture of *TP53* mutations in post–MPN-AMLs suggests that they play a prominent role in the transformation process. Mutations in p53 are rarely found in these diseases before acute leukemia transformation, but this does not rule out a role for p53 deregulation in disease progression. Furthermore, in a very recent study, Nakatake M. et al using Ba/F3-EPOR cells and ex vivo cultured CD34(+) cells from MPN patients have demostrated that expression of JAK2(V617F) affected the p53 response to DNA damage. They show that JAK2(V617F) mutation affects p53 response to DNA damage through the upregulation of La antigen and accumulation of MDM2. They also suggest that p53 functional inactivation accounts for the cytokine hypersensitivity of JAK2(V617F) MPN and might have a role in disease progression.[270]

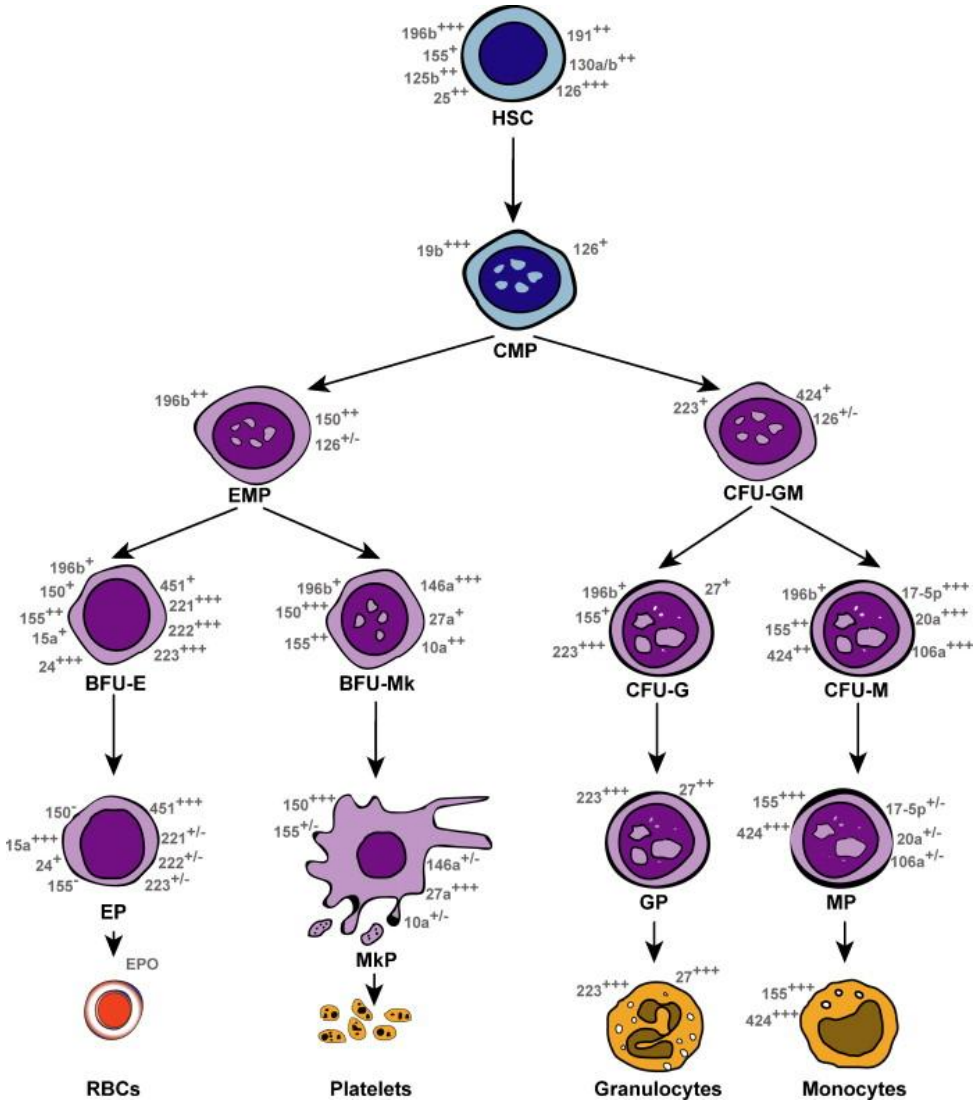
2.3.2 MUTATIONS AFFECTING THE SPLICING MACHINERY

During the last few years mutations affecting various proteins involved in RNA splicing have been discovered in almost patients 9.4 % of patients with MPNs.[205, 271] Overall, abnormalities have been identified in genes encoding the components of splicing machinery such as *SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF3A1* and, in most of the cases, mutations were found in a mutually exclusive manner. In the earlier studies *SF3B1* mutations were found, with high specificity in MDS patients, with increased ring siderloblasts (~80 % of the cases). Later, deeper analyses showed SF3B1 mutation in 4–6.5 % of patients with PMF and 3 % of patients with ET, but no association with clinical features or other mutations were clearly established although association with thrombotic events were proposed. [272-274] Intesrtigly, it was observed that increased ring siderloblasts was the only feature observed in MPN patients with this mutation [271]. *SRSF2* was found mutated in 17 % of patients with PMF and 18.9 % of post-MPN AML and was associated with *IDH1/2* mutations (28 % of *SRSF2* mutated cases carried *IDH1/2* mutations in addition).[275,276] *SRSF2* mutations target predominantly the P95 residue of the protein either by point mutations or insertion/deletions (P95H, P95L, P95R, etc.). Analysis of paired samples from leukemic and chronic phases showed that *SRSF2* mutations when present at transformation were also present during the chronic MPN phase of the disease . Moreover, these were associated with poor overall and leukemia-free survival.[275,276] Finally, mutations affecting *U2AF1* and *ZRSR2* were reported at low frequencies (~2 %) in MPN patients. The mechanism by which mutations in the splicing machinery lead to an impaired hematopoiesis is unclear. In vitro studies of U2AF1 mutants indicate that they are dominant negative, leading to aberrant splicing, inhibition of proliferation, and apoptosis induction[205,271].

2.4 MicroRNAs

Mature microRNAs are short (19-25 nucleotides) RNAs originated from the cleavage of a 70-100 nucleotide hairpin precursor (pre-microRNA) that hybridize to complementary mRNA targets and either lead to their degradation or prevent their translation of corresponding protein, at least in part depending on the degree of nucleotide complementarity. Expression of microRNAs is tissue- and developmental stage-specific, and as such they are involved in normal cell homeostasis; however, they are also being increasingly recognized as a new class of genes which are altered in human malignancies and can eventually function as oncogenes (onco-microRNAs). On the contrary, some microRNAs may have tumor-suppressor function, as argue by their down-regulation in certain tumors; for example, *let-7* and *mir15/mir-16* control the expression of *RAS* and *BCL-2*, respectively. A role of microRNAs in hematologic malignancies is supported by recent studies, and they have also been proposed as prognostic markers and therapeutic targets in chronic and acute leukemias. However, the fine mechanisms which tune the transcriptional expression of microRNAs are still largely unknown, and may depend on the binding of transcription factors to promoter as well as on differential methylation of the

microRNA gene or of putative 5' regulatory sequences.[231] Expanding knowledge of functional roles of miRNAs points at their potential involvement in human cancer. Several miRNAs are deregulated in primary human tumors[277]: some of them are located at genomic regions linked to cancer and , and may eventually act as regulators of proto-oncogenes, as is the case of *let-7* family for *RAS*. miRNA profile is characteristic of different cancer cells and reflects their developmental derivation and differentiation status, potentially allowing classification of poorly differentiated tumors better than messenger RNA profiling.[278,279,280] *miR-181a*, *-146*, and *-223,* are involved in murine B- and T-cell lymphopoiesis, respectively, while *miR-150* is initially upregulated during developmental stages of B and T cells, and then repressed during differentiation of naïve T cells into Th1 or Th2 effector cells. Furthermore, overexpression of *miR-150* prevented the formation of mature B cells in a murine transplant model. Others have reported a critical regulatory role for *miR-221*and *-222* in human erythropoiesis, which was exerted through downmodulation of c-Kit receptor; *miR-223* is highly expressed in murine bone marrow and in promyelocytic cell lines, and its expression is induced by granulocyte colony-stimulating factor or insulin-like growth factor-1 in differentiating 32D murine myeloid cells. Specific downregulation of a defined set of eight miRNAs (*miR-10a*, *-10b*, -30c, *-106*, *-126*, *-130a*, *-132*, and *-143*) occurred during in vitro differentiation of human $CD34^+$ cells toward the megakaryocytic lineage[281]. Finally, in a comparative study of BM and mobilized PB stem cell–derived normal human $CD34^+$ cells, 33 miRNAs were identified as potential regulators of hematopoietic differentiation; some of them were also differentially expressed between BM and PB stem cell–derived $CD34^+$ cells, and in particular, *miR-95*, *-190*, *-182*, and *-183* were considered as BM-restricted . In some instances, altered miRNA expression profile in a variety of solid cancers has been associated with clinical outcomes and response to treatment. Of particular interest is the *mir-17* microRNA cluster, which is located in a region on human chromosome 13 that is frequently amplified in B-cell lymphomas; we know that overexpression of the *mir-17* cluster cooperated with *Myc* to accelerate tumor development in a mouse B-cell lymphoma model, and *mir-17* itself was induced by overexpression of *Myc*[281,282]



Elvira Pelosi et al. Leukemia Research 2009

Figure 12. MicroRNAs in normal and malignant myelopoiesis . Outline of the expression of miRNAs during normal hematopoiesis. The expression of the main miRs involved in hematopoiesis is outlined. The level of expression is indicated as +++, ++, +, +/−, −. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; EMP, erythroid-megakaryocytic progenitior; CFU-GM, colony-forming unit-granulo-monocytic; BFU-E, burst forming unit-erythroid; BFU-Mk, burst forming unit-megakaryocytic; CFU-G, colony-forming unit-granulocytic; CFU-M, colony-forming unit-monocytic; EP, erythroid precursor; MkP, megakaryocytic precursor; GP, granulocytic precursor; MP, monocytic precursor

Downregulation of *miR-15* and *miR-16* has been demonstrated in the majority of patients with chronic lymphocytic leukemia[283], and a recent study performed by my lab. group has demonstrated that mature miR-16 levels are abnormally increased in CD34(+) cells of patients with polycythemia vera as a consequence of preferential expression of miR-16-2 on chromosome 3 rather than of miR-16-1 on chromosome 13.[284] In an earlier study,

performed by my group, the miRNA expression profile was characterized in cells from MPNs patients with the aim to identify a disease-associated expression profile, which might have diagnostic and, eventually, prognostic implication.
Twelve statistically significant deregulated miRNAs were identified and validated in PMF granulocytes; among these, *miR-95*, *-182*, and *-183* were upregulated while the others (*miR-190*, *-31*, *-150*, *-34a*, *-342*, *-326*, *-105*,*-149*, *-147*) displayed reduced levels compared to control granulocytes. Interestingly, the abnormally increased level of two of three overexpressed miRNAs (*miR182*- and *-183*) was found to linearly correlate with allelic burden of *JAK2*$^{617V>F}$ mutation. [285]

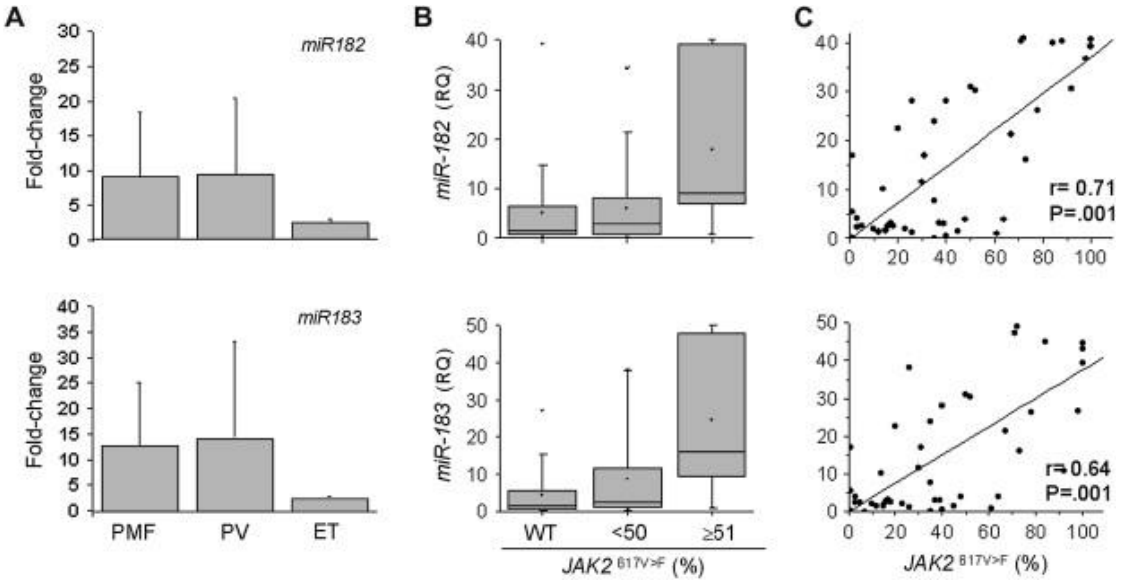


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Figure 13. Correlation between abnormal expression of miR-182 and -183 and JAK2$^{617V>F}$ mutational state. In (**A**), expression level of *miR-182* and *-183* in primary myelofibrosis (PMF), polycythemia vera (PV), or essential thrombocythemia (ET) granulocytes is presented as percent change using the mean value of control subjects as the reference value The correlation between microRNA expression level and *JAK2*$^{617V>F}$ mutational load is shown in (**B**). The level measured in patients with >51% mutant allele burden was significantly higher than that measured in wild-type patients or in those with <50% mutant allele burden ($p < 0.001$ for both). In (**C**), the correlation between *miR-182* and-*183* RQ and *617V>F* allele burden is presented; linear regression parameters are reported inside each plot.

In one of the latest study an aberrant expression of miRNAs 10a and 150 was demonstrated for essential thrombocythemia and PMF as well as for polycythemia vera and PMF, respectively. The expression of miR-150 was also shown to correlate with both JAK2 allele burden and peripheral blood counts. Furthermore, (RNAseq) led to the discovery that mature miRNA sequences are not invariant; rather, they constitute a mixture of miRNA variants: isoforms heterogeneous in 5'- and 3' ends or with

nontemplate 3’ additions of nucleotides, which are collectively called isomiRs. These variants, all potentially active, target only partially overlapping sets of coding mRNAs. Finally, a novel class of miRNA-related RNAs, micro-RNA offset RNAs (moRNAs), has been recently identified by RNAseq. moRNAs were first reported in the simple chordate ascidian *Ciona intestinalis* as approximately 20-nt-long RNAs that originate predominantly from the 5’-arm of the pre-miRNA, regardless of the position of the major miRNA, suggesting that moRNA and miRNA biogenesis may be linked but not necessarily interdependent. The expression of moRNAs in *C intestinalis* is developmental regulated, and their abundance sometimes exceeds the corresponding mature miRNA. Initially, *C intestinalis* moRNAs were considered byproducts of an miRNA processing machinery with intrinsic properties. However, shortly thereafter, Langenberger et al reported moRNAs, derived from 78 miRNA loci, as being weakly expressed in human prefrontal cortex, whereas Taft et al showed that moRNAs were enriched in the nucleus in the human leukemia cell line THP-1 and almost exclusively derived from the 5’-arm, regardless of the position of the prevalent miRNA. Some moRNAs were even more expressed than the prevalent miRNA of the same locus. Thereafter, moRNAs (eg, hsa-miR-410 5’moR, has-miR-326 5’moR) were discovered in solid tumors.

3. AIM OF THE STUDY

The exciting discovery of new genetic abnormalities in patients with myeloproliferative neoplasms holds the promise for advancing our understanding of the pathogenesis of these disorders as well as refining risk stratification and therapeutic management of patients. However, the lower frequency of these non-JAK2 mutations and the lack of large prospective cohorts of patients have prevented a clear understanding of their role in disease presentation and evolution.

Moving from this background, the aim of the project I followed in these three years of PhD fellowship was to better characterize genetic and epigenetic abnormalities in myeloproliferative neoplasms. As a matter of fact, although different studies have found statistically significant correlates between the presence (or allelic burden) of certain molecular abnormalities and clinical phenotype in MPN, particularly in myelofibrosis, these novel molecular information had minimal influence on risk stratification and treatment decisions. For this reason, this project had the general aim to translate information from the laboratory to the patient, in particular for diagnostic, prognostic stratification or therapeutic purposes. These goals have been addressed according to composite efforts that are briefly summarized below:

1) My first aim was to analyze the mutational status of selected genes in patients with myelofibrosis in order to characterize already reported genetic and epigenetic acquired somatic variants and, eventually, identify new somatic mutations. These molecular information were correlated with patient outcome data in order to establish whether particular combinations of mutations generated additional prognostic risk information and/or prognostically validate these mutations for overall and leukemia-free survival in the setting of current scoring systems (e.g., DIPSS-Plus). Considering that we are in the "era" of genomic-based approaches and high-throughput sequencing, to achieve this objective we have used a multi-faced approach based not only on conventional methods but also on new system of next-generation sequencing. Furthermore, this study was made possible by the availability of a large database of samples and by the collaborations with other italian and international research groups.
2) My second purpose was to gain insights into a possible role of microRNAs in myeloproliferative neoplasms. To achieve this objective, experiments were conducted accoridng to two investigation domains: i) Characterization of novel miRNAs in known hairpin precursors, isomiRNAs, and identification of moRNAs in SET2 cells, a *JAK2*V617F-mutated cell line; ii) identification of microRNAs whose activity was deregulated by the expression of JAK2V617F ("JAK2V617F miRNome") and, by performing integrative bioinfomatic analysis, correlation with target gene expression. These experiments were performed in tight collaboration with Dr. Stefania Bortoluzzi and her group of bioinformaticians (University of Padova) and Prof. Rossella Manfredini group (Centre for Regenerative Medicine, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena).

4. METHODS

4.1 PATIENTS AND SAMPLES

Patients diagnosed with PMF or PPV/PET-MF according to World Health Organization (WHO) and International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) criteria, respectively, were recruited for this study from the database at 6 hematology units: Florence, Italy; Pavia, Italy; Southampton, United Kingdom; Barcelona, Spain; Athens, Greece; and Mannheim, Germany. For PET-MF patients, only those who had a previous confirmed diagnosis of "true" ET as opposed to "prefibrotic" MF were considered for this study. Patients provided informed consent for the use of archival material for mutational analysis, and the study was performed under a Florence University Institutional Review Board–approved protocol in referring institutions. The study was conducted in accordance with the Declaration of Helsinki.

Samples had been collected at diagnosis of PMF or PPV/PET-MF, or no later than 1 year afterward provided the patient had remained free of cytotoxic treatment.

4.1.1 SAMPLE PREPARATION

Peripheral blood granulocytes were separated by differential centrifugation over a Ficoll-Ipaque gradient. Briefly, 20 ml of whole blood were collected into EDTA-containing polypropylene tubes and processed within 4 hours. Blood was diluted 1:1 with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, carefully layered over 15 ml of Ficoll-Ipaque in a 50-ml tube, and centrifuged at 800 × *g* at room temperature for 20 minutes. Both the upper fraction and the Ficoll layer were carefully removed, while the lower fraction was collected and transferred to a fresh tube. Lysis of red blood cells was performed by the addition of a 10X volume of 1X BD PharmLyse solution (Becton Dickinson BD, San Jose, CA), centrifugation of the tube after a 15-minute incubation at room temperature, and removal of the supernatant. This step was repeated twice. After two washes in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline, the dry granulocyte pellet was stored at −20°C until processed. Cytosmears of these cellular preparations routinely showed a granulocyte content greater than 95%. DNA was extracted using the QIAmp DNA blood kit (Qiagen, GmbH, Hilden, Germany) and quantified using the NanoDrop technology (ND-1000 spectrophotometer; NanoDrop Technology, Wilmington, DE). All samples used for real-time PCR had 260:280 ratios greater than 1.8

4.1.2 WHOLE GENOME AMPLIFICATION

DNA was purified using conventional methods and subjected to whole-genome amplification with the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare). It contains all of the components necessary for mini-scale whole genome amplification by isothermal strand displacement amplification which is carried out with very high fidelity due to Phi29 DNA polymerase proofreading activity. A typical DNA yield of 4 to 7 µg DNA can be achieved in less than two hours with little hands-on time.

Starting from 10 ng/µl of high quality genomic DNA template, 1 µl of DNA was heat-denatured at 95° for 3 min and then cooled in 9 µl of sample buffer containing random hexamers. A master-mix containing DNA polymerase (1 µl), additional random hexamers, nucleotides, salts and buffers (a total of 9 µl) was prepared and added to each cooled sample. An isothermal amplification was performed at 30°C for 1.5hr. After amplification, the enzyme was heat inactivated for 10min at 65°C.

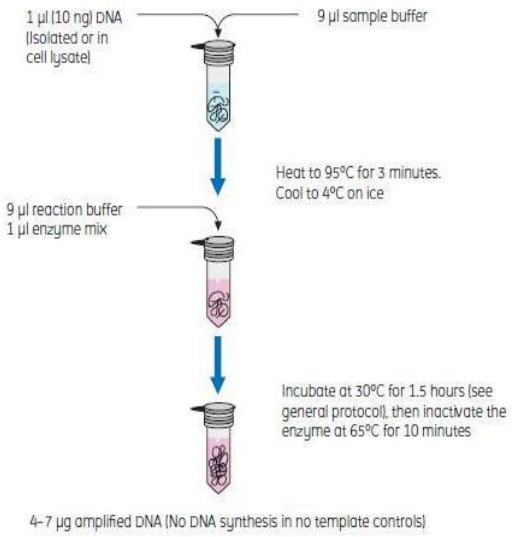


Figure 14. Whole Genome Amplification Protocol

PCR products were purified using Millipore plates (Millipore Corporation, Billerica, MA, USA).

4.2 GENOTYPING

We have considered several methods to either detect commonly known mutations or screening new ones in our large cohort of patients. Some of these methods were already reported in literature (PCR, real-time PCR and direct sequencing) and came into use in the last decade (High Resolution Melting Analysis). Moreover, we have developed new sophisticated methods (Next Generation Sequencing) also with the aim to preview their significance for routine laboratory and clinical work. An overviw of chronologically considered methods is reported below.

4.2.1 POLYMERASE CHAIN REACTION AND DIRECT SEQUENCING

A first set of experiments aimed to analyse the mutational status of selected genes (*ASXL1,CBL, DNMT3A, IDH1-2, SRSF2, TP53)* involved multiple steps: PCR-based amplification, DNA sequencing, and sequence interpretation (Figure 15). Reagents mix and cinetic steps of PCR reaction are listed in Table 8 and 9, respectively.

REAGENTS MIX	REACTION VOLUME (μL)
Buffer PCR 10X	2,5
$MgCl_2$	2,5
Forward primer	0,5
Reverse primer	0,5
dNTPs	0.5
DNA (~50ng/μl) + H_2O	18,3
Taq Gold	0.2

Table 8. PCR reagent mix

STEP	TAQ ACTIVATION	CYCLES (35 cycles)		
		DENATURATION	T° ANNEALING	EXTENSION
TEMPERATURE	95°C	95°C		72°C
TIME	13 min	30 sec	30 sec	30 sec

Table 9. PCR steps

Primers used to sequence target genes are listed in Table 10.

AMPLICON	FORWARD PRIMER	REVERSE PRIMER
ASXL1 (EXON 12)		
PCR1	AGGTCAGATCACCCAGTCAGTT	TAGCCCATCTGTGAGTCCAACTGT
PCR2	AGAGGACCTGCCTTCTCTGAGAA	TTCGATGGGATGGGTATCCAATGC
PCR3	ACTTGAAAACCAAGGCTCTCGT	GCAACCATCCCATCTGTCCTTGTA
PCR4	GGTGGACAAGGATGAGAAACCCAA	TGTCCTGTGACATAGCACGGACTT
PCR5	TGGATTCCAAAGAGCAGTTCTCTTC	CATGACAAAGGGCATCCCTTCCAA
PCR6	ACAGGAAAGCTACTGGGCATAGTC	CAAGAGTGCTCCTGCCTAAAGAGT
IDH1 (EXON4)	CGGTCTTCAGAGAAGCCATT	GCAAAATCACATTATTGCCAAC
IDH2 (EXON4)	GGGGTTCAAATTCTGGTTGA	TGTGGCCTTGTACTGCAGAG
CBL (EXON8)	TGTGGTTTCACTTTAAACCCTGGA	GCCAGGCCACCCCTTGTATC
CBL (EXON9)	GGCCTGGCTTTTGGGTTAGG	CACAATGGATTTTGCCAGTCTCC
SRSF2	GCCTTCGTTCGCTTTCAC	CGGACCTTTGTGAGGTCG
DNMT3A (EXON 15)	TTTCCATTCCAGGTAGCACACC	AGGCTCCTAGACCCACACACC
DNMT3A (EXON 16)	AGGGTGTGTGGGTCTAGGAGC	GCTGTGAAGCTAACCATCATTTCG
DNMT3A (EXON 17)	GACTTGGGCCTACAGCTGAC	TTTGCCCTTTACCCTCTCAA
DNMT3A (EXON 18)	ATAGGACAGTGGTGTGGCTCG	TTCTTCCTGTCTGCCTCTGTCC
DNMT3A (EXON 19)	GACAGCTATTCCCGATGACCC	TGCAGATGAGACAGGATGAAGC
DNMT3A (EXON 20)	GCCGGCGCTGTTTCATGC	CCACTATGGGTCATCCCACCTGC
DNMT3A (EXON 21)	CCTTCCCGCTGTTATCCAGG	CATCCTGCCCTTCCTTCTCC
DNMT3A (EXON 22)	TTTGGTAGACGCATGACCAG	AGCACAGCAATCAGAACAGC
DNMT3A (EXON 23)	TCCTGCTGTGTGGTTAGACG	ATGATGTCCAACCCTTTTCG
TP53 (EXON 1)	GGGTTGGAAGTGTCTCATGC	CCCCAACCCTTGTCCTTACCAG
TP53 (EXON 2)	GGGCTGAGGACCTGGTC	GAAGCCAAAGGGTGAAGAGG
TP53 (EXON 3)	GTGCACACCTATAGTCCCAGC	GTGAGGAATCAGAGGCCTGG

TP53 (EXON 4)	CTGGTTGCCCAGGGTCC	TCACGCCATTCTCCTTCC
TP53 (EXON 5)	TTGCCACAGGTCTCCC	AGAGGCAAGCAGAGGCTG
TP53 (EXON 6)	GGACCTGATTTCCTTACTGCC	GCATAACTGCACCCTTGGTC
TP53 (EXON 7)	CAAGGGTGCAGTTATGCCTC	GGCATTTTGAGTGTTAGACTGG
TP53 (EXON 8)	AAGACAATGGCTCCTGGTTG	AAGCAGGCTAGGCTAAGCTATTG
TP53 (EXON 9)	AACTTGAACCATCTTTTAACTCAGG	TAGGAAGGCAGGGGAGTAGG
TP53 (EXON 10)	AAGCATTGGTCAGGGAAAAG	AGACCCAAAACCCAAAATGG

Table 10. Primers used for sequencing *ASXL1,CBL, DNMT3A, IDH1-2, SRSF2, TP53.*

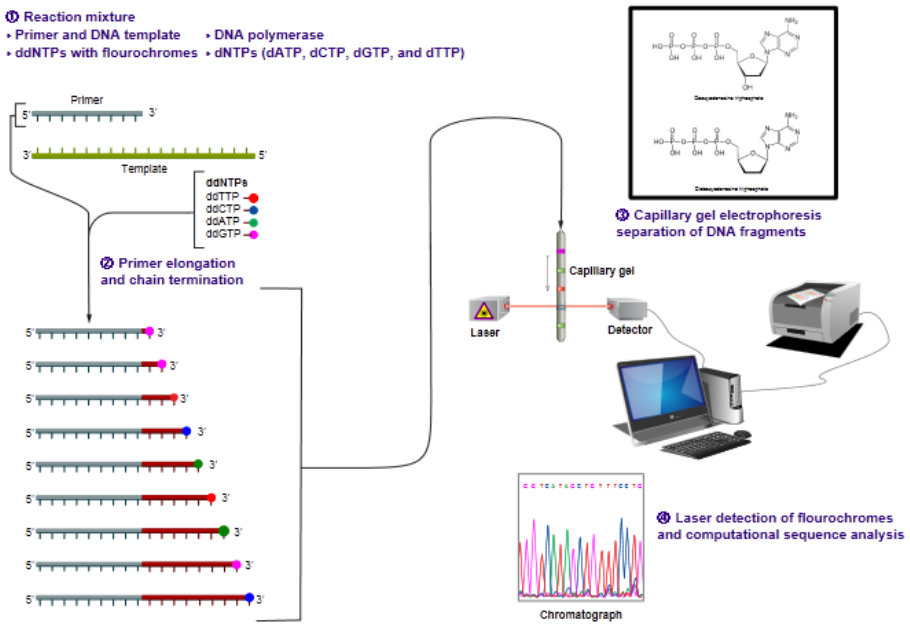


Figure 15. Direct Sequencing Workflow

4.2.2 QUANTITATIVE REAL-TIME PCR

Quantitative PCR (qPCR, also called real-time PCR) is precisely a method used to measure the quantity of a PCR product in a real-time using several kind of fluorescent dyes. We uses this approach to analyze the mutational status of JAK2 V617F and *MPL*W515L or W515K point mutations.

1. **Analysis of the JAK2^{V617F} mutation.** The mutant allele burden was measured by a quantitative real time (QRT)-PCR assay using 20 ng genomic DNA. PCR amplification and detection were performed on the StepOne real-time PCR system (Applied Biosystems, Foster City, CA) using the following cycling conditions: 10 minutes at 95°C followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. This method consists of two reaction: one performed using primers flanking the hot spot and specific for mutant allele (forward primer 5'-GCGCGGTTTTAAATTATGGAGTATGTT-3'; reverse primer 5'-GCGGTGATCCTGAAACTGAATTTTC-3') and the other one performed with primers

specific for wt allele (forward primer 5'-GCGCGGTTTTAAATTATGGAGTATGTG-3'; reverse primer 5'-GCGGTGATCCTGAAACTGAATTTTC-3'). In both reactions primers were employed together with Taqman probe 5'-TGGAGACGAGAGTAAGTAAAACTACAGGCT-6FAM-MGBNFQ. All samples were analyzed in duplicate (*JAK2*^{wt} and *JAK2*^{V617F}) and the amount of *JAK2*^{V617F} allele was calculated by comparison with serial dilutions of a specific plasmid (wt, 2.5% mut, 5%mut, 12.5% mut, 25% mut, 50% mut, 75% mut, 100% mut).

The mean of duplicate ΔCT determinations ($C_T^{JAK2V617F}$–C_T^{JAK2WT}) was used to calculate the percentage of mutant alleles. Positive and negative controls were included in each assay.

2. **Analysis of *MPL*W515L/K mutations.** This assay, developed in our laboratory (Pancrazzi A. et al J Mol Diagn. 2008) uses a single set of primers (Table 11), with the advantage that amplification of mutant and wild-type alleles has the same efficiencies, while specificities are obtained with different probes that were modified according to the LNA chemistry (Sigma-Proligo, Paris, France).

Real-time PCR primers/probes	Sequence
MPL forward primer	5′-agcctggatctccttggtgac-3′
MPL reverse primer	5′-accgccagtctcctgcct-3′
MPL wild-type probe	5′-ctgctg+Aggt+Ggc+Agtttc-3′
MPL 515W>L probe	5′-ctgc+Tgagg+T+Tgcag+T+Ttc-3′
MPL 515W>K probe	5′-tgc+Tgctgagg+A+Ag cagtttcc-3′

Table 11. Sequences of Primers and Probes Used for Real-Time PCR Assay of the *MPL*W515L or W515K Mutation

Real-time PCR was performed using the StepOne real-time PCR system (Applied Biosystems, Foster City, CA). Forty nanograms of granulocyte DNA were used in each real-time PCR assay. Three different real-time reactions were set up in triplicate (one each for W515L, W515K, and wild-type control) for each DNA sample. A 20-µl reaction contained 1X TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol/L each primer, and 200 nmol/L each LNA-modified probe. Control wells without template (NTC) were included in each assay. Amplification and detection were performed under the following conditions: initial hold at 50°C for 2 minutes, hold at 95°C for 10 minutes followed by 55 cycles at 95°C for 15 seconds and 66°C or 62°C for 1 minute for the case.

of *MPL* unmutated and W515L probe or W515K probe, respectively. The fluorescent signal intensities were recorded and analyzed during PCR amplification using the SDS software (Applied Biosystems). The mean ΔC_T of triplicate determinations (C_T*MPL*$^{W515L/K}$ – C_T^M*PL*$^{wild\text{-}type}$) was calculated, and the percentage of mutant alleles in the sample was obtained by comparison with a reference curve of serial dilutions of mutant plasmid mixtures in wild-type plasmid DNA. Both positive and negative controls were included in each assay.

4.2.3 HIGH RESOLUTION MELTING

High resolution melt (HRM) analysis is used to characterize DNA samples according to their dissociation behavior as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature (Figure 16).

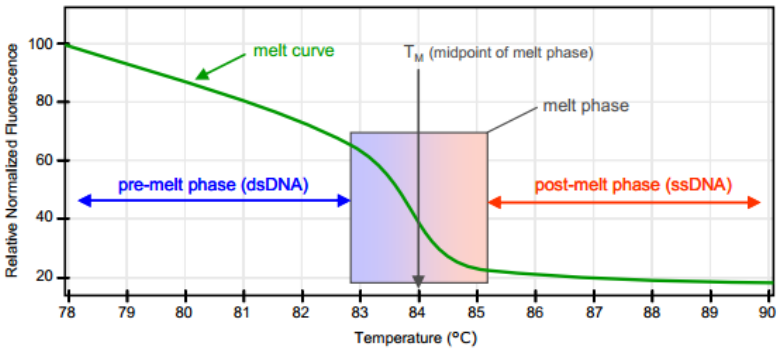


Figure 16. Melt curve

Prior to performing a HRM analysis, a target sequence must first be purified to high copy number. This is normally done using a DNA amplification reaction such as the PCR in the presence of a dsDNA intercalating fluorescent dye. The dye does not interact with ssDNA but actively intercalates with dsDNA and fluoresces brightly in this state. This shift in fluorescence can be used firstly to measure the increase in DNA concentration during a pre-HRM amplification reaction and then to directly measure thermally-induced DNA dissociation by HRM. Initially, fluorescence is high in a melt analysis because the sample starts as dsDNA, but fluorescence diminishes as the temperature is raised and DNA dissociates into single strands. The observed “melting” behavior is characteristic of a particular DNA sample. The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the temperature of melting (TM) of the particular DNA fragment under analysis. HRM is made possible by the introduction of third generation fluorescent dsDNA dyes. In our set of experiments we used LC Green (Idaho Technologies, Salt Lake City, UT). These dyes have lower toxicity in the amplification reaction than second generation dyes (such as SYBR Green) and can therefore be used at higher concentrations for greater saturation of the dsDNA sample.

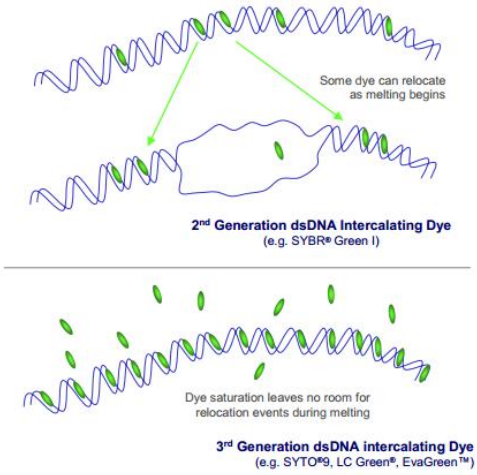


Figure 17. Characteristics of 2nd and 3rd generation intercalating dyes.

This lead to higher fidelity measurement of fluorescent signals because there is less dynamic dye redistribution tonon-denatured regions of the nucleic strand during melting and because dyes do not favor higher melting temperature products (Wittwer et al 2003). The use of combination of these characteristics provides greater melt sensitivity and higher resolution melt profiles.

Using HRM is possible to characterize samples based on sequence length, GC content, DNA sequence complimentarity and it can also be used to quantitatively detect a small proportion of variant DNA in a background of wild-type sequence at sensitivities approaching 5%. For these reasons *EZH2* and *TET2* mutation analysis were performed using HRM in a Rotor-Gene 6000 instrument (Corbett Life Science), using primer sets reported below (Table 12).

EZH2		
Exon	**Forward primer**	**Reverse primer**
2	GGTGATCATATTCAGGCTGG	AAACTTATTGAACTTAGGAGGGG
3	TTTCTCCTTTCCTCTCCTTCA	TCCAATAGCATAAACCAAAAGATG
4	GGCTACAGCTTAAGGTTGTCCT	CTGTCTTGATTCACCTTGACAAT
5	AAATCTGGAGAACTGGGTAAAGAC	TCATGCCCTATATGCTTCATAAAC
6	AGGCTATGCCTGTTTTGTCC	AAAAGAGAAAGAAGAAACTAAGCCC
7	CTGACTGGCATTCCACAGAC	AAGTGTAGTGGCTCATCCGC
8	CATCAAAAGTAACACATGGAAACC	TTGTAATAAATGATAGCACTCTCCAAG
9	TCCATTAATTGACTTTTCCAGTG	ACCTCCACCAAAGTGCAAAG
10	TTCTCTTCCATCAAAATGAGTTTTAG	TCCTCACAACACGAACTTTCAC
11	GAGTTGTCCTCATCTTTTCGC	CCAAGAATTTTCTTTGTTTGGAC
12	AAGAATGGTTTGCCTAAATAAGAC	CCTTGCCTGCAGTGTCTATC
13	TCTTGGCTTTAACGCATTCC	CAAATTGGTTTAACATACAGAAGGC
14	TGATCGTTTCCATCTCCCTG	AGGGAGTGCTCCCATGTTC
15	GAGAGTCAGTGAGATGCCCAG	TTTGCCCCAGCTAAATCATC
16	TTTTTGATGATGTGATTGTGTTTT	TGGCAATTCATTTCCAATCA
17	TTCTGTCAGGCTTGATCACC	CTCGTTTCTGAACACTCGGC

18	AGGCAAACCCTGAAGAACTG	TTCCAATTCTCACGTCAAAGGTA
19	CCGTCTTCATGCTCACTGAC	AAAAACCCTCCTTTGTCCAGA
20	CTTCAGCAGGCTTTGTTGTG	GGGGAGGAGGTAGCAGATG
TET2		
3,1	AACTAGAGGGCAGCCTTGTG	CTTGATCTGAAGGAGCCCAG
3,2	GAAGGGAAGCCAGAATAGTCG	ACTTTTCCCCTCCTGCTCAT
3,3	AACACATAACTGCAGTGGGC	CATCATCAGCATCACAGGC
3,4	ACTCACCCATCGCATACCTC	GGTGGTGTGGTAGTGGCAG
3,5	GTGGCAGCTCTGAACGG	CAGTCATTGTCCCTGCAGTC
3,6	GAGGGTAAACCTGAGGCACC	GCCTCATTACGTTTTAGATGGG
3,7	AGACAAGGAGCAAACACGAG	TGCCACACAGTGACTGCAC
3,8	CCAAGGTACAGTGGACCAAC	TCCTTCTCTTTGCTGATCATTG
3,9	TCTCCCTCAAAACCAGCAAC	TTGGAAAATATTGCATGTGATG
3.10	TTTCTTGTTCAAACAATACACACC	GTGTTTGCTGCTGTTCTTGC
3,11	CTGACCAGGGAGGAAGTCAC	AAACTGTGACTGGCCCTGAC
3.12	GCATCATTGAGACCATGGAG	TCCCATATCTGAAGATAAATTTGC
4	GCCCTTAATGTGTAGTTGGGG	TGCTTTGTGTGTGAAGGCTG
5	TGCCTCTTGAATTCATTTGC	AACCCAATTCTCAGGGTCAG
6	GTTTGGGATGGAATGGTGAT	ACCAAAGATTGGGCTTTCCT
7	TCCATAGCAATGAATTTGGTCTT	GCGATTATACATCAGGAAGTAAACAA
8	TTGTGTTTGGGATTCAAAATG	TGTTACAATTGCTGCCAATGA
9	TTTCGCATTCACACACACTTT	CAGCTGCTAAGCTGTCCTCA
10.1	TTGGGACCTGTAGTTGAGGC	GTTTCCTTTGTCGGCAAGTC
10.2	GAGTTTGGGAGTGTGGAAGC	CAGAACTTACAAGTTGATGGGG
11.1	TCTGTTCTCTCTTACCCTGTCCA	CCATTGCATTGATATGATGGAT
11.2	ATCCCATGAACCCTTACCC	TCTAGAGGTGGCTCCCATGA
11.3	GGGAAATTGCCTCCTTATCC	ACCAGACCTCATCGTTGTCC
11.4	AAAAGCAGCCATTGGCACTA	AGGCTCCCGTTTCACTTTTT
11.5	GCCCAGACTATGTGCCTCAG	TGCCCACGTCATGAGAACTA

Table 12. Primers used for screening *EZH2* and *TET2* through HRM.

Amplification conditions were as follows: 95°C for 5min, 45 cycles of 95°C for 15s, annealing at 72° C for 30s, 3 Hold steps at 72°C for 5min, 95° for 5s, 50°C for 30s, followed by HRM step.

In samples that showed a melt curve similar to control DNA, a subsequent analysis was performed by mixing test DNA (90%) with DNA from healthy controls (10%) with the aim to identify homozygous mutations. Products showing abnormal melt pattern were PCR amplified for direct sequencing in a total volume of 25 µl PCR mix containing 20 ng template DNA. PCR conditions were as follows: 95°C 13min; 35 cycles at 95°C for 30s, annealing temperature 72°C for 30s, extension at 72°C for 10min. Direct sequencing was performed using technology based on fluorescent dye terminators employing forward and reverse primers. Sequence analysis was performed using Mutation Surveyor (SoftGenetics, USA). All discovered mutations were further validated by repeating PCR and direct sequencing on genomic (i.e. not subjected to WGA) DNA from the archival sample.

4.2.4 NEXT GENERATION SEQUENCING

The discovery of the use of dideoxy nucleotides for chain termination by Sanger et al. [1977] marked a milestone in the history of DNA sequencing. This concept provided a basis for the development of automated Sanger sequencing (Smith et al. [1986], Ansorge et al. [1987]) which has been the method of choice for DNA sequencing for almost 20 years and the core technology of the Human Genome Project, which was funded in 1990 with the goal of determining all three billion base pairs making up the human genome. The project took ten years to produce first draft results (Lander et al. [2001], Venter et al.[2001]) and an additional three years to complete (Jasny and Roberts [2003]). During the project's final phase and early years there after numerous spin-off projects have been launched including the International HapMap Project and the prominent 1000 Genomes Project. The former project aimed at developing a haplotype map of the human genome which describes the common patterns of human DNA sequence variation (International HapMap Project [2006]). The latter concentrated on sequencing the genomes of at least one thousand anonymous participants from a number of different ethnic groups to provide a comprehensive resource on human genetic variation (1000 Genomes [2008]). Both projects were accompanied by the necessity for extensive sequencing. They led, together with a program aiming at the economic sequencing of complete, high-quality, mammal-sized genomes (Service [2006], Mardis [2006]), to the development of new sequencing technologies. These so-called next-generation sequencing (NGS) technologies allowed sequencing at unprecedented speed in combination with low costs per base. As a consequence, the number of sequencing related data stored in public available databases has increased significantly over the last years and is expected to grow even faster. Ion Torrent, a startup recently acquired by Life Technologies, has made significant progress in bringing to market a next-generation sequencing system that utilizes pH changes to detect base incorporation events (when a nucleotide is incorporated into a strand of DNA by a polymerase, a hydrogen ion is released as a by product) (Figure 18)

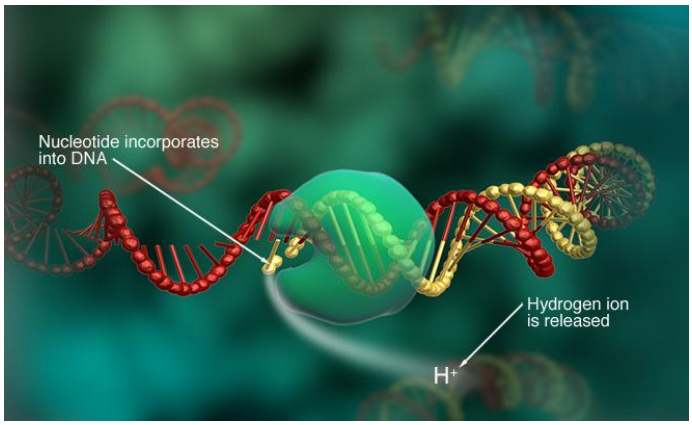


Figure 18. Ion Torrent next-generation sequencing system.

Field-effect transistors (FETs) are used to measure a change in pH in a microwell structure. To increase throughput, the Ion Torrent sequencing chip makes use of a highly dense microwell array in which each well acts as an individual DNA polymerization reaction chamber containing a DNA polymerase and a sequencing fragment. Just below this layer of microwells is an ion-sensitive layer followed by a sublayer composed of a highly dense FET array aligned with the microwell array. (Figure 19)

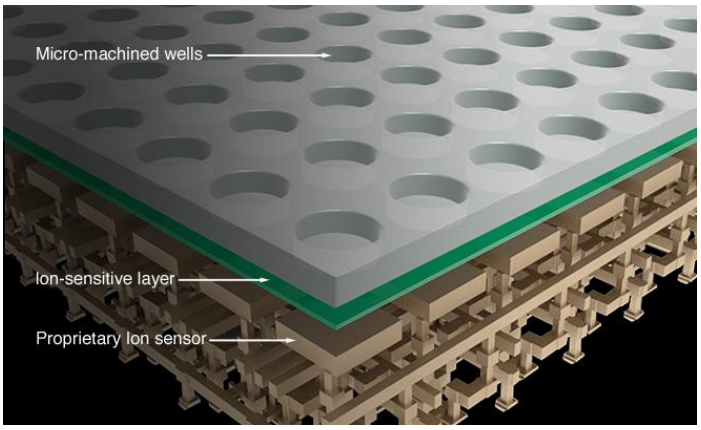
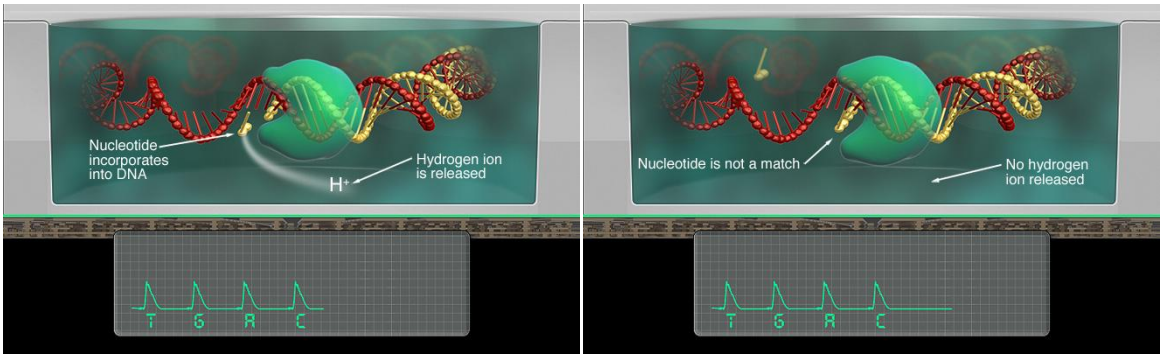


Figure 19. Ion Torrent sequencing chip structure

Sequential cycling of the four nucleotides into the microwells enables primary sequence resolution since the FET detector senses the change in pH created during nucleotide incorporation and converts this signal to a recordable voltage change (Fugure 20 A). Because the Ion Personal Genome Machine™ (PGM™) sequencer sequentially floods the chip with one nucleotide after another, if the next nucleotide that floods the chip is not a match, no voltage change will be recorded and no base will be called (Figure 20 B). If there are two identical bases on the DNA strand, the voltage will be double, and the chip will record two identical bases called (Figure 20 C).

A



B

C

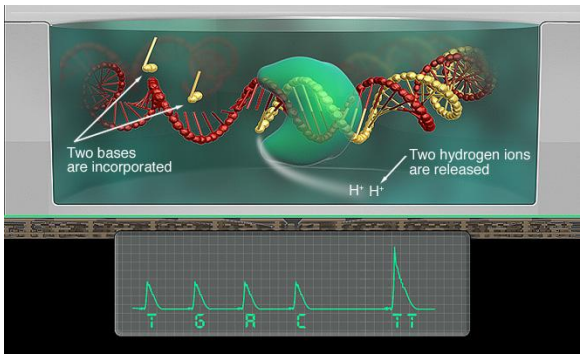


Figure 20. Sequential cycling of the four nucleotides into the microwells enables primary sequence resolution since the FET detector senses the change in pH created during nucleotide incorporation and converts this signal to a recordable voltage change (A). Because the Ion Personal Genome Machine™ (PGM™) sequencer sequentially floods the chip with one nucleotide after another, if the next nucleotide that floods the chip is not a match, no voltage change will be recorded and no base will be called (B). If there are two identical bases on the DNA strand, the voltage will be double, and the chip will record two identical bases called(C)

At present, Ion Torrent offers three generations of chips: the Ion 314, the Ion 316 and the Ion 318 sequencing chips. The 1.2 million microwells on the Ion 314 chip generates roughly 10 Mb of sequence information with average read lengths on the order of a 100 bases. To further increase throughput, the Ion 316 chip and the Ion 318 chip are being built with 6.2 million and 11.1 million microwells, respectively. The Ion 318 chip produces 1 Gb of sequencing data with average read lengths of 200-400 bases.

Because of many studies have focused on mutational “hotspots” or have used less sensitive techniques to identify new somatic mutations we have developed a new system of generation sequencing, Ion Torrent (Life Technologies) with the aim to perform even deeper mutational analyses. We have started to use Ion AmpliSeq™ Custom Panels (*Life Technologies*), pools of PCR primers specifically designed with Ion AmpliSeq™ Designer webhosted software, available at *www.ampliseq.com,* for amplification and coverage of large targets. We started from 10 ng of genomic DNA per amplification which was performed using 2 pools of primers targeting up to 600 amplicons. Before sequencing, each sample (corrisponding to each patient) was ligated with one Ion Xpress™ Barcode Adapter with the aim to prepare multiple barcoded libraries that could be combined and loaded onto a single Ion 316 Chip to minimize the sequencing run time and cost. After quantifying and diluting each library at the optimal input concentration, equal volumes of the barcoded libraries were combined in sets of 16 barcodes.

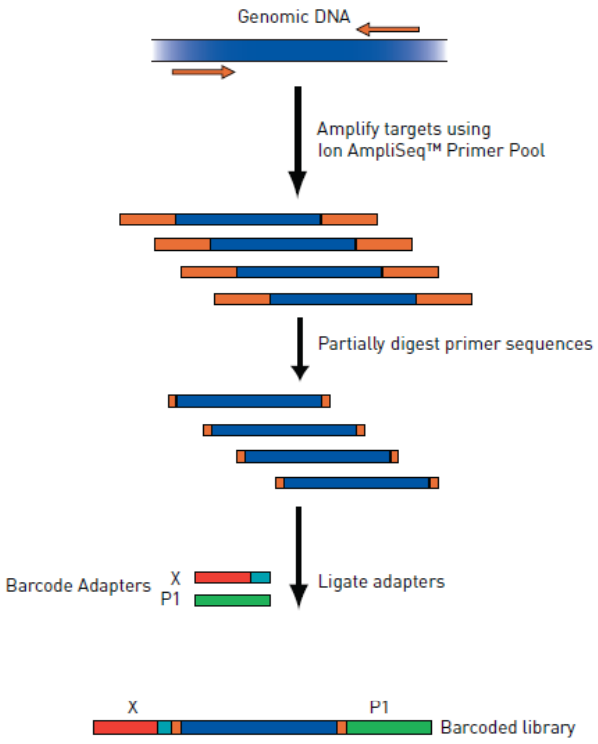


Figure 21. Ion Ampliseq 2.0 Protocol (Life Technologies)

4.3 STATISTICAL ANALYSIS

Our primary aim of our mutational analyses was to determine the correlation between mutational status and major outcome events, which included overall survival (OS) and transformation to acute leukemia. We also investigated whether mutational status was correlated with specific laboratory parameters or clinical features, including RBC indexes, leukocyte or platelet count, percentage of PB blasts, splenomegaly, constitutional symptoms, and the ranking of patients according to the International Prognostic Score System (IPSS) developed by the IWG-MRT. Constitutional symptoms included loss of 10% or more of body weight in the last 6-months, drenching night sweats, or unexplained fever. Splenomegaly was measured in centimeters from the left costal margin (LCM); we considered 2 groups of patients who presented a spleen enlargement smaller than or greater than 10 cm from the LCM, respectively.

The χ^2 or Fisher exact test (2 × 2 table) or the χ^2 test for trend (larger contingency table) were used as appropriate to compare the variables among the different patient groups that had been categorized according to mutational status. The analysis of continuous variables among the groups was performed using the Mann-Whitney *U* test (2 groups) or the Kruskal-Wallis test with the use of the Dunn method for multiple comparison. Kaplan-Meier analysis and the log-rank test were used to estimate OS. Cox regression models were used to perform multivariate analysis. $P < .05$ was considered to indicate statistical

significance; all tests were 2-tailed. Data were processed using SPSS Version 17.0 software (StatSoft)

4.4 COLONY ASSAY

Peripheral mononuclear cells (MNCs) from two PMF patients *JAK2*V617F^{mut}/ *EZH2*^{mut} were plated at 1 × 10^5 cells/mL in 35-mm-diameter dishes in quadruplicate in Methocult H4434 medium . (StemCell, Vancouver, Canada). The indicated concentration provided an optimal density of colonies for picking, without the danger of contamination by neighboring colonies. Cultures were incubated at 37°C in a humidified 5% CO2 atmosphere, and were scored at day 14. Single burst-forming units (BFUE) and colony-forming units granulocyte (CFU-G) were picked and analyzed individually for the presence of *EZH2* and *JAK2*- *V617F* mutations by DNA sequencing and allele-specific polymerase chain reacton (Baxter-method), respectively.

Figure 22. a) Single burst-forming units (BFUE) and b) colony-forming units granulocyte (CFU-G)

Baxter J. et al designed a three primer allele specific PCR with a reverse primer, a forward G>T mutation specific primer and a forward internal control primer. The mutation specific primer has an intentional mismatch at the third nucleotide from the 3' end, which improves specificity and amplifies a 204-bp product as compared to the internal control primer which generates a 364-bp product. Both forward primers were used in a concentration of 0.5µmol/L, whereas the concentration of the reverse primer was 1.0 µmol/L. A Hotstar Taqman polymerase was used in a 20 µl volume of 15 mM MgCl2 buffer (Qiagen, Hamburg, Germany) and dNTP. The PCR conditions were: 36 cycles of denaturation at 95° C for 15 seconds, annealing at 58° for 30 seconds and extension at 72° for 30 seconds. All reactions were performed in duplicates on a GeneAmp© PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Fifteen µl of the PCR product was loaded on a 1% Agarose gel, run at 100 Volts for 30 minutes and analyse by Kodak 1 DLE 3.6.

4.5 ISOLATION OF MURINE BONE MARROW-DERIVED TER119+ AND GR1+ CELLS

The analysis of miRNA expression profile was performed on cells purified from *Jak2*^{V617F} KI animals kindly concessed Dr. Jean Luc Villeval (Mouse Clinical Institute, Illkirch, France)where the model was generated. The animal model was developed as

follows: a fragment encompassing *Jak2* exon 13 was amplified by polymerase chain reaction (PCR) on 129S2/SvPas mouse embryonic stem (ES) cell genomic DNA to introduce the GTC > TTC point mutation (617V > F) and subcloned in a vector with a floxed neomycin resistance (NeoR) cassette resulting in a step1 plasmid. A 5' homologous arm was amplified by PCR and subcloned in step1 plasmid to generate a step2 plasmid. Finally, a 3' homologous arm was subcloned in step2 plasmid to generate the final targeting construct. Targeted 129S2/SvPas ES clones were confirmed by PCR and Southern blot and injected into C57BL/6J blastocysts to generate chimeric mice. Chimeras (L2) were crossed with flippase (FLP) TG C57BL/6 mice to excise the FRT site-flanked NeoR cassette on F1 progenies (L-). Finally, F1 animals (L-) were crossed with 129Sv mice to generate F2 animals.

Mice belonging to L- progenies (n=2) and L2 progenies (n=2) were sacrified and then bone marrow cells were purified from both femour and tibia. Bones were transfered to 50mL Falcon tube containing sterile PBS. Immediately after, we removed a bone from the PBS with tweezers and cut the ends. We flushed out the bone marrow into the 50mL Falcon tube by inserting a 20 mL syringe filled with PBS at the knee side of both types of bone. After passing the PBS through the bone, the colour of the bone turned from red to white, indicating that all the marrow was expelled. When all bones were washed of marrow, we centrifuged cells at 1500rpm for 5 minutes. $TER119^+$ and $GR1^+$ cells were obtained by immunomagnetic separation using anti-TER119-mouse and anti-GR1-mouse antibodies (Milteny Biotec). In adult mice, Anti-Ter-119 reacts with 20–25% of bone marrow cells and approximately 50% of spleen cells, but not with thymocytes or lymphonode cells. The mouse Anti-Gr-1 antibody reacts with Ly-6G, a 21-25 kDa, GPI-anchored cell surface protein, previously defined as the granulocyte-differentiation antigen-1 (Gr-1). Gr-1 is expressed on mature granulocytes in bone marrow and peripheral tissues. The Anti-Gr-1 antibody also stains monocytes transiently during their differentiation in bone marrow and at low levels plasmacytoid dendritic cells in lymphoid tissues. The quality of purified $TER119^+$ and $GR1^+$ populations was evaluated by flow citometer.

4.6 miRNA EXPRESSION PROFILE

The analysis of miRNA expression profile of (n=2) $TER119^+$ and (n=2) $GR1^+$ cells purified from either L2 and L- mice was performed using miRCURY LNA™ Universal RT microRNA PCR KIT (EXIQON). The miRCURY LNA™ Universal RT microRNA PCR system is a microRNA-specific, LNA™-based system designed for sensitive and accurate detection of microRNA by quantitative real-time PCR using SYBR®Green. The method is based on two steps: 1) universal reverse transcription (RT) followed by 2) real-time PCR amplification with LNA™ enhanced primers (Figure 23).

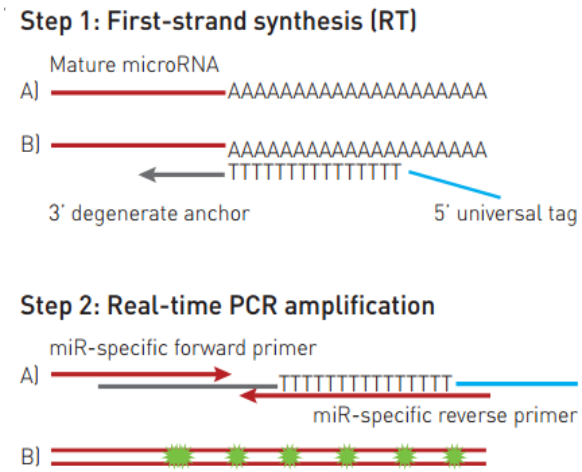


Figure 23. A poly-A tail is added to the mature microRNA template (step 1A). cDNA is synthesized using a poly-T primer with a 3' degenerate anchor and a 5' universal tag (step 1B). The cDNA template is then amplified using microRNA-specific and LNA™-enhanced forward and reverse primers (step 2A). SYBR® Green is used for detection (step 2B).

Starting from 20 ng of total RNA, the analysis of miRNA expression profile was performed using a pre-defined Mouse&Rat panel designed for targeting 384 know microRNAs. The experiments were accpmplished on ABI 7900 instruments (Applied Byosistem). After performing an initial data analysis using the software supplied with the real-time PCR instrument to obtain raw Ct values,the statistical analysis of our results was carried out with the collaboration of the bioinformatic group of Biology Department of University of Padova, under the supervision of Dr S. Bortoluzzi (AGIMM). Data of miRNA expression profile were integrated with those of gene expression profile performed, on the same set of samples, by Prof. Rossella Manfredini Lab. group (Dep. of Rigenerative Medicine, University of Modena and Reggio Emilia, AGIMM).

4.7 STATISTICAL ANALYSIS

After signal reconstruction with RMA, gene expression profiles with VC (variation coefficient) values included in the lowest 33% (expression profiles less informative and poorly variable across samples) were filtered out, obtaining 11,505 genes. miRNA expression data matrix (2E-DeltaCt values) was calculated from Ct row data and then normalized to make comparable the two set of samples, using quantile-quantile normalization. We considered the contrasts:

L2 VS L- GR1 L2 VS L- TER119 L2 VS L- ALL

For each contrast we selected those miRNAs with |logFC(KI/WT)|>2 AND maximum expression level in contrasted samples over the III quartile calculated on the corresponding matrix (most of the values in the matrix are low, so the max over III

quartile is a good filtering criteria, that eliminates only miRNAs with only low expression values). miRNA Target predictions were collected according to Targetscan 6.1. For each miRNA, predictions consisted of predicted target sites in human transcript sequences. For each predicted site, a "context score" was given, that was considered the most important feature to predict functional targets, as well as information about site evolutionary conservation. We selected top 25 predictions according to context score. A gene was considered target of a miRNA if at least one transcript of the gene carries a selected target site. After calculating all the pairwise Spearman correlations between miRNAs differentially expressed and predicted target genes we selected two sets of relations supported by expression data analysis, using different thresholds on correlation strength: the Spearman correlation =1 and the less stringent Spearman Correlation <-0.9

The reconstructed regulatory networks were drawn by Cytoscape.

4.8 RNA ISOLATION, SHORT RNA LIBRARY CONSTRUCTION, AND SEQUENCING *(Stefania Bortoluzzi, Andrea Bisognin et al. Blood 2012)*

Total RNA from SET2 cells (DSMZ, Braunschweig, Germany) was size-fractionated on a 15% trisborate-EDTA-Urea polyacrylamide gel, RNA fragments 15-50 nts length were isolated from the gel, quantified, and ethanol precipitated. The SRA 5' adapter (Illumina) was ligated to the RNA fragments with T4 RNA ligase (Promega), followed by 15% tris-borate-EDTA-Urea polyacrylamide gel size-fractionation. The RNA fragments of ~41-76 nts length were isolated. The SRA 3' adapter (Illumina) ligation was then performed, followed by a second size-fractionation and recovery of RNA fragments of ~64-99 nts length. The ligated RNA fragments were reverse transcribed to singlestranded cDNAs using M- MLV (Invitrogen) with RT-primers recommended by Illumina. The cDNAs were amplified with pfx DNA polymerase (Invitrogen) in 20 cycles of PCR using Illumina's small RNA primers set. PCR products were then purified on a 12% TBE polyacrylamide gel and a slice of gel of ~80-115 bps was excised. This fraction was eluted and the recovered cDNAs were precipitated and quantified on Nanodrop (Thermo Scientific) and on TBS-380 mini-fluorometer (Turner Biosystems) using Picogreen® (Invitrogen). Details about raw data processing and filtering, reads mapping, espressed miRNA indentification and new miRNA target prediction were available in Supplemental Methods of the published manuscript. *(Stefania Bortoluzzi, Andrea Bisognin et al. Characterization and discovery of novel miRNAs and moRNAs in* JAK2*V617F-mutated SET2 cells. Blood March 29, 2012vol. 119 no. 13 e120-e130)*

4.8.1 miRNA EXPRESSION PROFIILING IN SET2 CELLS TREATED WITH JAK1/JAK2 INHIBITOR INC424/RUXOLITINIB *(Stefania Bortoluzzi, Andrea Bisognin et al. Blood 2012)*

Exponentially growing SET2 cells were cultured in medium supplemented with 10% fetal bovine serum in the presence or the absence (control cells) of 160, 800 e 1,600 nM

concentrations of the JAK1 and JAK2 inhibitor INC424/Ruxolitinib for 3 and 6 h. Triplicate cultures were prepared for each drug concentration and culture time. Total cellular RNA, including small RNAs, was extracted using RNeasy Micro kit (Qiagen, Valencia, CA). Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit, Agilent Technologies, Waldbrunn, Germany) were used to determine the purity/integrity of RNA samples using Agilent 2100 Bioanalyzer. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to evaluate the RNA sample concentration, and 260/280 and 260/230 nm ratios were considered to verify the purity of total RNA. Total RNA (500ng for samples) was labeled using the FlashTag® Biotin HSR kit (Genisphere) and hybridized to the Affymetrix Genechip miRNA array 2.0 using manufacturer's protocols. Image files from scanning (.dat and .cel files) were generated with Affymetrix Expression Console package. Signal reconstruction from raw array data and data normalization were obtained using RMA (Affy library of Bioconductor) with a custom procedure (background subtraction step performed considering all species and controls of the chip; normalization and summarization steps considering only human probesets). An expression matrix including 1,100 human microRNAs was obtained and used to identify those miRNAs whose expression level was affected by the dose and time factor. A repeated measure ANOVA model was fitted for each miRNA with dose (levels of INC424/Ruxolitinib) as a between-groups factor and time (3h and 6 h) as a within-group factor. miRNAs for which the dose factor resulted significant in a F test at $\alpha<0.05$ were selected and contrasts were examined with Tukey Honest Significance Difference method.

5. RESULTS

When I started working on my PhD thesis project, and quite soon after the discovery of JAK2V617F mutation, it became increasingly appreciated that additional genetic events were involved in the clonal proliferation of myeloproliferative neoplasms. Genetic studies, in fact, were revealing that a spectrum of somatic mutations might be acquired at various disease stage, including leukemic transformation. Several of these mutations affect genes encoding epigenetic modifiers (*IDH1*, *IDH2*, *DNTM3A*, *ASXL1*, *EZH2*, and *TET2*), splicing regulating factors (SRSF2) as well as genes involved in JAK/STAT signaling pathway (CBL, LNK) other than JAK2 itself (Figure 24)

However, preliminary data only were available and many questions were still to be solved.

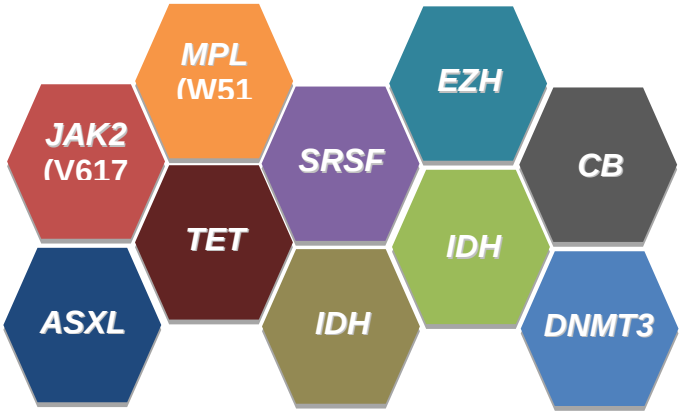


Figure 24. Selected genes

5.1 EZH2 MUTATIONAL STATUS IN PATIENTS WITH MYELOFIBROSIS

Data from the Nick Cross lab in Southampton University showed novel loss-of-function mutations in *EZH2* in 1 of 30 (3%) PV and in 4 of 30 PMF patients (13%), as well as in 11%-25% of patients with myelodysplastic syndromes (MDS) and in 10% of patients with MDS/MPN.[249] Mutations were spread throughout the gene and included missense, nonsense, and premature stop codons; both monoallelic and biallelic mutations were described. Among patients with MDS/MPN, survival was significantly worse in those with *EZH2* mutation. Furthermore, subjects with homozygous mutations had a trend toward inferior survival compared with heterozygous patients.[249] Several different *EZH2* mutations were also reported in an independent series of 102 patients with MDS,[248] whereas a functionally distinct heterozygous missense point mutation at codon Y641 of *EZH2* was described in patients with follicular lymphoma (7%) and diffuse, germinal center-origin, large B-cell lymphomas (22%).[287]

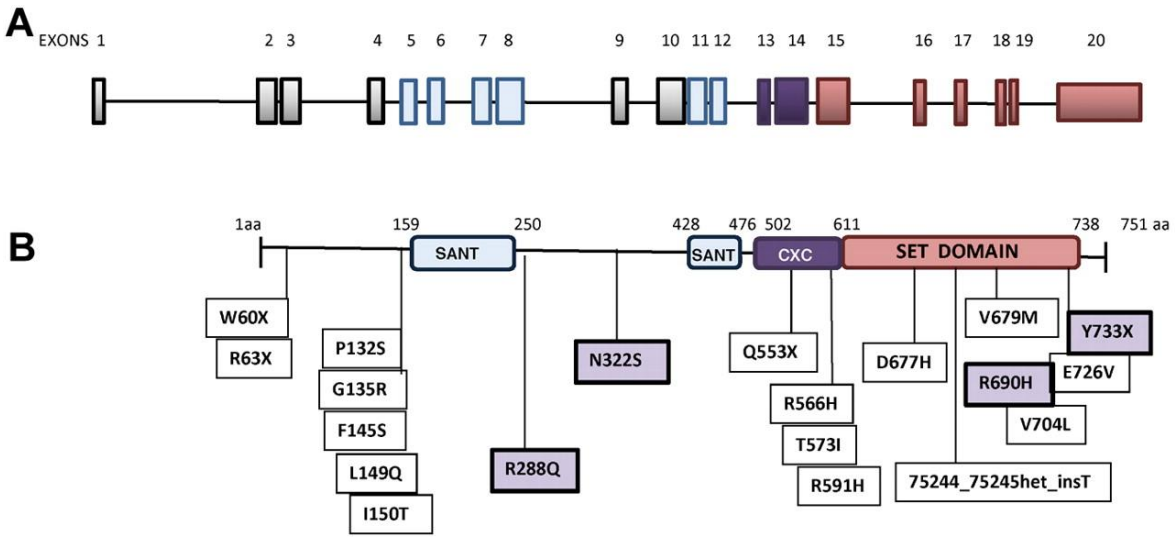
Thus, the aim of my first set of experiments was to determine the frequency and characteristics of *EZH2* mutations in a large series of patients with primary myelofibrosis and PPV/PET MF and to analyze the prognostic relevance of a mutated *EZH2* status. We included in the study 518 patients: 370 subjects with PMF, 84 with PPV-MF, and 64 with PET-MF. A total of 321 subjects were *JAK2*V617F mutated (62%): 213 with PMF (58%), 78 with PPV-MF (93%), and 30 with PET-MF (47%). The median V617F allele burden was 42% (range 3%-100%) in PMF, 69% (range 11%-100%) in PPV-MF, and 48% (range 2%-100%) in PET-MF. Considering only patients with PMF, 31 (14.5%) had a V617F allele burden < 25%. The *MPLW515L/K* mutation was found in 18 patients (3.8%): 13 with PMF (3.7%) and 5 (10.6%) with PET-MF. Results of cytogenetic analysis at diagnosis were available in 188 patients; of these, 21 with PMF (15.3% of evaluated), 7 with PPV-MF (21.2%), and 6 with PET-MF (33.3%) had unfavorable cytogenetic abnormalities (ie, complex karyotype or single or 2 abnormalities including −8, −7/7q−, i(17q), −5/5q−, 12p−, inv(3), or 11q23 rearrangement). The stratification of PMF patients according to the IPSS reflected that reported in large patients series, with 30.7%, 25.5%, 22.4%, and 21.4% in the low-risk, intermediate 1–risk, intermediate 2–risk, and high-risk categories, respectively, indicating that our patient population was well representative of all of the different IPSS risk categories. We screened all of the 20 coding exons of *EZH2* using High Resolution Melting Analysis followed by direct-sequencing confirmation. A total of 29 patients (5.6% of total) were *EZH2* mutated; of these, 22 had PMF (5.9% of all PMF), 1 had PPV-MF (1.2%), and 6 had PET-MF (9.4%). Because only 1 of 84 PPV-MF patients was *EZH2* mutated, we carefully reviewed his records and confirmed the original diagnosis of PV using the 2008 WHO criteria[3] and the diagnosis of progression to PPV-MF according to the IWG-MRT criteria.[159] This was a 62-year-old man who had a 14-year-long history of heavily phlebotomized PV treated with hydroxyurea at the time he was diagnosed as PPV-MF. Hematologic and clinical features are listed in Table 13.

		PMF	PPV-MF	PET-MF
N.		370	84	64
FU (mo), (N=500)[§], Median (range)		39.6 (1-340)	38.8 (1-234)	24.2 (1-176)
Age, Median (range)		60.0 (14-90)	63.0 (32-84)	62.0 (33-83)
Male Gender, (N=303) [§], No.(%)		226(61.1)	41 (48.8)	36 (56.3)
Leukocytes x10^6/L, (N=500)[§], Mean±SD		12.7±13.3	16.4±16.2	11.5±9.6
Hb g/L x10^6/L, (N=497)[§], Mean±SD		114±26	121±26	103±22
Platelets x10^6/L, (N=501)[§], Mean±SD		423±345	377±322	419±304
Peripheral blast cells >1%, (N=329)[§], No. (%)		52 (21.7)	5 (10.2)	7 (17.5)
Constitutional symptoms, (N=333)[§], No.(%)		75 (29.2)	24 (58.5)	12 (34.3)
Splenomegaly, (N=494)[§], No.(%)	0	92 (26.1)	7 (9.0)	12 (20.3)
	1	150 (42.6)	32 (41.0)	27 (42.2)
	2	110 (31.3)	39 (50.0)	24 (37.5)
Unfavorable Karyotype, (N=188)[§], No.(%)		21 (15.3)	7 (21.2)	6 (33.3)
***JAK2*V617F (N=517)[§], No.(%)**		213 (57.7)	78 (93.0)	30 (46.9)
% *JAK2*V617F allele burden, Mean±SD		45.7±24.9	67.4±23.5	54.1±23.8
***MPL* W515L/Kpos , (N=466)[§], No.(%)**		13 (3.7)	0 (0.0)	5 (10.6)
***EZH2* mut, (N=518)[§], No.(%)**		22 (5.9)	1 (1.2)	6 (9.4)
IPSS, (N=192)[§], No.(%)	Low	59 (30.7)	----	----
	Int-1	49 (25.5)	----	----
	Int-2	43 (22.4)	----	----
	High	41 (21.4)	----	----
Progression to AL, (N=433)[§], No.(%)		64 (18.6)	8 (14.3)	9 (21.4)
Death, (N=494)[§], No.(%)		99 (28.1)	18 (23.1)	11 (17.2)

§ number of patients for whom information was available. Hb, hemoglobin, AL; acute leukemia. Splenomegaly: 0= not palpable; 1= palpable at <10 cm from left costal margin; 2= palpable at >10 cm from left costal margin.

Table 13. Hematologic and clinical features of patients population

We identified a total of 25 different mutations, of which 20 were exonic and 5 intronic. Seven mutations (in 9 patients) were located in the suppressor of variegation3-9, enhancer of zeste and trithorax (SET) domain and 4 (in 5 patients) in the CXC domain; other mutations were located in exons 3, 5, 8, and 9(Figure 25).



Guglielmelli P., Biamonte F., Score J., et al. Blood 2011

Figure 25. *EZH2* schematic structure and localization of mutations. **(A)** Blue and purple bars correspond to exons encoding the SANT-DNA-binding domain and the SET domain, respectively. **(B)** Domain structure of EZH2 and positions of mutations carried by subjects. Missense mutations highlighted in the violet boxes (R288Q, N322S, R690H, and Y733X) were already described previously.

Most exonic mutations were heterozygous missense changes caused by single-nucleotide substitution; in 5 patients (17%; patient numbers 2, 15, 17, 26, and 28 in Table 14), a homozygous mutation was detected, including one intronic change (patient number 26 in Table 14), and 1 patient presented with 2 different mutations (patient number 19 in Table 14). Five intronic mutations were also identified: of these, 3 (patient numbers 25, 26, and 28) affected the absolutely conserved AG/GT exon flanking splice sites and are therefore very likely to be causative. The changes in patients 27 and 29 were of uncertain significance, but were considered as causative for the correlative analysis. Mutations were confirmed as somatic for 5 of 5 patients with available germline DNA extracted from buccal epithelial cells: 74938G > GA/G > A (patient numbers 1 and 2), 54557T > TA (patient number 7), 73990G > C (patient number 10), and 70231C > CT (patient numbers 11 and 12) abnormalities. All the other patients were denoted as having mutations based on the exclusion of known single nucleotide polymorphisms in published databases (Ensembl and National Center for Biotechnology Information). Later, we also genotyped patients for additional mutations, including *JAK2*, *MPL*,*IDH1*, *IDH2*, and *ASXL1* and we found that twelve *EZH2*-mutated subjects (41.4%) harbored the *JAK2V617F* mutation; of these, 9 had PMF, 1 PPV-MF, and 2 PET/MF. On the other hand, no *EZH2*-mutated patient had mutations in *IDH1/IDH2*, whereas 6 of 27 evaluated subjects (22.2%) concurrently harbored an *ASXL1* exon 12 mutation. At the time, the simultaneous occurrence of the *EZH2*, *JAK2*V617F, and *ASXL1* mutations had not been documented yet. Finally, no concurrent *EZH2* and *MPL*W515L/K mutation was detected

ID patient	gender	Age	Diagnosis	EZH2 EX	EZH2 mutation type	JAK2	MPL W515K/L	ASXL1
1	M	68	PMF	EX18	74938G>GA:679V>V/M	WT	WT	78558G>GA:1397G>G/S
2	F	56	PMF	EX18	74938G>A:679V>M	WT	WT	76092C>CT:575Q>Q/X
3	F	53	PET	EX9	64693A>AG:322N>N/S	WT	WT	WT
4	M	90	PMF	EX9	64693A>AG:322N>N/S	V617F (34%)	WT	WT
5	M	55	PMF	EX9	64693A>AG:322N>N/S	V617F (48%)	WT	WT
6	F	57	PMF	EX8	57825G>GA:288R>R/Q	WT	WT	76446C>CT:693R>R/X
7	F	56	PET	EX5	54557T>TA:149L>L/Q	WT	WT	WT
8	M	67	PMF	EX18	75013G>GC:704V>V/L	V617F (29%)	WT	WT
9	M	61	PPV	EX5	54560T>TC:150I>I/T	V617F (82%)	WT	WT
10	M	65	PMF	EX17	73990G>C:677D>H	V617F (15%)	WT	WT
11	F	77	PMF	EX15	70231C>CT:573T>T/I	WT	WT	77037C>CT:890L>L/F 78117T>TC:1250S>S/P
12	F	38	PMF	EX15	70231C>CT:573T>T/I	V617F (49%)	WT	WT
13	F	45	PMF	EX18	74972G>GA:690R>R/H	WT	WT	n/a
14	M	78	PET	EX18	74972G>GA:690R>R/H	Positive	WT	WT
15	M	77	PET	EX5	54514G>A:135G>R	V617F (100%)	WT	76435C>CG:689S>S/X
16	M	41	PMF	EX15	70210G>GA:566R>R/H	WT	WT	n/a
17	M	79	PMF	EX3	37794C>T:63R>X	Positive	WT	WT
18	M	70	PMF	EX5	54505C>CT:132P>P/S	Positive	WT	WT
19	M	72	PMF	EX3 EX19	37786G>GA:60W>W/X 75234A>AT:726E>E/V	WT	WT	WT
20	M	81	PMF	EX5	54545T>TC:145F>F/S	WT	WT	WT
21	M	51	PMF	EX15	70195G>G/A:591R>R/H	WT	WT	WT
22	M	53	PMF	EX14	69394C>CT:553Q>Q/X	Positive	WT	WT
23	F	72	PMF	EX19	75244_75245het_insT	WT	WT	WT
24	M	78	PMF	EX19	76620C>CA:733Y>Y/X	WT	WT	WT
25	M	70	PMF	INT 3-4	51571A>AG	V617F (35%)	WT	WT
26	M	57	PMF	INT 19-20	75253G>A	WT	WT	77037C>CT:890L>L/F 77675G>GT:1102E>E/D
27	F	52	PET	INT 5-6	54608C>CT	WT	n/a	WT
28	F	47	PMF	INT 6-7	55584G>T	WT	WT	WT
29	M	57	PET	INT 16-17	72749_72753het_delCTTTA	WT	WT	WT

n/a : not available

Table 14. Details of EZH2 mutated patients, and concurrent genotyping for JAK2V617F, MPL W515K/L, ASXL1 and ASXL1.

The comprehensive mutational frequency of *EZH2* mutated patients reported in Figure 26.

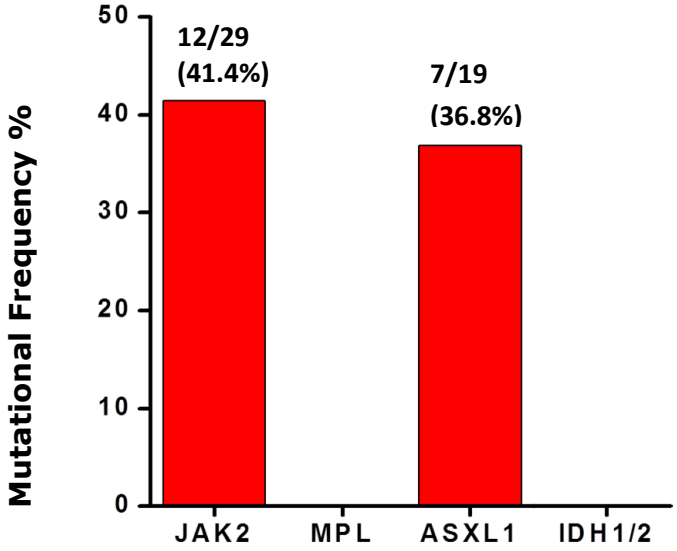


Figure 26. Comprehensive mutational analysis in *EZH2* mutated patients

With the specific aim to understand wether EZH2 mutations can be acquired during follow up, we also analyzed a prospective cohort of 118 PMF patients who had *EZH2* wild-type genotype at diagnosis and for whom we had stored at least 1 additional blood sample collected after a minimum of 1 year from diagnosis (median 38; range 12-84 months). We found that only one patient acquired *EZH2* mutation at 32 months after diagnosis; she has been followed for an additional 24 months without obvious changes in her illness. Finally, in 1 *EZH2/ASXL1*–mutated PMF patient who evolved to leukemia after 17.6 months from diagnosis (patient number 1 in Table 14), the blast cells showed maintenance of both mutations. In this patient, granulocytes collected at diagnosis and blast cells at leukemic transformation tested negative for *JAK2*V617F, *MPL*W515L/K,and *IDH1/IDH2* mutations. Conversely, none of 7 *EZH2* wild-type PMF patients at diagnosis who later evolved to leukemia acquired an *EZH2* mutation. Of these, 5 were *JAK2*V617F mutated at chronic phase and maintained this mutation in leukemic blasts.

then I evaluated the correlation, if any, of *EZH2* mutation with clinical characteristics. To this end we compared *EZH2*-mutated PMF patients with their wild-type counterparts (Table 15). We found that there was no difference in age, sex, hemoglobin level, platelet count, or occurrence of constitutional symptoms between *EZH2* mutated and wild-type patients. The proportion of the *JAK2*V617F mutation in the 2 groups was also similar (40.9 vs 58.8%). PMF patients harboring an *EZH2* mutation (including the 5 intronic variants) displayed at diagnosis a higher leukocyte count (median 17.8; range 3.5-47.9 vs 8.5 × 10^9/L; range 1.4-106, P = .001), more frequently had a blast count > 1% (52.6% vs 20.7%, P = .002), and presented with a larger spleen (the proportion of those with

palpable spleen at > 10 cm from the LCM was 54.5% vs 29.7%, *P* = .016). We found that variables associated with leukocytosis in multivariate analysis were: age > 65 years (*P* = .030), presence of constitutional symptoms (*P* = .018), and *EZH2* mutation (*P* = .023). Factors associated with > 1% blast cells in univariate analysis were *ASXL1* mutation (*P* = .001), hemoglobin < 100 g/L (*P* = .001), leukocytosis (> 25 × 10^9/L) (*P* = .001), and*EZH2* mutation (*P* = .003); however, in multivariate analysis, *EZH2* lost its significant association in favor of the others. Finally, variables associated with splenomegaly > 10 cm in univariate analysis were: the presence of constitutional symptoms (*P* = .027), a > 25% V617F allele burden (*P* = .014), and *EZH2* mutation (*P* = .019); however, all of these variables lost their significant association in multivariate analysis.

The analysis of the PPV/PET-MF patients did not reveal any significant differences in terms of hematologic and clinical parameters that could be meaningfully associated with their *EZH2* mutational status (Table 16).

To assess whether *EZH2* status was correlated with IPSS prognostic score, we evaluated the distribution of PMF patients in the different IPSS risk categories.We observed that most *EZH2*-mutated patients (52.6%) clustered in the high-risk category compared with the low-risk group (21.1%; *P* = .002). The low number of *EZH2*-mutated patients who had cytogenetic analysis available (n = 11) did not allow us to ascertain the correlation of *EZH2* mutational status with the Dynamic IPSS Plus (DIPPS Plus) score. However, the proportion of patients with unfavorable karyotype was double among *EZH2*-mutated versus wild-type subjects (27.3% vs 14.3% for PMF and 66.7% vs 22.9% in PPV/PET-MF patients, respectively), although the difference was not statistically significant possibly because of the low number of patients.

	PMF		***P***
	EZH2		
	Wild-type	**Mutated**	
N	348	22	
Median follow-up, mo, (range) (n = 500)*	39.567 (1-340)	28.183 (8-183)	.365
Median age, y (range)	60.0 (14-90)	66.0 (38-90)	.135
Male sex, no. (%) (n = 303)*	221 (60.6)	15 (68.2)	.481
Leukocytes, × 106/L, mean ± SD (n = 500)*	12.3 ± 13.3	18.7 ± 11.5	**.001**
Hb, g/L, mean ± SD (n = 497)*	114 ± 27	112 ± 19	.700
Platelets, × 106/L, mean ± SD, (n = 501)*	341.0 ± 345.6	405.0 ± 258.7	.831
Peripheral blast cells, %, mean ± SD (n = 329)*	0.7 ± 2.1	1.6 ± 2.1	**.002**
Constitutional symptoms, no. (%) (n = 333)*	67 (28.2)	8 (42.1)	.198
Splenomegaly, no. (%) (n = 494)[*†]			**.016**
0	91 (27.6)	1 (4.5)	
1	141 (42.7)	9 (40.9)	
2	98 (29.7)	12 (54.5)	
Abnormal karyotype, no. (%) (n = 195)*	26 (20.2)	4 (36.4)	.209
Unfavorable karyotype, no. (%) (n = 188)*	18 (14.3)	3 (27.3)	.252
JAK2V617F, no. (%) (n = 518)*	204 (58.8)	9 (40.9)	.100
*JAK2*V617F allele burden, %, mean ± SD	45.7 ± 22.9	42.2 ± 22.1	.603
MPL W515L/K, no. (%) (n = 466)*	13 (3.9)	0 (0.0)	.344
IWG-MRT score, no. (%) (n = 192)*			**.002**
Low	55 (31.8)	4 (21.1)	
Int-1	44 (25.4)	5 (26.3)	
Int-2	43 (24.9)	0 (0.0)	
High	31 (17.9)	10 (52.6)	
Progression to acute leukemia, no. (%) (n = 443)*	57 (17.6)	7 (31.8)	.098
Death, no. (%) (n = 494)*	86 (26.0)	13 (61.9)	<**.001**

- *P* values in bold indicate statistical significance.
- * Number of patients for whom information was available.
- † Splenomegaly: 0 = not palpable; 1 = palpable at < 10 cm from left costal margin; and 2 = palpable at > 10 cm from left costal margin.

Table 15. Hematologic and clinical characteristics of PMF patients stratified according to *EZH2* mutational status

	PPV/PET-MF		*P*
	EZH2		
	Wild-type	**Mutated**	
N	141	7	
Median follow-up, mo, (range) (n = 500)*	29.7 (1-234)	16.8 (1-70)	.256
Median age, y (range)	62.0 (32-84)	62.0 (52-78)	.731
Male sex, no. (%) (n = 303)*	73 (51.8)	4 (57.1)	.781
Leukocytes, × 106/L, mean ± SD (n = 500)*	14.3 ± 14.1	13.8 ± 7.0	.360
Hb, g/L, mean ± SD (n = 497)*	114 ± 26	100 ± 18	.153
Platelets, × 106/L, mean ± SD, (n = 501)*	404.1 ± 317.6	232.1 ± 198.0	.097
Peripheral blast cells, %, mean ± SD (n = 329)*	0.5 ± 2.0	0.4 ± 0.9	.959
Constitutional symptoms, no. (%) (n = 333)*	34 (47.9)	2 (40.0)	.733
Splenomegaly, no. (%) (n = 494)[*†]			**.059**
0	17 (12.6)	3 (42.9)	
1	58 (43.0)	1 (14.3)	
2	60 (44.4)	3 (42.9)	
Abnormal karyotype, no. (%) (n = 195)*	17 (32.7)	2 (66.7)	.229
Unfavorable karyotype, no. (%) (n = 188)*	11 (22.9)	2 (66.7)	.092
JAK2V617F, no. (%) (n = 518)*	105 (74.5)	3 (42.9)	.066
*JAK2*V617F allele burden, %, mean ± SD	64.1 ± 23.4	61.1 ± 52.6	.747
MPL W515L/K, no. (%) (n = 466)*	5 (4.7)	0 (0.0)	.586
IWG-MRT score, no. (%) (n = 192)*			-
Low			
Int-1			
Int-2			
High			
Progression to acute leukemia, no. (%) (n = 443)*	16 (17.2)	1 (20.0)	.872
Death, no. (%) (n = 494)*	28 (20.6)	1 (16.7)	.816

- *P* values in bold indicate statistical significance.
- * Number of patients for whom information was available.
- † Splenomegaly: 0 = not palpable; 1 = palpable at < 10 cm from left costal margin; and 2 = palpable at > 10 cm from left costal margin.

Table 16. Hematologic and clinical characteristics of PPV/PET patients stratified according to *EZH2* mutational status

We also stratified PMF patients according to 4 different categories defined by their *EZH2* and *JAK2*V617F mutational status (Table 17 and 18). Among *EZH2* wild-type subjects, the presence of the *JAK2*V617F mutation was associated with significantly older age, higher leukocyte and hemoglobin levels, and a lower frequency of the *MPL*W515 mutation; conversely, there was no difference with regard to sex, platelet count, PB blast cell count, constitutional symptoms, splenomegaly, IPSS score, or proportion of patients

progressing to leukemia (Table 17). In *EZH2*-mutated patients, the concurrent presence of the *JAK2*V617F mutation did not affect the hematologic or clinical phenotype at all (Table 18).

	***EZH2* wild-type**		***P***
	JAK2		
	Wild-type	**V617F**	
N	143	204	
Follow-up, mo (range) n = 354)*	49.4 (1-340)	37.9 (1-282)	**.020**
Median age, y, (range)	55.0 (14-88)	63.0 (18-90)	**.001**
Male sex, no. (%) (n = 369)*	90 (62.9)	120 (58.8)	.440
Leukocytes, × 106/L, mean ± SD (n = 354)*	11.3 ± 13.0	13.1 ± 13.5	**.005**
Hemoglobin, g/L, mean ± SD (n = 352)*	109 ± 24	118 ± 28	**.002**
Platelet, × 106/L, mean ± SD (n = 355)*	447.0 ± 403.2	408.7 ± 308.0	.840
Peripheral blast cells, %, mean ± SD (n = 240)*	1.0 ± 2.6	0.5 ± 1.7	.090
Constitutional symptoms, no. (%) (n = 257)*	26 (28.3)	41 (28.1)	.976
Splenomegaly, no. (%) (n = 352)[*†]			.747
0	41 (29.7)	50 (26.0)	
1	58 (42.0)	83 (43.2)	
2	39 (28.3)	59 (30.7)	
Unfavorable karyotype, no. (%) (n = 137)	8 (12.1)	10 (16.7)	.466
*JAK2*V617F allele burden, %, mean ± SD		45.9 ± 23.0	
MPL W515L/K, no. (%) (n = 354)*	12 (8.8)	1 (0.5)	<**.0001**
IWG-MRT score, no. (%) (n = 192)*			.484
Low	26 (36.6)	29 (28.4)	
Int-1	14 (19.7)	30 (29.4)	
Int-2	18 (25.4)	25 (24.5)	
High	13 (18.3)	18 (17.6)	
Progression to acute leukemia, no. (%) (n = 345)*	26 (19.3)	31 (16.5)	.520
Death, no. (%) (n = 352)*	38 (27.7)	48 (24.7)	.541

- *P* values in bold indicate statistical significance.
- * Number of patients for whom information was available.
- † Splenomegaly: 0 = not palpable; 1 = palpable at < 10 cm from left costal margin; and 2 = palpable at > 10 cm from left costal margin.

Table 17. Hematologic and clinicalc characteristics of EZH2-wt PMF subjects stretified according to *JAK2*V617F mutational status

	***EZH2* mutated**		***P***
	JAK2		
	Wild-type	**V617F**	
N	13	9	
Follow-up, mo (range) n = 354)*	25.7 (8-182)	30.7 (11-84)	.616
Median age, y, (range)	57.0 (41-81)	70 (38-90)	.324
Male sex, no. (%) (n = 369)*	8 (61.5)	7 (77.8)	.421
Leukocytes, × 106/L, mean ± SD (n = 354)*	16.2 ± 11.5	22.4 ± 11.1	.117
Hemoglobin, g/L, mean ± SD (n = 352)*	107 ± 22	118 ± 14	.171
Platelet, × 106/L, mean ± SD (n = 355)*	392.8 ± 255.0	422.5 ± 278.6	.764
Peripheral blast cells, %, mean ± SD (n = 240)*	1.4 ± 2.4	1.8 ± 1.8	.299
Constitutional symptoms, no. (%) (n = 257)*	3 (27.3)	5 (62.5)	.125
Splenomegaly, no. (%) (n = 352)[*†]			.518
0	1 (7.7)	0 (0.0)	
1	6 (46.2)	3 (33.3)	
2	6 (46.2)	6 (66.7)	
Unfavorable karyotype, no. (%) (n = 137)	2 (28.6)	1 (25.0)	.898
*JAK2*V617F allele burden, %, mean ± SD		42.2 ± 22.1	
MPL W515L/K, no. (%) (n = 354)*	0 (0.0)	0 (0.0)	
IWG-MRT score, no. (%) (n = 192)*			.247
Low	3 (27.3)	1 (12.5)	
Int-1	4 (36.4)	1 (12.5)	
Int-2	0 (0.0)	0 (0.0)	
High	4 (36.4)	6 (75.0)	
Progression to acute leukemia, no. (%) (n = 345)*	6 (46.2)	1 (11.1)	.083
Death, no. (%) (n = 352)*	7 (53.8)	6 (75.0)	.332

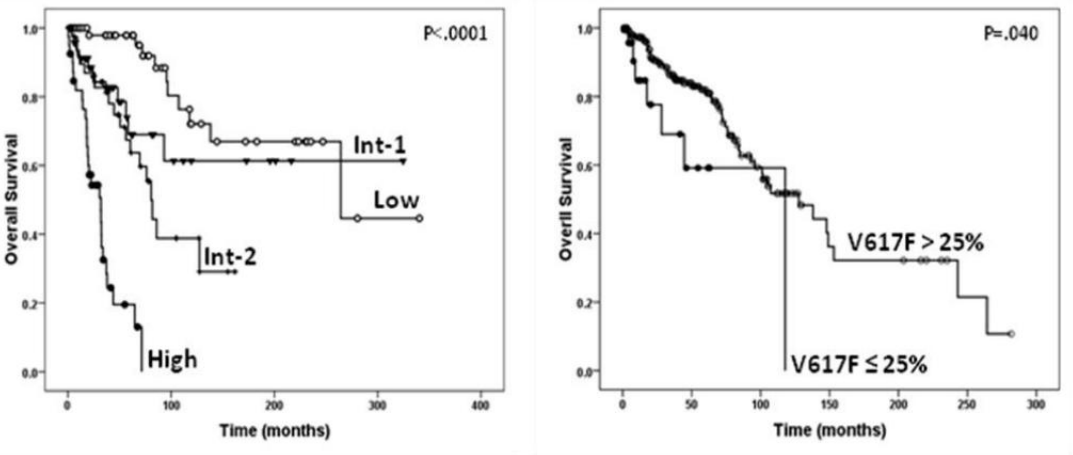
- *P* values in bold indicate statistical significance.
- * Number of patients for whom information was available.
- † Splenomegaly: 0 = not palpable; 1 = palpable at < 10 cm from left costal margin; and 2 = palpable at > 10 cm from left costal margin.

Table 18. Hematologic and clinicalc characteristics of EZH2-mutated PMF subjects stretified according to *JAK2*V617F mutational status

Finally, i aimed at determining the correlation between *EZH2* mutational status and the disease outcome. Information about progression to acute leukemia and death were available in 443 and 494 patients, respectively. After a median follow-up of 39 months (range 1-340), 128 patients (25.9%) died; of these, 99 had PMF (28.1% of all PMF), 18 had PPV-MF (23.1%), and 11 had PET-MF (17.2%). The median survival was 128 months in

PMF patients (95% confidence interval [95% CI], 92-163) and 103.3 months (95% CI, 79-128) in PPV/PET-MF.

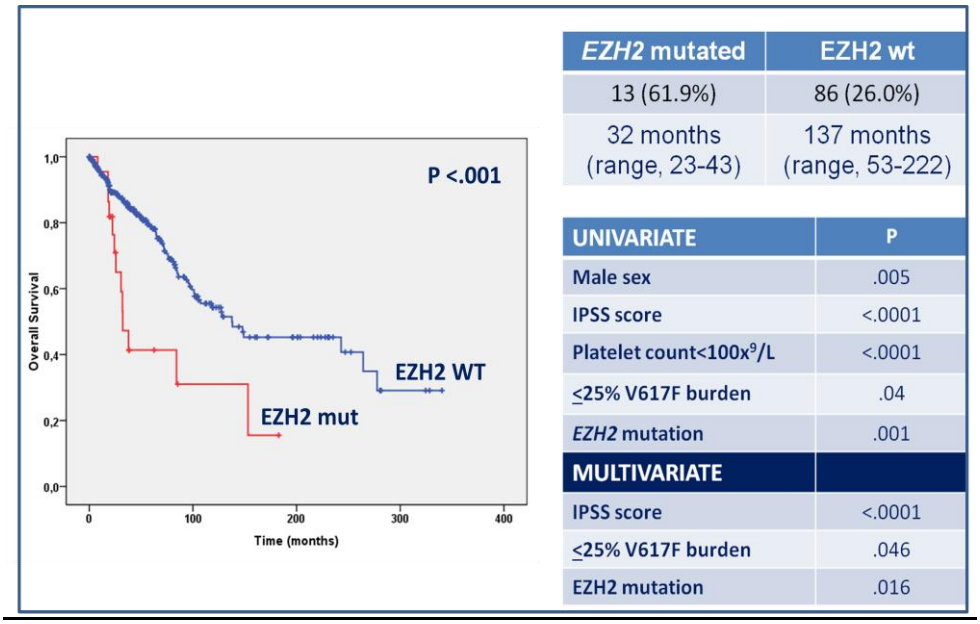
Considering PMF patients only, survival varied according to the 4 IPSS risk categories (Figure 27A); the median survival was 264 months (95% CI, 50-478) in the low-risk category, was not reached in the intermediate 1–risk category, 80 months (95% CI, 71-90) in the intermediate 2–risk category, and 32 months (95% CI, 18-46) in the high-risk category patients ($P < .001$). As reported previously, survival was also significantly reduced in *JAK2*V617F-mutated PMF patients, who presented an allelic burden < 25% compared with higher allelic burden quartiles (Figure 27B). *JAK2*V617F allele burden had no impact on survival in PPV/PET-MF patients (not shown), confirming previous findings.



Guglielmelli P., Biamonte F., Score J., Blood 2011

Figure 27. A) Survival data of the cohort of patients with PMF (n= 343) according to the four risk categories of the IPSS is shown. B) The impact of a low JAK2V617F allele burden (<25%) on OS in PMF patients

Among patients who died, 14 (13 with PMF and 1 with PET-MF) and 114 were *EZH2* mutated or wild-type, respectively, corresponding to 51.9% and 24.4% of their respective categories ($P < .001$). The median OS was significantly shortened in *EZH2*-mutated PMF patients (31.6 months; 95% CI, 23-43) compared with wild-type (137 months; 95% CI, 53-222; $P < .001$;)(Figure 28). We found that variables associated with reduced survival among PMF patients in univariate analysis were sex, IPSS score, low platelet count, low (< 25%) *JAK2*V617F allele burden, and *EZH2*-mutated status. On the other hand, on multivariate analysis, OS was predicted by IPSS score ($P < .0001$), a < 25% *JAK2*V617F allele burden ($P = .046$), and *EZH2*-mutated status ($P = .016$). Finally, the fact that only one event was recorded in the PPV/PET-MF group prevented statistical analysis of survival between *EZH2*-mutated and wild-type patients (data not shown).

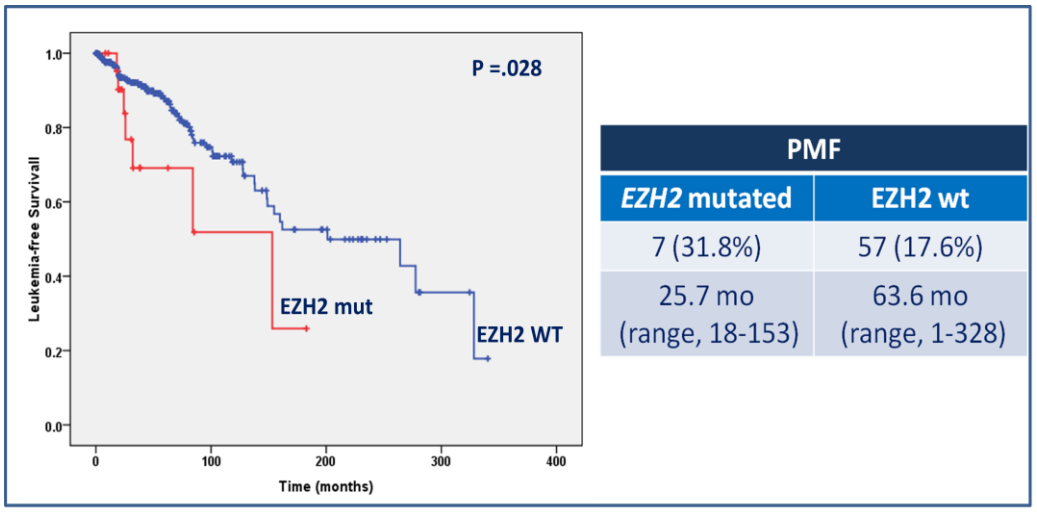


EZH2 mutated	EZH2 wt
13 (61.9%)	86 (26.0%)
32 months (range, 23-43)	137 months (range, 53-222)

UNIVARIATE	P
Male sex	.005
IPSS score	<.0001
Platelet count<100x^9/L	<.0001
≤25% V617F burden	.04
EZH2 mutation	.001
MULTIVARIATE	
IPSS score	<.0001
≤25% V617F burden	.046
EZH2 mutation	.016

Figure 28. Reduced OS in *EZH2* mutated subjects with PMF; variables associated with reduced survival among PMF patients in univariate and multivariate

Acute leukemia occurred in 81 patients (18.3%), corresponding to 19%, 14%, and 21% of PMF, PPV-MF, and PET-MF patients, respectively. Among these, 73 were*EZH2* wild-type (17.5%) and 8 *EZH2* mutated (26.9%). We found that leukemia-free survival (LFS), measured from diagnosis to the time of leukemia transformation, was significantly shorter in *EZH2*-mutated PMF patients (n = 7; 153.1 months, 95% CI, 42-264) compared with *EZH2* wild-type patients (n = 57; 201.07 months, 95% CI, 103-299; *P* = .028) (Figure 29). Unfortunately, the low number of cases harboring the *EZH2* mutation prevented any multivariate analysis of variables associated with reduced LFS. Due to low number of events, we were also unable to determine the statistical significance of *EZH2* mutational status for LFS in the PPV/PET-MF group.



PMF	
EZH2 mutated	EZH2 wt
7 (31.8%)	57 (17.6%)
25.7 mo (range, 18-153)	63.6 mo (range, 1-328)

Figure 29. Reduced LFS in *EZH2* mutated subjects with PMF

At the end of this study we have concluded that *EZH2* mutations are usually already present at the time of diagnosis and, at least in some cases, are maintained in leukemic blasts at the time of leukemia transformation. On the contrary, we found no evidence for *EZH2* mutations being acquired at the time of leukemia transformation in any of the 8 patients who were wild-type at diagnosis. The analysis of hematologic-clinical correlates highlighted only subtle differences associated with the *EZH2* mutation in PMF patients, such as more pronounced leukocytosis, larger spleens, and higher circulating blast cells. Therefore, we concluded that the *EZH2* mutation does not contribute a specific phenotypic signature in patients with primary and PPV/PET PMF. Conversely, we found that *EZH2* mutational status had a significant negative impact on disease outcome among PMF patients. This is supported by the following findings: (1) *EZH2*-mutated patients preferentially clustered in the IPSS high-risk category; (2) both OS and LFS were shortened in *EZH2*-mutated subjects compared with their wild-type counterparts; and (3) in a multivariate analysis, *EZH2* mutational status maintained a negative prognostic significance together with the IPPS score and a low *JAK2*V617F allele burden. On the other hand, the low number of events (death and leukemia) recorded in the PPV/PET-MF group prevented statistical testing of a possible impact of *EZH2* mutational status on OS and LFS.

All the results obtained in this study, and reported in this thesis, were published in *Blood.* : *Guglielmelli P, Biamonte F, Score J, Hidalgo-Curtis C, Cervantes F, Maffioli M, et al. EZH2 mutational status predicts poor survival in myelofibrosis. Blood. 2011; 118(19): 5227-34*

5.2 PROGNOSTIC IMPACT OF MUTATIONS IN A LARGE SERIES OF PATIENTS WITH MYELOFIBROSIS

Although the identification of single gene mutations with impact on outcome is of clinical and biologic importance, we thought that the main goal of mutational studies should be to inform and improve prognostic algorithms in MPNs. For this reason and given the increasing number of genetic abnormalities that have been identified in PMF patients, we decided to perform a more extensive mutational analysis focusing on this subset of patients, with the aim to determine whether mutational status for specific mutations or for combinations of mutations affect outcome. Given the established importance of some of these genes, we evaluated the prognostic impact of known mutations (with a reported $\geq$2% frequency), affecting *JAK2*-V617F, *MPL* K515L, *TET2*, *ASXL1*, *DNMT3A*, *CBL, SRSF2*, *EZH2*, *IDH1*, and *IDH2,* detected at diagnosis in a large multicenter (Florence, Pavia, Athens, Barcelona, Mannheim, Salisbury, Southampton) international series of 483 patients with primary myelofibrosis. A diagnosis of PMF was made according to the WHO 2008 criteria.[3] Genotyping was performed using samples of DNA from granulocytes (n= 415) or whole blood (n= 68) collected at, or within 6 months from, diagnosis in the absence of cytotoxic treatment. PMF patients were included in the study only if a minimum set of clinical information and an adequate follow-up were available. Clinical and hematological characteristics of PMF patients are listed in Table 18.

We considered a total of 483 patients between the ages of 14 and 90 years with a median follow-up of 3.7 years. Results of cytogenetic analysis at diagnosis were available in 266 patients; of these, 13 with PMF (6.3% of evaluated), had unfavorable cytogenetic abnormalities (ie, complex karyotype or single or 2 abnormalities including −8, −7/7q−, i(17q), −5/5q−, 12p−, inv(3), or 11q23 rearrangement).

Characteristics	
FU (yrs), median (range)	3.7 (0.02-27.9)
Age (yrs), median (range)	61 (14-90)
Male, N (%)	296 (61.3%)
WBC x10^9/L, median (range)	9.0 (1.4-106.0)
Hb g/L, median (range)	114 (44-165)
Plt x10^9/L, median (range)	135 (7-3279)
Peripheral Blast count ≥1%, N(%)	80 (16.6%)
Abnormal Karyotype, N (% of 266)	53 (23.5%)
Unfavorable *	13 (6.3%)
Constitutional symptoms (%)	137 (28.1%)
Splenomegaly , (%)	355 (74.7%)

* +8, -7/7q, i(17q), inv(3), -5/5q-, 12p-, 11q23 rearr.

Table 18. Characteristics of the study patient population

The stratification of PMF patients according to the IPSS reflected that reported in large patients series, with 166 (34.4%), 146 (30.2%), 104 (21.5%), and 67 (13.9%) in the low-risk, intermediate 1–risk, intermediate 2–risk, and high-risk categories, respectively, indicating that our patient population was sufficiently well representative of all of the different IPSS risk categories. The median overall survival was 9.7 yrs (95%CI, 7.9-12.2yrs): 157 patients died (32%), of these 75 (15.5%) because of leukemia.

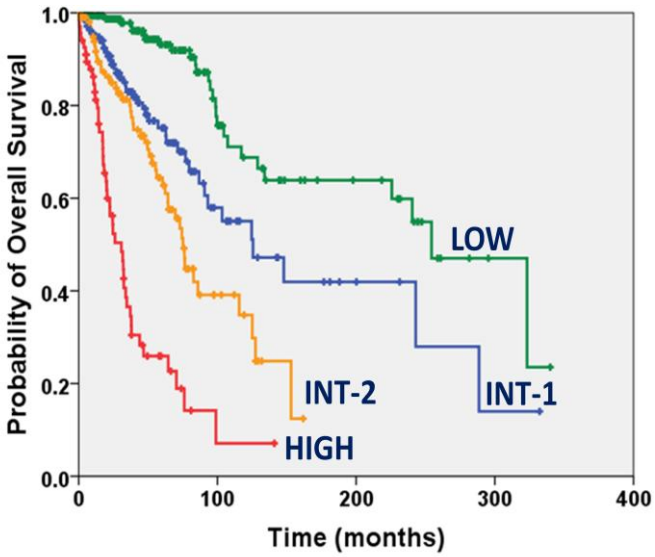


Figure 30. Overall Survival According to the IPSS

Moving from knowledge gained from several already published studies, we focused on mutational "hotspots" for all the target genes and we analyzed all coding sequence only for *EZH2* and *TET2; EZH2* and *TET2* mutation analysis were performed using high-resolution melting (HRM) in a Rotor-Gene 6000 instrument (Corbett Life Science), using primer sets as described previously (see Methos session). Products showing abnormal melt pattern were subjected to bidirectional direct sequencing and sequence analysis was performed using Mutation Surveyor (SoftGenetics). The presence of the *JAK2*V617F mutation and the mutated allele burden, as well as *MPL*W515L/K mutations, were determined by quantitative real-time PCR (QRT-PCR) as described previously. Mutational analysis of all the other genes were performed by direct sequencing (Sanger). All mutations were confirmed at least twice and in case of novel mutations, SNPs were excluded by database searching (i.e. NCBI, Ensemble, 1000 Genomes) and when feasible by germline DNA genotyping. Overall we found that 382 (79.1%) of patients presented at least one somatic mutation. Mutations in *JAK2* and *ASXL1* were most common, occurring in 59.2% and 21.7% of cases, respectively. Mutations in the other target genes occurred at a frequency of less than 10% (Table 19).

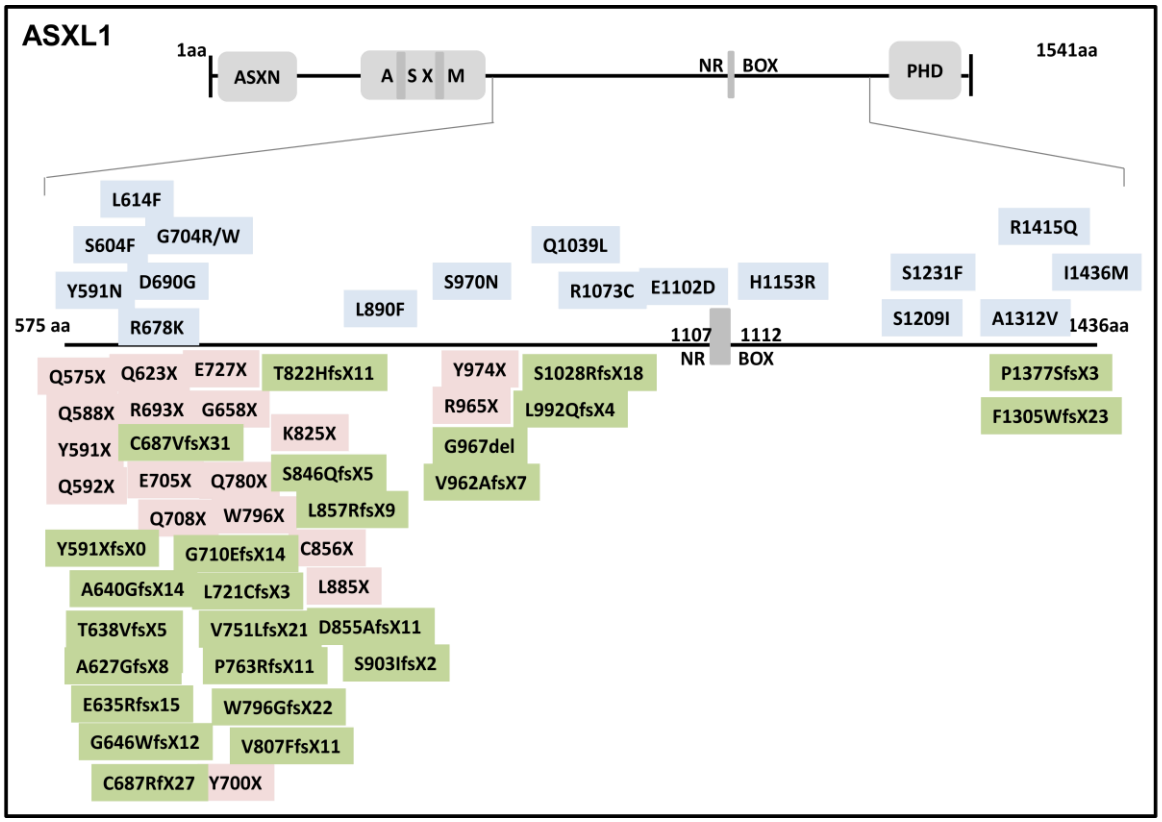
Gene	Overall Frequency (%)
JAK2	59.2
ASXL1	21.7
TET2	9.7
SRSF2	8.5
DNMT3A	5.7
MPL	5.2
EZH2	5.1
CBL	4.4
IDH1/2	2.6

Table. 19 *JAK2*-V617F*, MPL* K515L*, TET2, ASXL1*, *DNMT3A*, *CBL*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2* mutation frequency

The majority of mutations found were heterozygous frameshift, nonsense and missense mutations. Interestingly, the target gene affected by the highest number of different mutations, even if sequenced only in the hotspot, was *ASXL1.* Conversely*, IDH1, IDH2* and *SRSF2* resulted the target genes with the lowest number of somatic variants that affected one single aminoacid in all three. The mutation frequencies found in this study match very well with previous reports . Frameshift and nonsense mutations are supposed to result in C-terminal truncation of the protein, in some cases upstream of the functional domain (i.e. PHD and SET domains for *ASXL1* and *EZH2*, respectively) (Figure 31A and B). The functional relevance of reported missense mutations is not clear. One of the most controversial mutation affecting *ASXL1*, the duplication of a guanine nucleotide (c.1934dupG) leading to a frameshift (p.Gly646TrpfsX12), has been described as a polimorphism by Abdel-Wahab O. et al (Leukemia 2010) because it was found in germ-line DNAs and control DNAs. On the other hand, several other studies generally consider it to be a *bona fide* mutation. In our analysis, since it was not feasible to check for Gly646TrpfsX12 mutation in germline DNA we considered it as somatic mutation.

Schematic structure and mutations localizations of ASXL1, EZH2, TET2, DNMT3A, and CBL, IDH1, IDH2 and SRSF2 are reported in figure 31A,B,C,D,E respectively.

A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ASXN
A S X M
NR BOX
PHD
1541aa
L614F
S604F
G704R/W
Y591N
D690G
R678K
575 aa
L890F
S970N
Q1039L
R1073C
E1102D
H1153R
1107
1112
NR
BOX
R1415Q
S1231F
I1436M
S1209I
A1312V
1436aa
Q575X
Q623X
E727X
T822HfsX11
Y974X
S1028RfsX18
P1377SfsX3
Q588X
R693X
G658X
R965X
L992QfsX4
F1305WfsX23
Y591X
C687VfsX31
K825X
G967del
Q592X
E705X
Q780X
S846QfsX5
V962AfsX7
Q708X
W796X
L857RfsX9
Y591XfsX0
G710EfsX14
C856X
A640GfsX14
L721CfsX3
L885X
T638VfsX5
V751LfsX21
D855AfsX11
A627GfsX8
P763RfsX11
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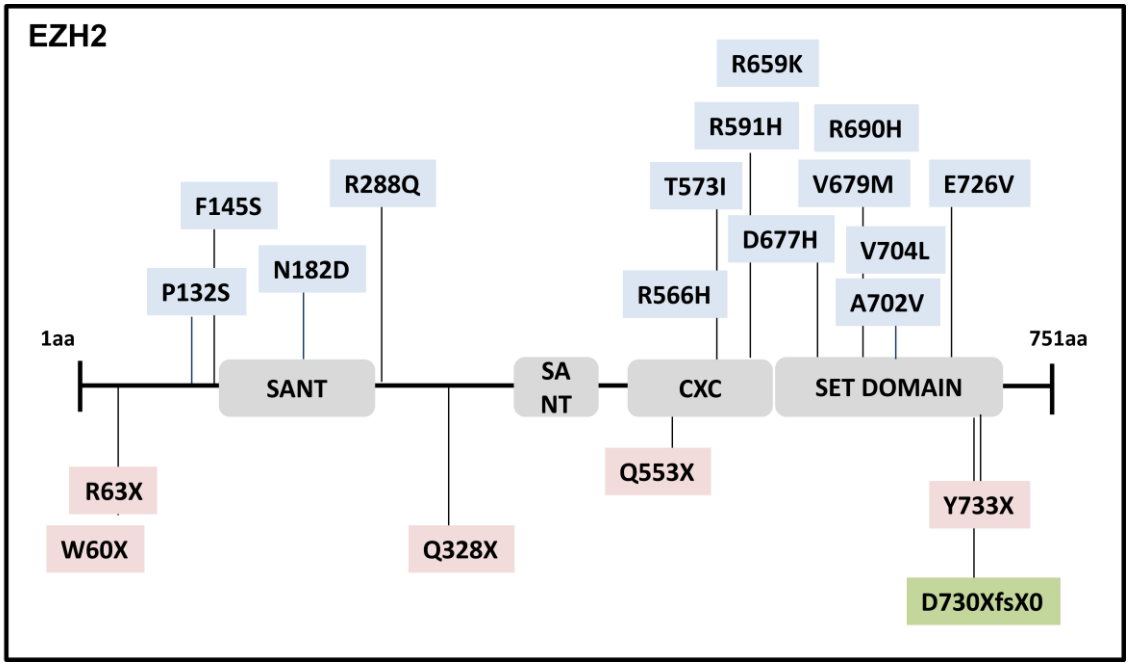
C687RfX27
Y700X

B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R591H
R690H
T573I
V679M
E726V
F145S
R288Q
D677H
V704L
N182D
P132S
R566H
A702V
1aa
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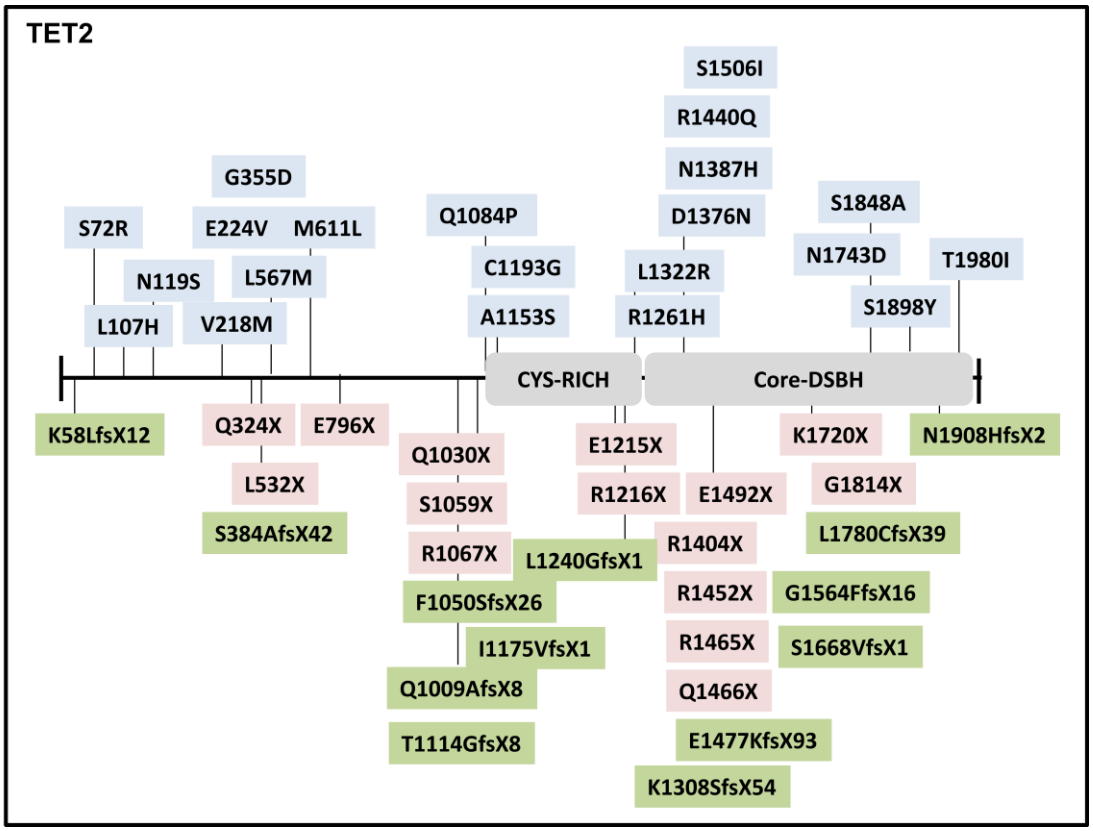
Q553X
Y733X
D730XfsX0

C


TET2
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D1376N
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E224V
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C1193G
L1322R
N1743D
T1980I
N119S
L567M
A1153S
R1261H
S1898Y
L107H
V218M
CYS-RICH
Core-DSBH
K58LfsX12
Q324X
E796X
Q1030X
E1215X
K1720X
N1908HfsX2
L532X
S1059X
R1216X
E1492X
G1814X
S384AfsX42
R1067X
L1240GfsX1
R1404X
L1780CfsX39
R1452X
G1564FfsX16
F1050SfsX26
I1175VfsX1
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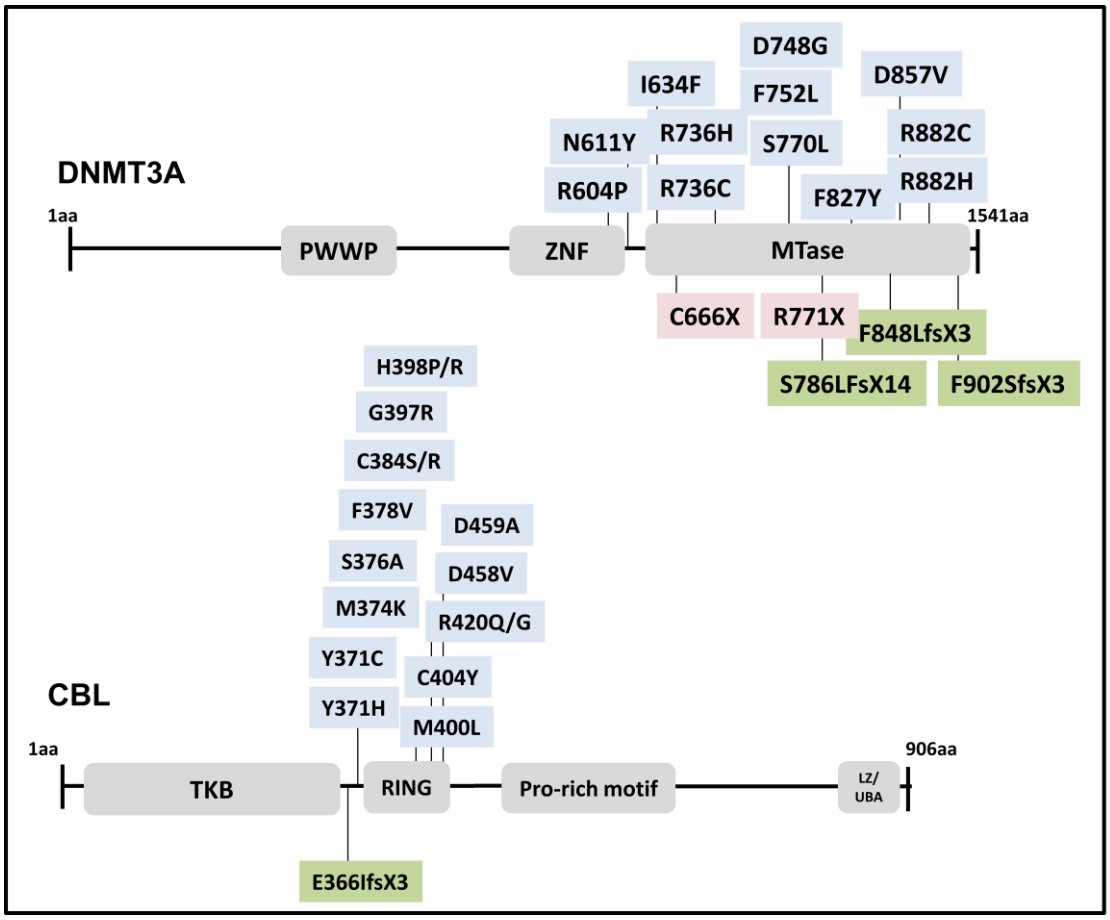
E1477KfsX93
K1308SfsX54

D


D748G
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D857V
F752L
N611Y
R736H
S770L
R882C
DNMT3A
R604P
R736C
F827Y
R882H
1aa
1541aa
PWWP
ZNF
MTase
C666X
R771X
F848LfsX3
S786LFsX14
F902SfsX3
H398P/R
G397R
C384S/R
F378V
D459A
S376A
D458V
M374K
R420Q/G
Y371C
C404Y
CBL
Y371H
M400L
1aa
906aa
TKB
RING
Pro-rich motif
LZ/
UBA
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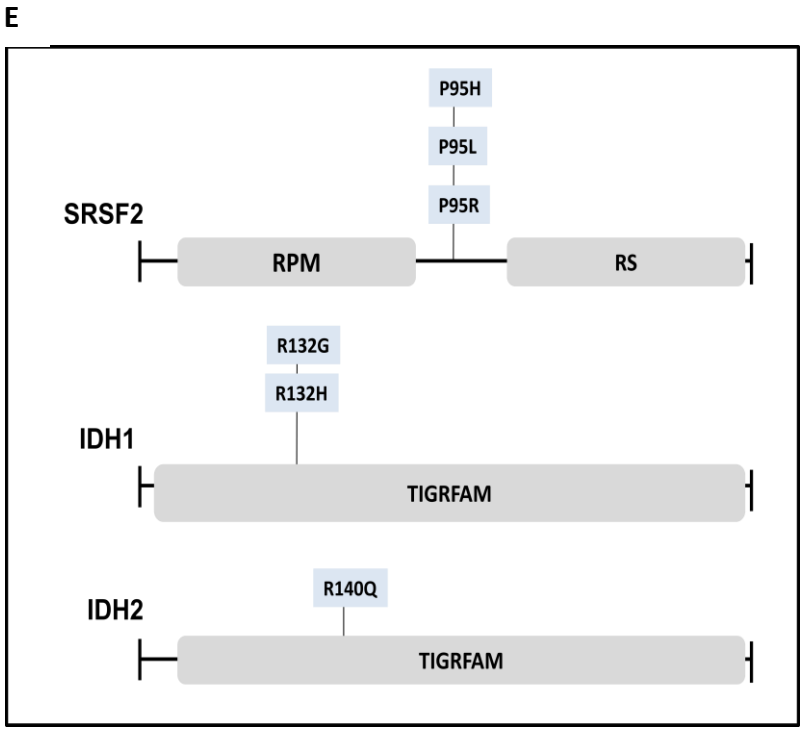


Figure 31. Schematic structure and mutations localizations of A) ASXL1, B) EZH2, C) TET2, D) DNMT3A and CBL, E) SRSF2, IDH1 and IDH2. Light blue, pink and green boxes represent missense, nonsense and frameshift mutations, respectively.

Then, I explored the possible association, co-occurrence and/or mutual exclusion between gene mutations. Interestingly, we observed that *ASXL1* more frequently associated with *EZH2* (P<0.002), *TET2* (P=0.043), *DNMT3A* (P=0.02), *CBL* (P<0.001) and *SRSF2* (P=0.035). *IDH1* and *IDH2* mutations were mutually exclusive and preferentially clustered with DNMT3A (P<0.001) and *SRSF2* (P<0.001) mutations. Furthermore we found that the majority of *MPL* mutated patients were *JAK2*^{wt} (12%) (vs 0.7% V617F^{+};P<0.001). Considering mutations in all genes investigated in our study, 31 cases (6.4%) had $\geq$3 somatic point mutations in the same sample. Associations between genes mutations are represented by a Circos diagram. (Figure 32).

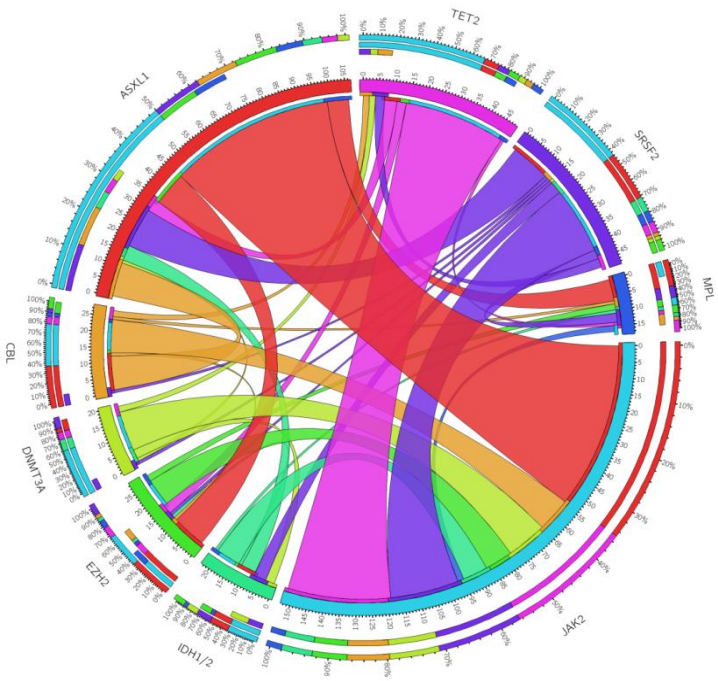


Figure 32. Circos diagram

Once investigated the frequency and the association between genes mutations we focused on our main endpoint which was to explore the prognostic impact of these molecular abnormalities on patient outcome. Overall survival (OS) end points, measured from the date of first sample collection, were death and alive at last follow-up. Time to AML progression was measured from date of first sample collection to the time of AML diagnosis. Progression to AML was defined according to the 2008 WHO classification.[3] Primary analysis was performed on OS and time to AML progression. The Kaplan-Meier method, log-rank test, and Cox proportional hazards models were used to estimate the distribution of OS and time to AML progression and to compare differences between survival curves, respectively. The prognostic impact of each mutated gene was calculated separately. Multivariate analysis demonstrated that patients with EZH2 (hazard ratio [HR] = 1.91; 95% confidence interval [95% CI], 1.08-3.36; *P* = .0008), ASXL1 (hazard ratio [HR] = 2.21; 95% confidence interval [95% CI], 1.57-3.11; *P* < .0001) and *SRSF2* mutations (hazard ratio [HR] = 2.60; 95% confidence interval [95% CI], 1.63-4.16; *P* < .0001) had significantly shorter OS. (Figure 33)

We did not observe any significant correlation with all the other genes.

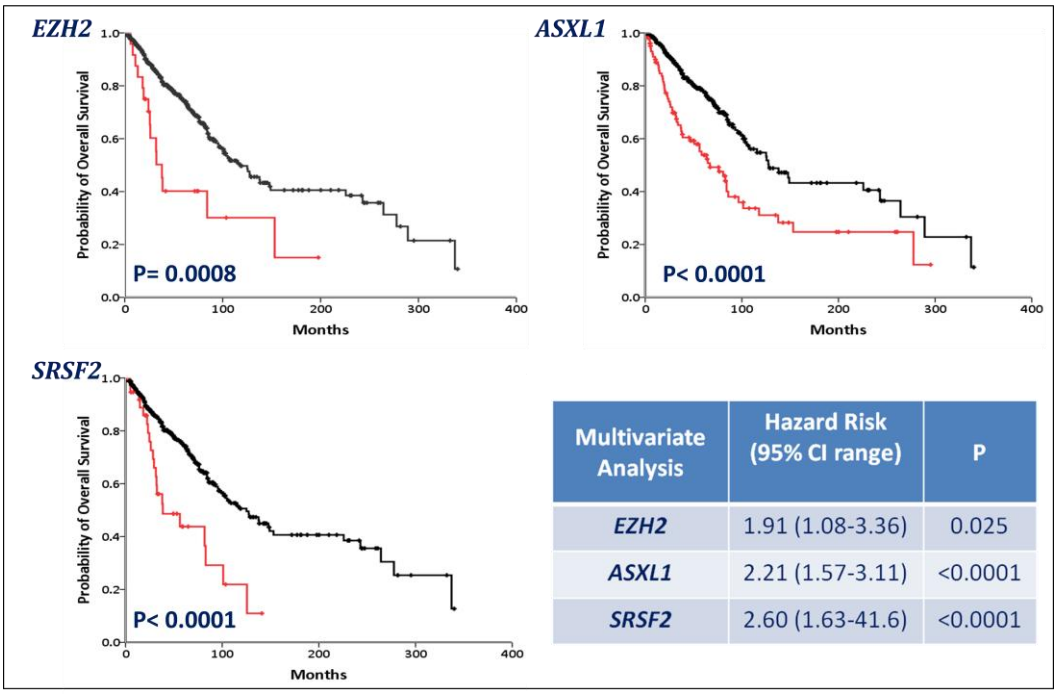


Figure 33. Impact of Mutations on Overall Survival

The analysis of prognostic impact in terms of leukemia-free survival (LFS), measured from diagnosis to the time of leukemia transformation, revealed that *EZH2*, *ASXL1*, *SRSF2* and *IDH* mutations significantly predicted for leukemia transformation indipendently from each other. (Figure 34) Multivariate analysis demonstrated that patients with EZH2 (hazard ratio [HR] = 1.98; 95% confidence interval [95% CI], 0.88-4.46; *P* = .003), ASXL1 (hazard ratio [HR] = 2.5; 95% confidence interval [95% CI], 1.51-4.13; *P* < .0001), *SRSF2* mutations (hazard ratio [HR] = 2.73; 95% confidence interval [95% CI], 1.34-5.55; *P* = .007) and IDH mutations (hazard ratio [HR] = 2.66; 95% confidence interval [95% CI], 1.10-6.47; *P* < .0001) had significantly shorter LFS.

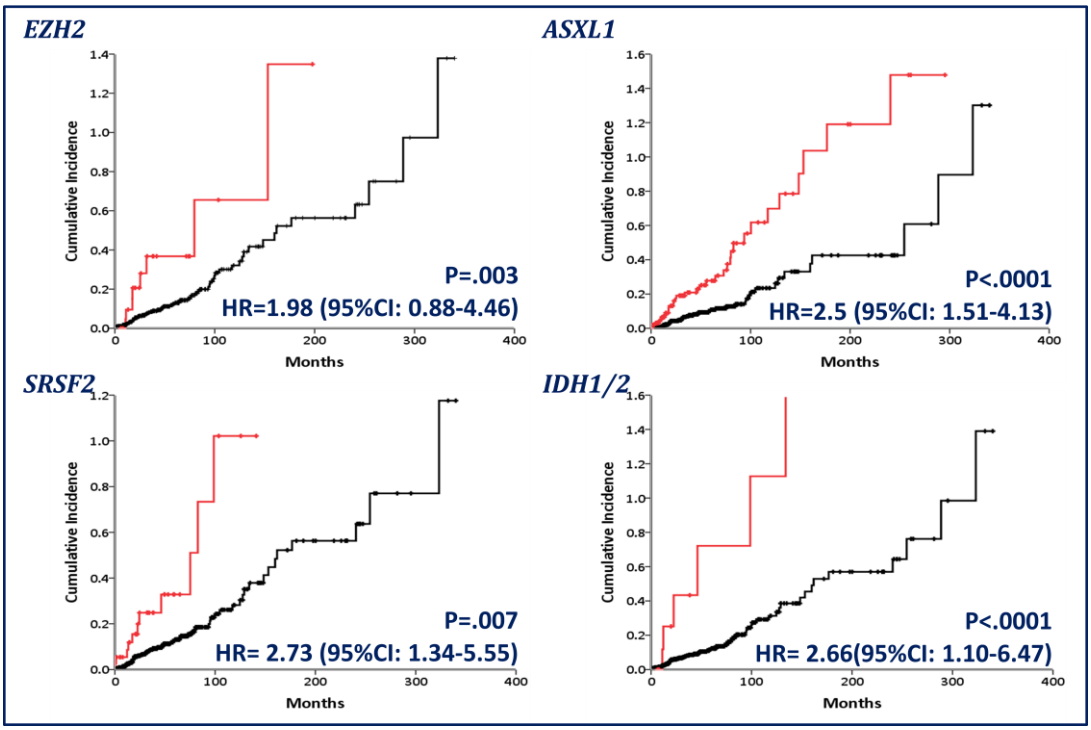


Figure 34. Impact of Mutations on Risk of Acute Leukemia

Once identified the four genes whose mutations significantly impacted on patients outcome in terms of both OS and LFS, we decided to accordingly classify the entire patient cohort into those who displayed at least one ("*molecularly high-risk*") or ("*molecularly low risk*") none of the four mutations. It was very interesting to find that in the "molecularly high-risk" category, overall survival was 81 months (range: 61.9-99.5) compared with 148 months (range: 52.5-243.2) in the "molecularly low-risk" category (P<0.0001). Moreover in the "molecularly high-risk" category, leukemia-free survival was 129 months (range: 90-168) compared with 323 months (range: 194-452) in the "molecularly low-risk" category (P<0.0001) (Figure 35)

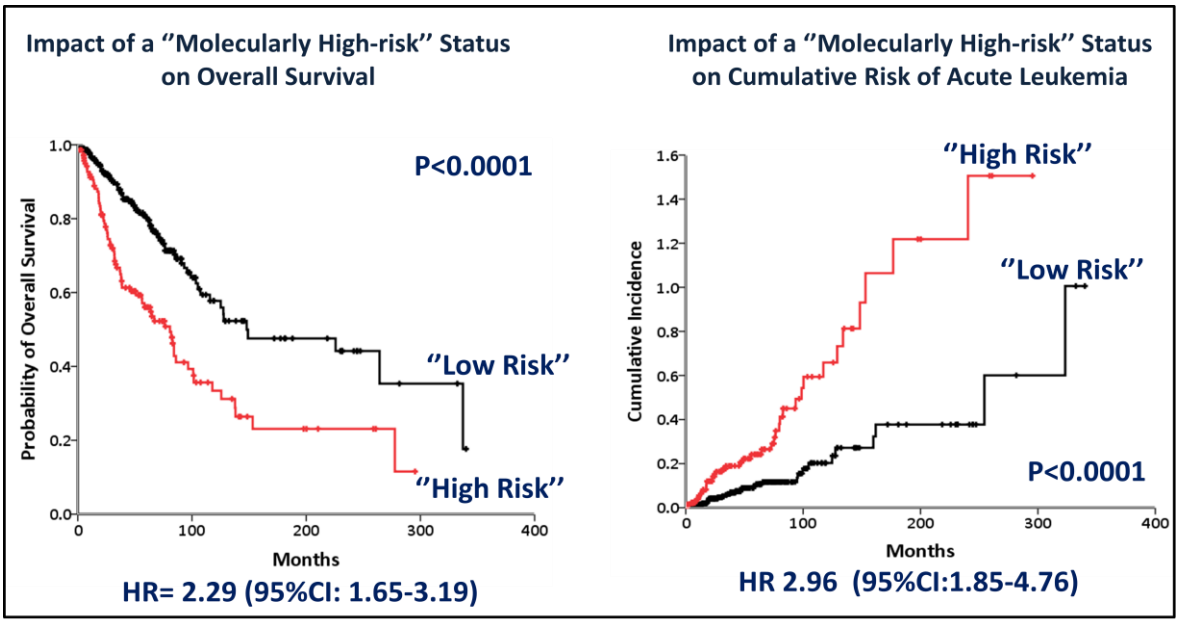
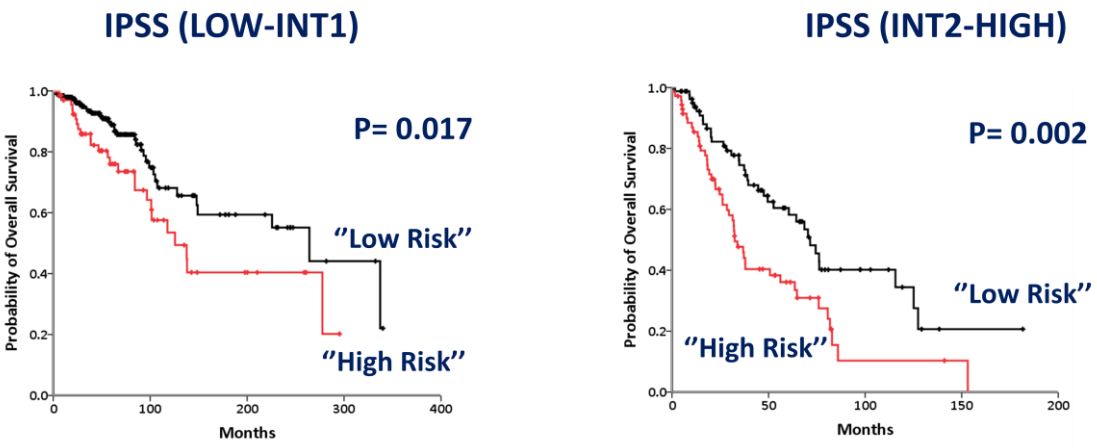


Figure 35. Impact of a ''Molecularly High-risk'' Status on Overall Survival and Cumulative Risk of Acute Leukemia

Finally, with we asked whether the molecular characterization of patients accoridng to the mutational status could refine the prognostication categorization of PMF patients according to IPSS risk categories. We found that subjects with mutationally defined low risk had a better outcome comared with patients clustering in the same risk category according to IPSS. In fact, we observed that in ”molecularly low-risk” patients comprised withihn the LOW-INT1 IPSS category, overall survival was 125.6 months compared with 264.2 months in those “molecularly low-risk” withihn the same IPSS category (P<0.017). Patients classified as INT-2/HIGH according to IPSS and belonging to “molecularly high-risk” and “molecularly low-risk” had an OS of 32.4 months and 71.5 months, respectively (P<0.002). (Figure 36)

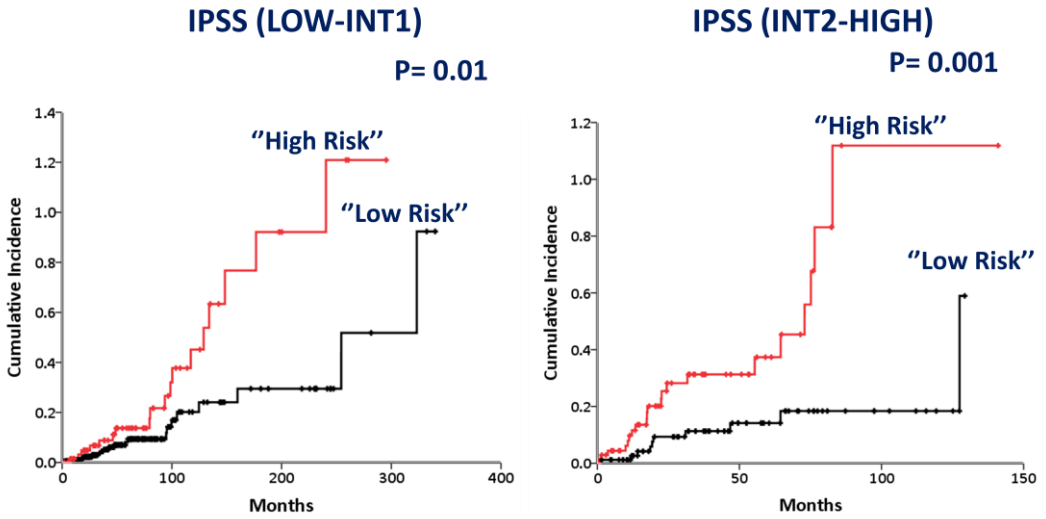


Molecular Risk category	Median Overal Survival, months (range)
LOW	264.2 (184.7-343.6)
HIGH	125.6 (77.4-173.8)

Molecular Risk category	Median Overal Survival, months (range)
LOW	71.5 (59.2-23.7)
HIGH	32.4 (26.6-38.3)

Figure 36. The ''Molecularly High-Risk'' Status Refines IPSS Prognostication for Overall Survival

Furthermore multivariate analysis demonstrated that "molecularly high-risk" patients in the IPSS (LOW-INT1) (hazard ratio [HR] = 2.28; 95% confidence interval [95% CI], 1.20-4.36; *P* = .01) and in IPSS (INT2-HIGH) (hazard ratio [HR] = 3.22; 95% confidence interval [95% CI], 1.52-6.83; *P* = .001), had significantly shorter LFS compared to "molecularly low-risk" patients of the same IPSS category . (Figure 37)



IPSS category	Hazard Risk (95%CI,range)
LOW-INT1	2.28 (1.20-4.36)
INT2-HIGH	3.22 (1.52-6.83)

Figure 37. The ''Molecularly High-Risk'' Status Predicts for Leukemia within IPSS Categories

In a second set of experiments i went to evaluate the prognostic significance of p53 mutational status on leukemia trasformation. Several previous studies have demonstrated that *TP53* mutations are not associated with the chronic phase of MPNs.[268] Mutations in *TP53* are rarely found in these diseases before acute leukemia transformation, but this does not rule out a role for *TP53* deregulation in disease progression As a matter of fact, mutated *TP53* have been found with a 20% frequency in post–MPN-AML patients[269] suggesting that it plays a prominent role in the transformation process. Thus, in order to understand whether TP53 mutations were present also in the chronic phase and could therefore predict for leukemia, , we decided to analyze mutational status of *TP53* using samples at diagnosis in selected patients (N=81) who evolved to leukemia. We found only four patients (5%) who were TP53 mutated (Table 20) , confirming that *TP53* mutations were not associated with the chronic phase of MPNs.

ID PATIENT	***TP53* MUTATIONS**
1	C242W
2	N131I - G245S
3	S116F
4	S261T

Table 20. *TP53* mutations found in the subgroup of PMF patients evolved to AML

To explore if, in the same subset of patients, mutations were acquired during follow-up, we analyzed TP53 mutation status using DNA purified from samples collected at the time of AML transformation (were available N=35). Possibly due to low number of events, we were unable to reveal any novel mutations.

5.3 CLONAL ANALYSIS OF *EZH2* AND *JAK2* V617F MUTATIONS SUGGESTS THAT EZH2 CAN BE A LATE EVENT IN THE PROGRESSION OF MPN

To study the order of events in the clonal evolution of *EZH2* and *JAK2* mutations, we examined individual colonies derived from peripheral blood of two PMF patients with *EZH2* mutations (*EZH2* V704L and *EZH2* A702V) that were identified by HRM anlaysis followed by direct sequencing. Both patients were also positive for *JAK2*-V617F in granulocytes. Mononuclear cells from peripheral blood were grown in methylcellulose. Single burst-forming units erythroid (BFU-E) and colony-forming units granulocyte (CFU-G) were picked and analyzed individually for the presence of *EZH2* and *JAK2*-V617F mutations. We picked and avarage of 60 colonies per sample: during the analysis we excluded all those colonies that showed an ambiguous genotype because of contamination by neighboring colonies. JAK2-V617F mutation analysis was performed by allele-specific polymerase chain reaction using HEL (JAK2 V617F positive cell line) and

K562 cell line (JAK2 V617F positive cell line) as positive and negative control, respectively. PCR product was loaded and analyzed on a 3% Agarose gel (Figure 38).

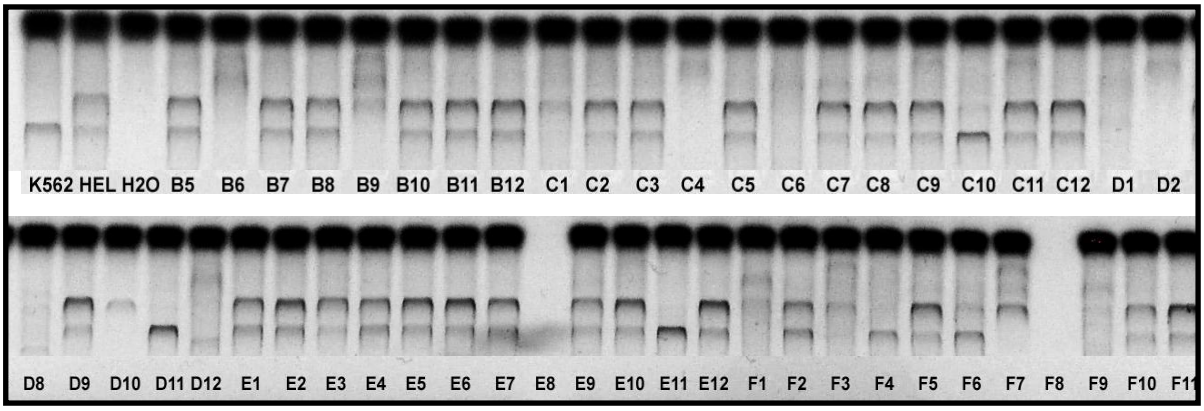


Figure 38. Example of allele-specific PCR. K562 DNA, negative control, results as one PCR product. HEL DNA, positive control, results as two PCR products. Colonies that resulted in two PCR products were considered *JAK2* V617F mutated (i.e B5, B7, B8); colonies that resulted in one PCR product were considered *JAK2* V617F negative (i.e C11, D11, E11, F4); colonies with uncertain genotype, such as B9, D10, F1, F7, were not considered.

On the same set of colonies we analyzed the mutional status of *EZH2* by performing HRM (see Method session). Products showing abnormal melt pattern (Figure 39) were PCR amplified for direct sequencing (Figure 40).

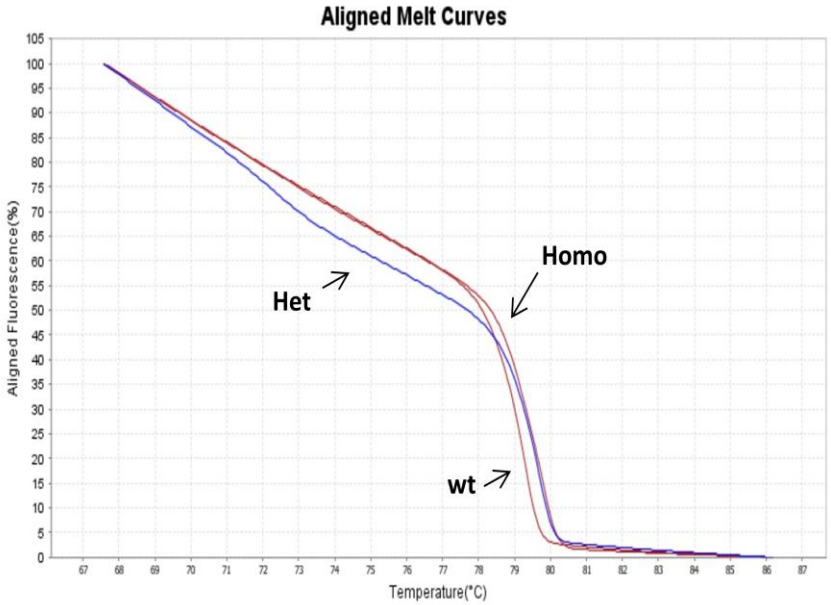


Figure 39. Example of melt curves obtained by HRM analysis of three different genotypes: Homozygous, Heterozygous and wild-type for *EZH2* V704L mutation.

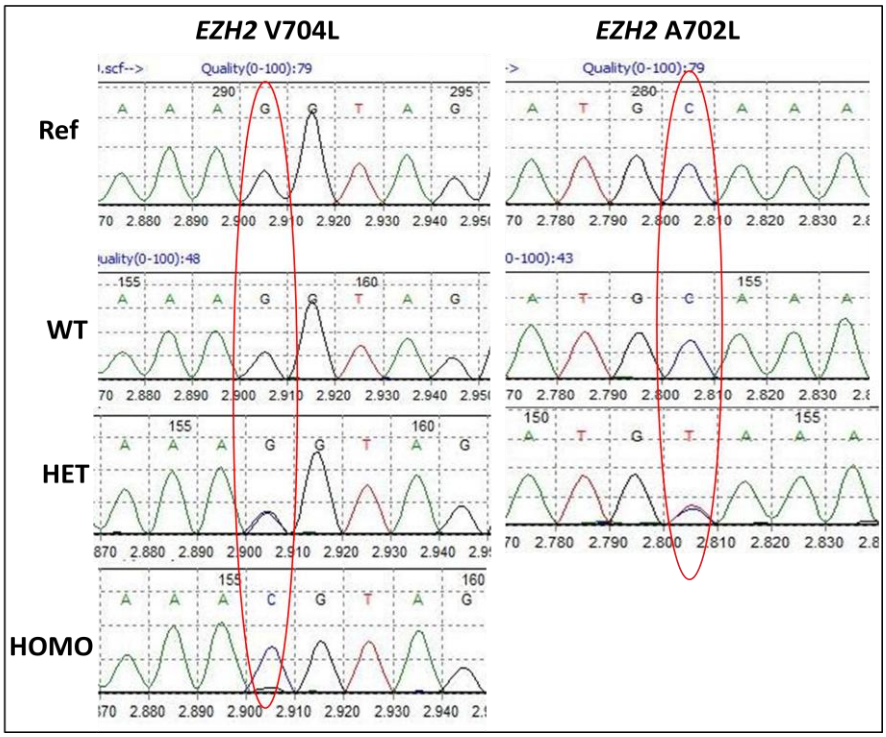


Figure 40. *EZH2* V704L and A702L sequences analyzed using Mutation Surveyor Software.

Mutational analysis of single colonies allowed us to distinguish 3 different patterns of mutationsin both samples (Figure 41): we found that some colonies with mutated *JAK2* carried wild-type *EZH2*, whereas others were *EZH2* mutatedOther colonies were negative for both mutations. We didn't find colonies positive for *EZH2* that were also negative for *JAK2,* indicating that *EZH2* occurred after the acquisition of *JAK2*-V617F.

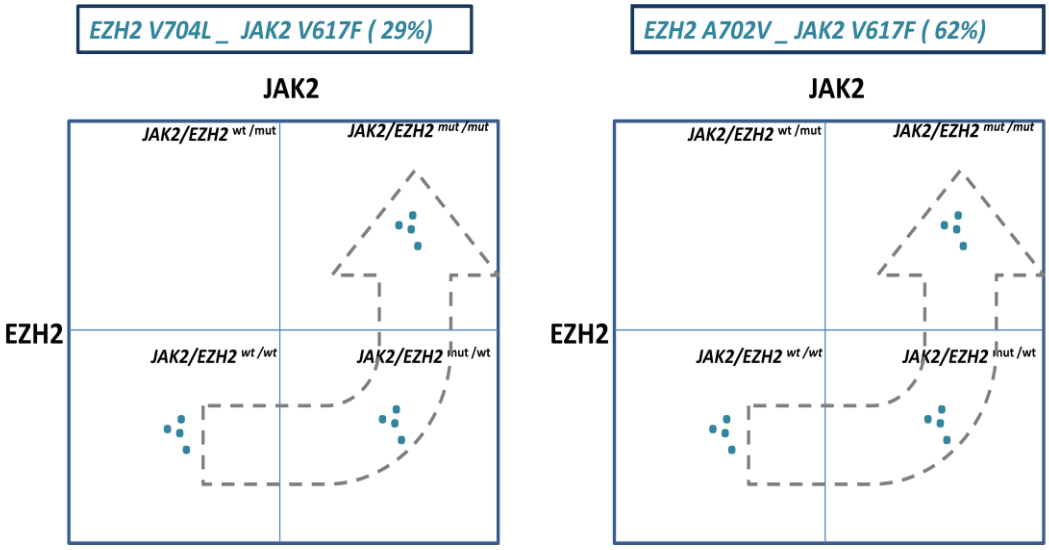


Figure 41. Patterns compatible with *EZH2* mutations occurring after *JAK2*-V617F. Each colony is represented by a dot that is placed into one of 4 quadrangles representing the

four possible genotypes: wild-type (wt)/mutated (mut) for *EZH2*on the vertical axis, and for *JAK2*-V617F on the horizontal axis. The *EZH2* mutation and the allelic ratio of JAK2-V617F in purified granulocytes (%) are shown above the corresponding boxes. Grey arrows indicate the suggested order of mutation events.

5.4 ANALYSIS OF miRNAs CONTRIBUTION TO THE PATHOGENESIS OF MPNs

5.4.1 CHARACTERIZATION AND DISCOVERY OF NOVEL miRNAs AND moRNAs IN JAK2V617F-MUTATED SET2 CELLS.

Sequencing of the small RNA from SET-2 cells, a JAK2 V617F cell line, produced 22,167,999 filtere reads (68% of raw data) that were mapped to "extended hairpins" to identify and quantify known miRNAs and for discovery and characterization of novel isomiRs and other miRNA-associated expressed RNAs. A total of 652 known miRNAs were found expressed in SET2 cells, with expression levels ranging from 10 to 2,268,333 (mean, 29 830; median, 613); Twenty-one only highly expressed miRNAs accounted for 70% of known miRNA expression and were thus predicted to account for most of miRNA-mediated gene repression in SET2 cells. Target genes of these 21 most expressed miRNAs are genes involved in pathways that were already anticipated to have functional relevance for MPN-associated cellular abnormalities, such as the MAPK signaling pathway, TGF-β signaling pathway, mTOR signaling pathway, and Wnt signaling pathway. It was interesting to find that the majority of expressed mature miRNAs were not represented by a unique sequence corresponding to that annotated in miRBase. So, we evaluated the whole group of reads belonging to each miRNA, including the "classic" mature sequence annotated in miRBase ("exact" alignment) as well as those reads perfectly matching the precursor but overlapping the mature position by 3 nt (longer/shorter), those presenting 1 mismatch (1-Mismatch), and those presenting 2 mismatches at the 3'-end (2-3'-Mismatches; supplemental Methods). Considering the whole set of variants presenting at least 10 reads each, 636 miRNAs were identified: 232 (36%) appeared "invariant," whereas the remaining 404 (64%) represented a mixture of 2 to 6 sequence variants. We considered as "biologically meaningful" sequence variants (isomiRs) accounting for at least 10% of the total per miRNA read count, calculated over all variants. In this way, of 644 miRNAs represented, 224 miRNAs (35%) were "invariant" and 420 (65%) were associated with 2 to 6 isomiRs each. Nevertheless, it was interesting that some of miRNAs considered "invariant" were associated with an unique major sequence as well as to other sequences that weren't annotated because none of them satisfied the required number of reads. We reasoned that the presence of isomiRs could be particularly relevant for miRNAs expressed at the highest level. Specifically, the contribution of the major isomiR averaged 52% in case of miRNAs with more than one isomiRs and 83% in "invariant" miRNAs. As known, 5'- and 3'-regions of mature miRNA

sequence play a different role in target recognition: in case of canonical target sites, the seed region, whose pairing to the target is crucial, is included in the 5′ of mature miRNA. It is conceivable that isomiRs with different seed sequence recognize and regulate different targets. Thus, for each MEm, we considered the 5′- and 3′-half sequences separately and classified expressed isomiRs according to the observed difference between the isomiR sequence and the "classic" mature miRNA (mismatch or length difference) in the involved region. Of 819 isomiRs, 648 (79%) differed in the 3′-region, 64 in the 5′-region (8%), and 107 in both (13%). No "Mismatch" isomiRs were observed regarding the 5′-region. Figure 42 reports the relative contribution of isomiRs for the 21 most expressed miRNAs in SET2 cells.

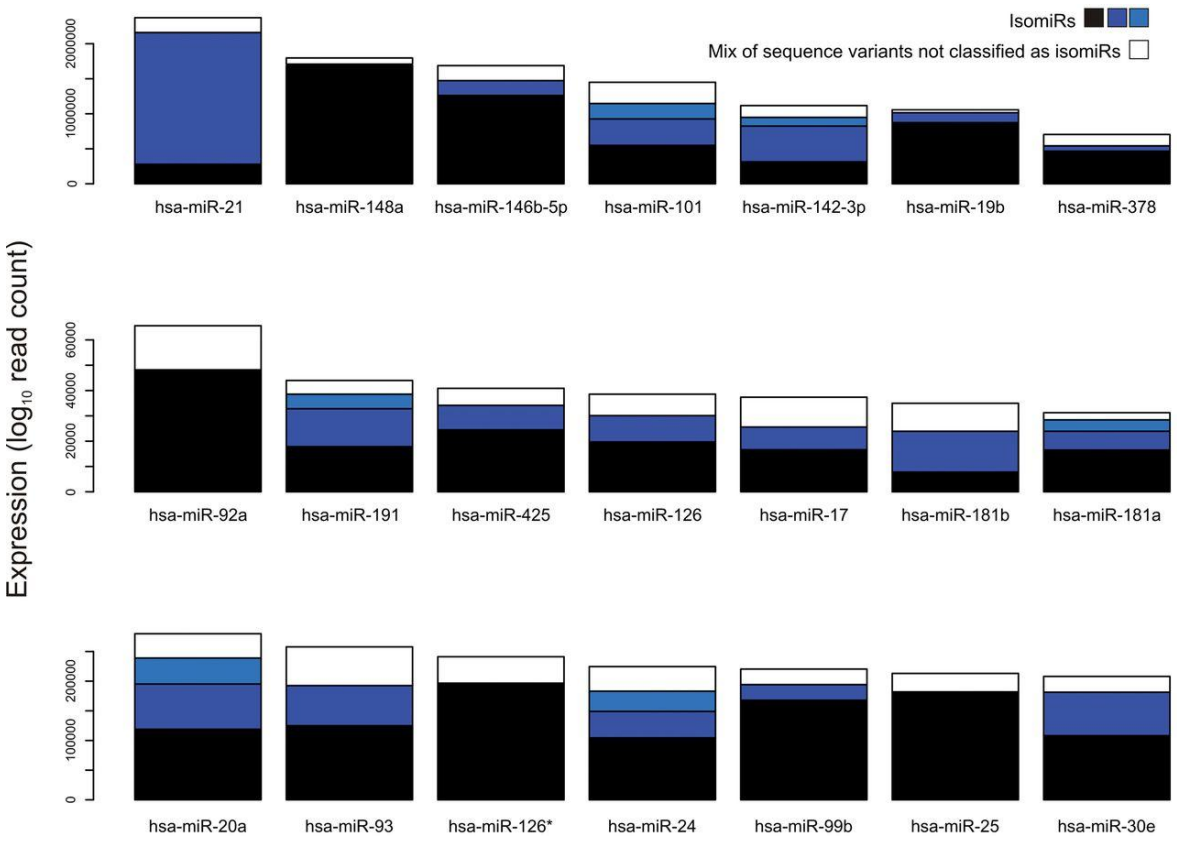


Figure 42. Relative isomiR contribution to the total read count of 21 most expressed miRNAs in SET2 cells. The total read count belonging to sequences not passing the stringent threshold for being considered as isomiRs is indicated in the white rectangle.

In the MEm set, 323 isomiRs were identified: 140 (43%) were coincident with the miRNA sequence reported in miRBase (classic isomiR), 154 (48%) represented sequence variants with longer or shorter 5′- and 3′-ends, and 29 (9%) showed 1-nt difference versus the classic isomiR. Interestingly, we found that the major isomiR was not coincident with the classic sequence annotated in miRBase in 53 of 161 highly expressed miRNAs (33%).

Besides, the isomiR corresponding to the sequence annotated in miRBase was not detected in 21 (13%) of the miRNAs expressed in SET2 cells.

We then considered 113 highly expressed miRNAs associated with more than one isomiR to understand how many mismatch alignments contributed to sequence variability. We found that 87 of these (77%) did not include isomiRs with single nucleotide substitutions respective to the sequence annotated in miRBase, whereas only a minority, which may arise from SNPs, RNA editing, and sequencing errors, included one or 2 isomiRs aligning with one mismatch to the hairpin precursor (24 and 2 miRNAs, respectively). In terms of total expression, isomiRs with mismatch alignments contributed only to less than 3% of read count. Classic isomiRs and isomiRs showing 5′- and 3′-length variability almost equally contribute to 97% of total read counts of highly expressed miRNAs. Therefore, we concluded that the most important sequence variation is represented by 5′- and 3′-length variability, possibly occurring as a consequence of alternative, noncanonical, regulated processing of the precursor sequence.

5.4.2 NOVEL miRNAs EXPRESSED IN SET2 CELLS WERE DISCOVERED IN KNOWN HAIRPIN PRECURSORS

For novel miRNA discovery, we operationally defined as “expressed RNA elements” those discrete hairpin regions covered by overlapping reads with a minimum count of 10 and a start position within 4 nt each from the following one. We found that a discrete number of regions located outside known mature miRNAs were expressed from detectable to high level; a number of expressed RNA elements able to pair with known miRNAs in the most probable duplex produced by Dicer processing of the hairpin structure were identified. Specifically, we considered 943 hairpins associated with only one annotated mature miRNA, whereas an additional 478 included 2 known sister mature miRNAs.

The analysis of known hairpin precursors associated with only one known miRNA produced a set of 78 novel miRNAs expressed in SET2 cells: of these the 4 most expressed new miRNAs (hsa-miR-1307*, hsa-miR-376a-2*, hsa-miR-382*, and hsa-miR-539*) are shown in Figure 43. Expression levels of new miRNAs ranged from 10 to 72 670 (mean, 1471; median, 63) and were significantly lower than known miRNAs (2-sample *t* test of mean equality, $P = 6.521 \times 10^{-06}$; supplemental Figure 3). Nevertheless, 11 new miRNAs (14%) showed an expression level higher than the median value observed for known miRNAs. In particular, hsa-miR-1307*, hsa-miR-376a-2*, and hsa-miR-382* resulted very highly expressed, at a level even more than 75% of known miRNAs.

Specifically, conserved and nonconserved target sites were predicted using TargetScan and filtered according to the context score. Only conserved target sites associated with top 25% scores and nonconserved sites included in top 5% scores were reported.

Considering known and new miRNAs expressed in SET2 cells as a whole, we found that both miRNA and miRNA* were expressed concurrently in 260 hairpins, corresponding to approximately one-half of those with at least one miRNA espresse. miRNA and miRNA* of

the same hairpin, called a sister miRNA pair, have different sequences, thus targeting different sets of coding RNAs and contributing uniquely, but possibly in a coordinate way, to transcriptional regulation. When increasing thresholds of read count were applied to consider a miRNA as being "expressed," the fraction of hairpins producing concurrently miRNA and miRNA* decreased, whereas the fraction of hairpins producing a meaningful quantity of both miRNAs remained considerable at all thresholds. We observed no strand prevalence in expressed miRNAs). Of 277 hairpins expressing only one miRNA, 47% and 53% expressed only the 5'- or 3'-miRNA (130 and 147), respectively. Regarding 260 hairpins expressing both sister miRNAs, the most expressed miRNA was the 5'- or 3'-form in 135 and 125, respectively. A considerable fraction of concurrently expressed miRNA pairs showed comparable levels: 66 (25%) and 38 (14%) of concurrently expressed pairs were associated with an absolute $\log_2$(ratio) of expression values not higher that 2 and 1, respectively.

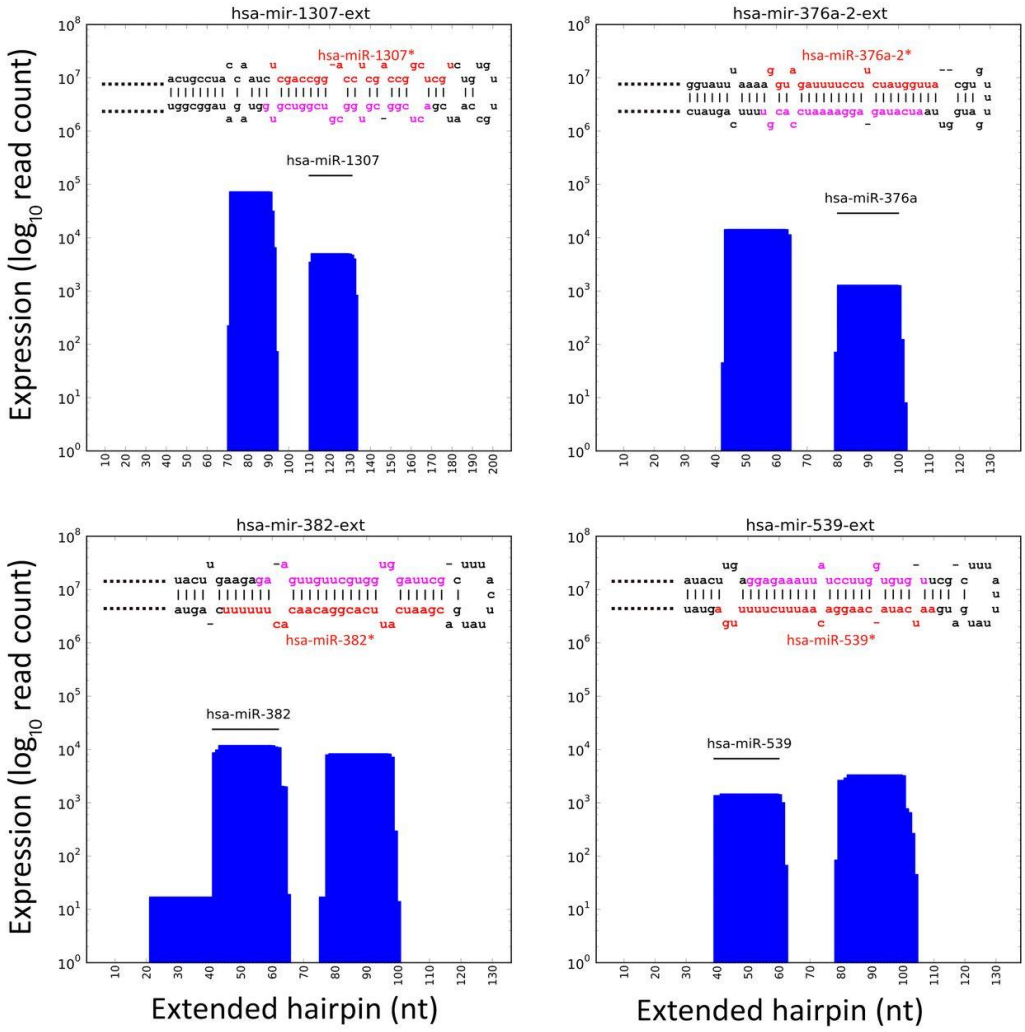


Figure 43. Examples of new miRNAs discovered. Plots show the number of reads per nucleotide position mapping in extended hairpin loci for hsa-mir-1307, hsa-mir-376a-2, hsa-mir-382, and hsa-mir-539, expressing both known and new miRNAs. Known miRNA

position respective to the extended hairpin is indicated in the plot. In the top part of panel, the sequence of mature miRNAs is shown (red indicates known miRNA; and pink, new miRNA).

5.4.3 moRNA IDENTIFICATION IN KNOWN HAIRPIN PRECURSORS

We discovered ERE also outside known and novel miRNAs. These were classified as 5'-moRNAs, 3'-moRNAs, and expressed loops (Figure 44). In particular, we identified 58 moRNAs expressed from 56 hairpins, at moderate to high level (mean 127, median 52). Three hairpins were associated each with 2 moRNAs expressed (Figure 44); of these, hsa-mir-106b is an example of a "5-phased" precursor, with detected expression for 5 different regions.

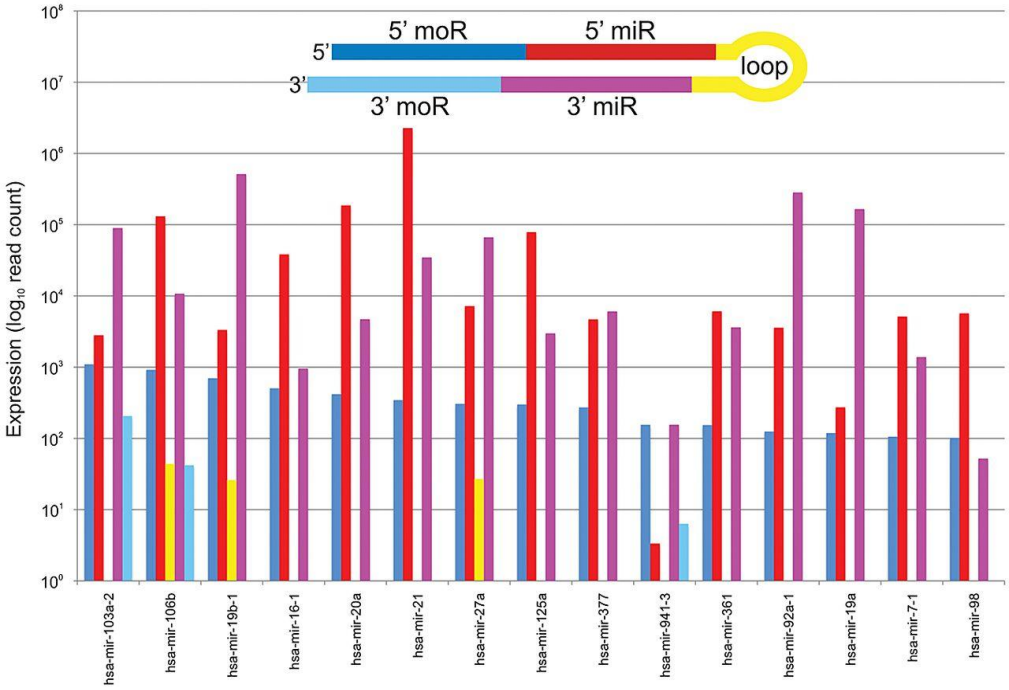


Figure 44. Two miRNAs and 2 moRNAs may be produced by transcription and processing of a single miRNA locus. The plots show expression levels of miRNAs, moRNAs, and loops from 15 hairpins corresponding to most expressed moRNAs.

To derive insights concerning moRNA biogenesis, we considered the position of moRNA sequences relatively to both the miRNA hairpin precursor (Figure 45A) and the mature miRNA from the same hairpin arm (Figure 45B). Regarding the region of the hairpins from which moRNAs derived, we noticed that 8 of 55 5'-moRNAs were included in the "classic" hairpin precursor sequence, whereas only one belonged to the region of 30 nt flanking the hairpin and 46 (84%) were partially overlapping the hairpin 5'-border. This indicates that moRNA sequence spanned the canonical Drosha cutting site and supports a role for noncanonical Drosha cleavage in moRNAs biogenesis. Moreover, we considered the positions of 50 couples of concurrently expressed 5'-moRNAs and 5'-miRNAs in extended

hairpins. It is noteworthy that 5'-moRNAs and 5'-miRNAs were adjacent in 27 pairs (54%): the miRNA sequence started exactly after the moRNA end. In this case, the 2 elements might derive from a single cutting step of a common precursor sequence. Conversely, in only 1 pair (2%), the 2 sequences were disjointed and the miRNA sequence started at least 1 nt after the moRNA end. Finally, 22 moRNAs (44%) overlapped with the nearby miRNA sequence for 1 to 8 nt, indicating the presence of pairs of moRNAs and miRNAs that were probably produced alternatively from the processing of the same precursor.

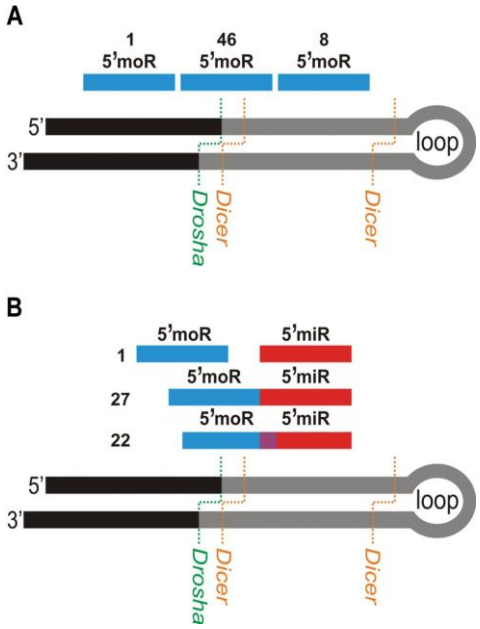


Figure 45. 5'-moRNA position relative to miRNA hairpin precursor (A) and to the mature miRNA expressed from the same hairpin arm (B).

With the aim to identify a set of miRNas that lie in the JAK2 regulatory pathway, SET2 cells were treated with the JAK2 inhibitor INC424/ruxolitinib and subjected to miRNA quantification using microarrays. Cells were exposed to INC414/ruxolitinib at 160, 80, and 1600nM for 3 and 6 hours and then processed for miRNA expression analysis by microarray. The concentration of 160nM was selected as the IC50 concentration in a proliferation assay of SET2 cells (not shown). We found that INC424/ruxolitinib caused dose-dependent reduction of the level of phosphorylated STAT5 (measured by FACS) and Pim-1 mRNA (measured by quantitative real-time PCR), indicating effective inhibition of JAK/STAT signaling (top panels Figure 46). Using stringent selection criteria we identified 9 microRNAs among those identified by RNASeq in untreated cells that showed significant dose-dependent modulated expression (hsa-let-7c, hsa-miR-1299, hsa-miR323-3p were up-regulated, while hsa-miR-330-5p, hsa-miR-548, hsa-miR-665, hsa-miR-935u, hsa-miR-381, hsa-miR-92a-1* were down-regulated; (Figure 46 Bottom panels).
Dose effects were statistically significant ($P < .05$).

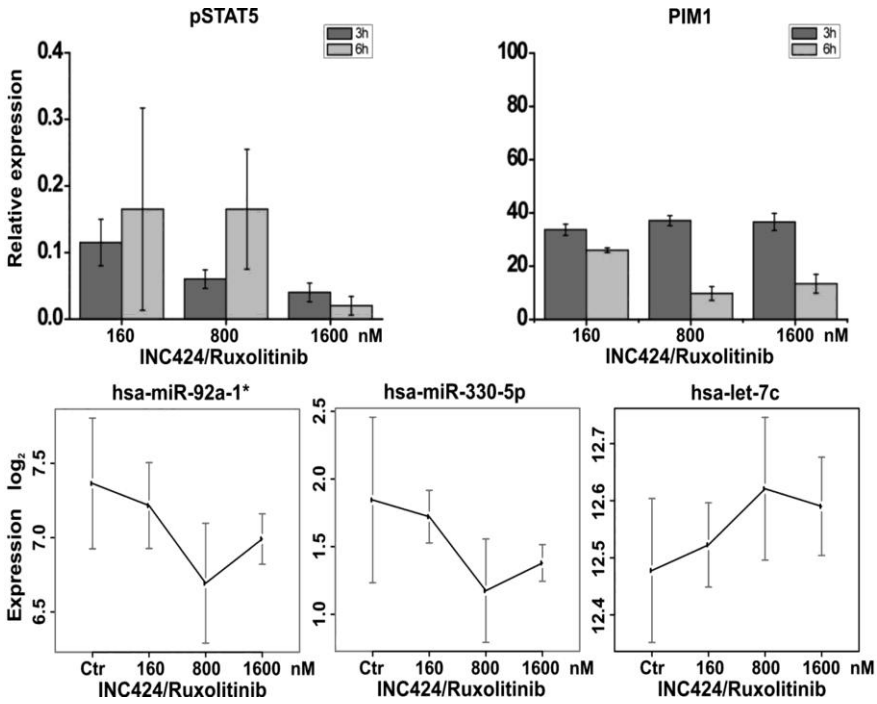


Figure 46. Modulation of miRNAs by INC424/ruxolitinib treatment of SET2 cells. Modulation of miRNAs by INC424/ruxolitinib treatment of SET2 cells. Cells were exposed to INC414/ruxolitinib at 160, 80, and 1600nM for 3 and 6 hours and then processed for miRNA expression analysis by microarray. The concentration of 160nM was selected as the IC50 concentration in a proliferation assay of SET2 cells (not shown). INC424/ruxolitinib caused dose-dependent reduction of the level of phosphorylated STAT5 (measured by FACS) and Pim-1 mRNA (measured by quantitative real-time PCR), indicating effective inhibition of JAK/STAT signaling (top panels). (Bottom panels) Examples of INC424/ruxolitinib-induced miRNA level modulation, as measured using miRNA array, are shown. The levels of hsa-miR-92a-1* and hsa-miR-330-5p dose-dependently decreased, whereas those of has-miR-let-7c increased compared with untreated control cells. Dose effects were statistically significant ($P < .05$).

All the results obtained in this study, and reported in this thesis, were published in *Blood.* : *Stefania Bortoluzzi, Andrea Bisognin, Marta Biasiolo, Paola Guglielmelli, Flavia Biamonte, Ruggiero Norfo, Rossella Manfredini and Alessandro M. Vannucchi. Characterization and discovery of novel miRNAs and moRNAs in JAK2V617F-mutated SET2 cells. Blood March 29, 2012vol. 119 no. 13 e120-e130*

5.4.4 CHARACTERIZATION AND VALIDATION OF INTEGRATED miRNA AND TRANSCRIPTOMIC PROFILES IN JAK2^{V617F} KI AND JAK2^{wt} MICE.

The aim of this study was to identify microRNAs whose activity was deregulated by the expression of JAK2V617F ("JAK2V617F miRNome") and, by performing integrative bioinfomatic analysis, to correlate them with target gene expression so as (i) to produce a list of validated deregulated microRNAs and target genes in a JAK2V617F overexpressing murine model; (ii) to explore the relevance, using functional approach, of selected target genes/regulatory pathways predicted by *in silico* reconstruction to be involved in MPNs. We thought that as a whole, these information could help to clarify the contribution of microRNAs to the pathogenesis of JAK2V617F-positive MPNs and hopefully allow to identify novel putative targets for therapy.

As genes and microRNAs are usually expressed in a lineage-specific manner we performed both miRNA and gene expression profiling (GEP) in erythroid (TER119+) and myeloid (GR1+) cells purified from bone marrow of JAK2^{V617F} KI (n=2) and JAK2^{wt} (n=2) mice by using a combination of immunomagnetic and cell sorting approaches (Figure 47A and B).

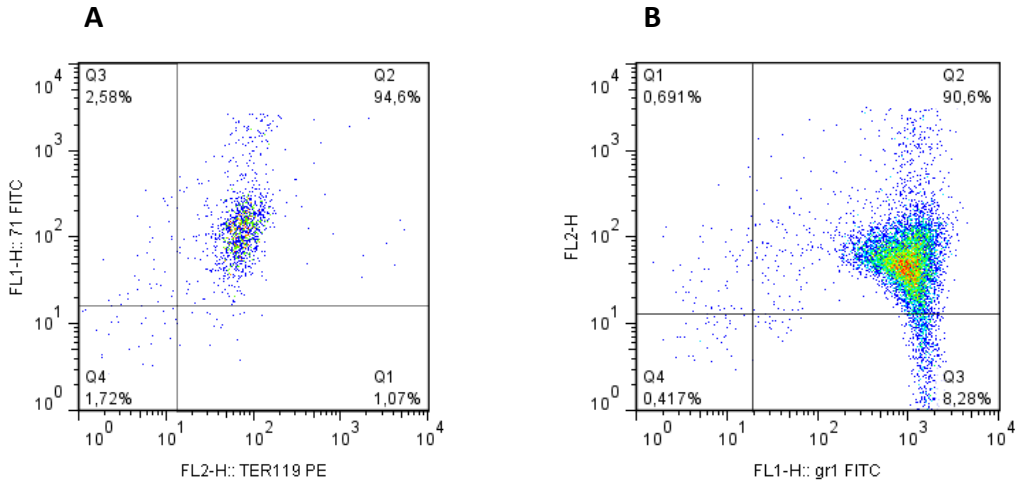


Figure 47. Flow cytometry data plots of A) TER119^{+}and B) GR1^{+}.

Only samples with a purity greater than 90% were analyzed. In order to obtain sufficient quantity of miRNA for subsequent analysis it was necessary to extract total RNA from both L2 (TER119^{+}and GR1^{+}) cells and L- (TER119^{+}and GR1^{+}) . Total RNA fraction was used to determine the RNA Integrity Number (RIN), which was in the range of 7.4 to 9.6, and to assess RNA concentration and rRNA ratio [28 s/18 s] by means of a bioanalyzer (Agilent). Once samples passed this quality control, the analysis of micro-RNA expression profile was performed by miRCURY LNA™ Universal RT microRNA PCR KIT (EXIQON) that includes 384 miRNAs covering most well-characterized miRNAs in the miRNA Registry v14

(http://microrna.sanger.ac.uk). Gene expression profiling (GEP) was carried out by Prof. Rossella Manfredini lab. Group (University of Modena and Reggio Emilia) on the same set of cell preparation using the Affymetrix Mouse Genome 430 2.0 Array.

We performed an initial data analysis using the software supplied with the real-time PCR instrument to obtain raw Ct values. Then, because integrative data analysis with gene expression profile requires sophisticated biocomputational procedures, the statistical analysis of our results was carried out with the collaboration of the bioinformatic group of Biology Department of University of Padova, under the supervision of Dr S. Bortoluzzi (AGIMM). We decided to study three kind of comparisons:

L2 VS L- (GR1) L2 VS L- (TER119) L2 VS L- (ALL)

miRNA expression data matrix (2E-DeltaCt values) was calculated from Ct row data and then, with the aim to focus on the most relevant miRNA-gene networks we selected only miRNAs with |log Fold Change(L-/L2)|>2

The numbers of modulated miRNAs per contrast are reported in Table 21:

	L2 VS L- GR1	**L2 VS L- TER119**	**L2 VS L- ALL**
UP	10	11	11
DW	10	12	13
Total	20	23	24

Table 21. Modulated miRNAs per contrast. UP (miRNAs up regulated); DW (miRNAs down regulated).

We found that 20, 23 and 24 miRNAs were modulated in L2 VS L- (GR1), L2 VS L- (TER119) and L2 VS L- (ALL), respectively (Figure 48A and B). In particular 20 miRNAs were differentially modulated in myeloid GR1+ cells (10 up- and 10 down-regulated) and 23 in erythroid TER119+ cells (11 up- and 12 down-regulated). Only 9 miRNAs resulted modulated in both GR1 and TER119 cells : of these five were up-regulated (miR-27b, miR-106a, miR-26a, miR-301a, miR-16) and four down-regulated (miR-350, miR-33, miR-150, miR-let-7f).

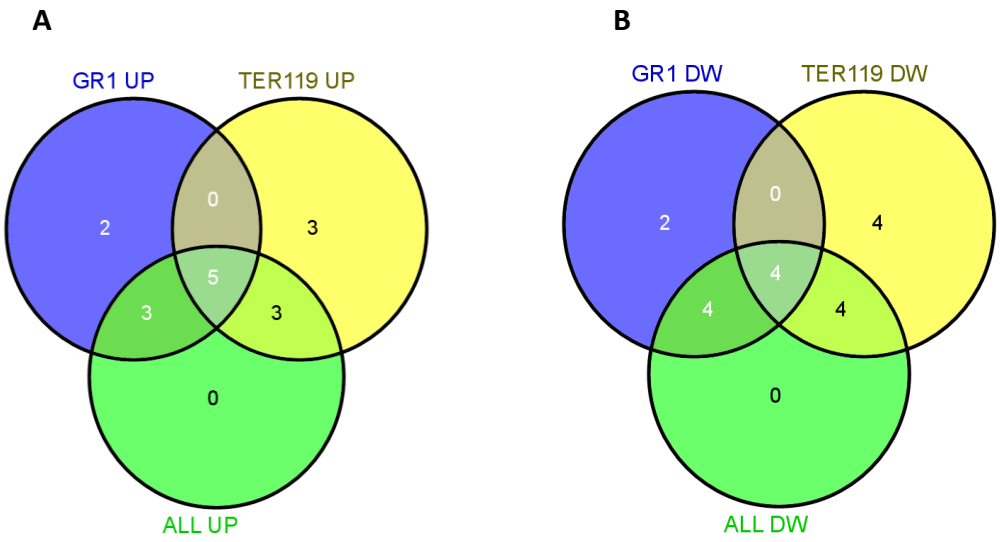


Figure 48. Number of modulated miRNA for each contrast. miRNA up-regulated (A) and down-regulated (B)

A summary of differentially expressed miRNAs is reported in Figure 49.

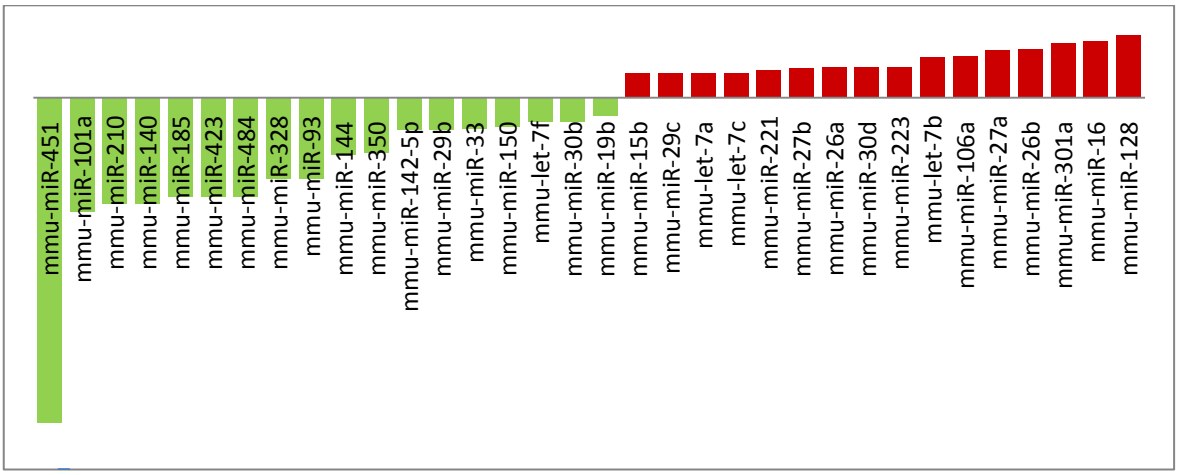


Figure 49. modulated miRNAs

It was very encouraging to find that two of differentially expressed (DEMs) miRNAs (miR-16 and miR-150) have already been described as being deregulated in MPNs. A paper from Bruchova H. and colleagues, as well as a paper from Guglielmelli P. et al who performed a gene expression profiling of micro-RNAs (miRNAs) in granulocytes from MPNs patients demonstrated that mir-16 was overexpressed in PV granulocytes. In

another study Bruchova *et al*. demonstrated dow-regulation of mi-150 in ET or PMF patients.

Lately, an integrative analysis of gene and miRNA expression was performed by using a combination of miRNA target prediction systems (PITA, miRanda and targetScan) in order to construct bipartite regulatory networks of the most likely functional miRNA-target interactions. For each miRNA, predictions consisted of predicted target sites in human transcript sequences. For each predicted site, a "context score" was given, that was considered the most important feature to predict functional targets, as well as information about site evolutionary conservation. miRanda computes optimal sequence complementarity between a set of mature microRNAs and a given mRNA using a weighted dynamic programming algorithm. Moreover the TargetScan Human resource provides miRNA target predictions based on sequence complementary to target sites with emphasis on perfect base-pairing in the seed region and sequence conservation. These web-based computational tools are based on different algorithms which are based on several parameters calculated individually for each miRNA.

One limitation of this kind of study is the difficulty of correlating deregulated expression of miRNAs with that of target genes, since there is a pleiotropy of putative target genes. We found 6,367 genes predicted to be target of at least one miRNA but, with the aim to focus only on the most meaningful relations, we calculated the pairwise Spearman correlations between DEMs using a threshold =-1 on correlation strength. Using such a stringent correlation we identified 398 target genes and 555 putative regulatory networks. Overall these included genes involved in pathways having functional relevance for MPN such as cell cycle (e.g. *CDC25a* and *DUSP1*), survival and apoptosis (e.g. *PIM-1* and*E2F*), epigenetic regulation (e.g. *EZH2* and *HDAC10*), signal transduction and transcriptional modulation (e.g. *GATA2, MAPK6* and *SMAD3*).

It is still premature to conclude whether and how aberrant expression of miRNAs concurs with genetic lesions to modify disease presentation, and also if it is related to *JAK2* V617F mutation. Moreover, the demonstration that expression level of some genes, selected by in silico analysis as possible targets of the deregulated miRNAs, was modulated in $TER119^+$ and GR^+ cells of L- mice compared to those of L2 mice does not represent a formal proof of a cause-effect relationship. A clarification of the role of abnormally expressed miRNAs will require manipulation of endogenous miRNA levels in hematopoietic stem cells and/or their descendants obtained from patients with MPNs.

6. DISCUSSION

The discovery of *JAK2V617F* as a common, recurrent, genetic lesion in three related but phenotypically distinct diseases, ET, PV and PMF, raised the question of how does a single point mutation contribute to the pathogenesis of three different disease entities. Several scenarios have been discussed to explain the phenotypic heterogeneity including mutational dosage, cytokine receptor expression patterns in different lineages, additional genetic hits and acquisition of mutations at distinct differentiation states (progenitor *versus* stem cell). A strong line of evidence supports the notion that the relative proportion of mutated and wild-type JAK provides an important contribution.[19-22] However, it also became soon clear that JAK2V617F mutation can not explain the whole story, and the theory of both a ''pre-*JAK2*'' and "later-occurring" mutational events has emerged.[26,27] Subsequent discoveries of several novel mutations occurring in MPNs are in support of this theory, although it must be acknowledged that a definite picture has not yet emerged. Therefore, a clear picture of what is the clonal hierarchy of MPN is still an unmet objective. Furthermore, the significance of JAK2V617F and other mutations in terms of clinical phenotype and particularly prognosis is known still imperfectly for most of the genes. On the other hand, this represents an important field of research, particularly in PMF, that is associated with poorer survival compared with other classic *BCR-ABL*–negative chronic MPNs,[34] where the identification of variables associated with prognosis might help to refining risk stratification and support therapeutic decisions, particularly concerning the choice between drug therapy and hematopoietic stem cell transplantation, which remains the only potentially curative therapeutic approach. Therefore, we thought to focus this project with a double aim, the first to search and characterize novel genetic abnormalities in MPN (including gene mutations, epigenetic modifications and miRNAs deregulation), where possible also determining their phylogenetic relationships with JAK2 mutation the second to ascertain the relevance of those genetic abnormalities for disease progression,leukemia transformation and overall survival more in general, particularly in PMF. Elucidation of these issues would facilitate the understanding of genotype-phenotype correlations and produce clinically relevant new information.

Initally I focused on *EZH2* mutations in a large cohort of 518 patients with MF. We found *EZH2* in approximately 6% of PMF patients, 9% of PET-MF patients, and only in only 1 PPV-MF patient (1.2%). In a prospective cohort of 118 PMF patients I found that *EZH2* mutations were maintained in leukemic blasts at the time of leukemia transformation while no *EZH2* mutations were acquired at the time of leukemia in any of the 8 patients who were wild-type at diagnosis. I found that *EZH2* mutation does not contribute a specific phenotypic signature in patients with primary and PPV/PET PMF highlighting only subtle differences, such as more pronounced leukocytosis, larger spleens, and higher circulating blast cells (these associations were appreciable only in

PMF patients). However, the main findings of this study was that *EZH2* mutational status had a significant negative impact on disease outcome among PMF patients. In fact, I found, that *EZH2*-mutated patients preferentially clustered in the IPSS high-risk category, that both overall survical (OS) and leukemia-free survival (LFS) were shortened in *EZH2*-mutated subjects compared with their wild-type counterparts; in a multivariate analysis, *EZH2* mutational status maintained a negative prognostic significance together with the IPPS score and a low *JAK2*V617F allele burden. On the other hand, the low number of events (death and leukemia) recorded in the PPV/PET-MF group prevented statistical testing of a possible impact of *EZH2* mutational status on OS and LFS;[217] data from a larger series of patients are needed before any firm conclusion can be drawn. To get insights in the order of events in the clonal evolution of *EZH2* and *JAK2* mutations I performed mutational analysis of single colonies derived from peripheral blood of two PMF patients that concurrently expressed *EZH2* mutations (*EZH2* V704L and *EZH2* A702V) and *JAK2*-V617F in granulocytes. The single-cell clonal analysis revealed three different patterns of mutation appearance in both patients: colonies with mutated *JAK2* and wild-type *EZH2*, colonies with mutated *JAK2* and mutated *EZH2,* other colonies negative for both mutations. I did not find colonies positive for *EZH2* that were also negative for *JAK2,* thus suggesting that*EZH2* mutations occurred after the acquisition of *JAK2*-V617F. However, the number of samples analyzed was limited, and a larger series of experiments are necessary to validate these preliminary data. At the conclusion of this study, we decided to perform a more extensive mutational analysis focusing only on PMF patients, with the aim to determine whether mutational status for specific mutations or for combinations of mutations affected outcome. Given the established importance of some of these genes, I focused on known mutations affecting *JAK2*-V617F, *MPL* K515L, *TET2*, *ASXL1*, *DNMT3A*, *CBL,* SRSF2, *EZH2*, *IDH1*, and *IDH2,* using samples collected at diagnosis in a multicenter (Florence, Pavia, Athens, Barcelona, Mannheim, Salisbury, Southampton) series of 483 patients with primary myelofibrosis. Overall I found that 382 (79.1%) of patients presented at least one somatic mutation. It was very supportive to find that mutations frequencies in our study population matched very well with previous studies: *JAK2* and *ASXL1* were most common, occurring in 59.2% and 21.7% of cases, respectively; mutations in the other target genes occurred at a frequency of less than 10%; *IDH1, IDH2* and *SRSF2* resulted the target genes with the lowest number of somatic variants. No specific association between two or more mutations have been highlighted. The analysis of prognostic impact in terms of overall servival and leukemia-free survival measured from diagnosis to the time of leukemia transformation revealed that *EZH2*, *ASXL1*, *SRSF2* were the only genes significantly predicting for reduction of OS indipendently from each other. The same three genes, together with *IDH* mutations, were found predictive of progression to acute myeloid leukemia. The statistical significance was manteined in both univariate and multivariate analysis. Once identified the four genes whose mutations significantly impacted on patients outcome in terms of both OS and LFS, we decided to accordingly classify the entire patient cohort into those who displayed at least one ("*molecularly high-risk*") or ("*molecularly low risk*") none of the four mutations. It was

very interesting to find that subjects who were defined at mutational “low risk” had a better outcome compared to those defined as “high risk” even if clustering in the same risk category according to IPSS. The correlation between the mutational status and the disease outcome was significant in terms of both overall servival and leukemia-survival.
Results of this study made a string case that mutations in *EZH2*,*IDH*,*SRSF2* and *ASXL1* provide an important contribution to the prognostication of patients with PMF, even beyond the classical IPSS risk categories, thus deserving translational relevance. Validated reproducibility, low cost and preferentially high throughput are prerequisites for novel diagnostic tests to be implemented for clinical purposes. Next-generation sequencing (NGS) has recently reduced costs and time for entire genome analyses with deep coverage. Since a “next-generation sequencing” system (Ion Personal Genome Machine, Ion Torrent, Life technolgies) has become available in our laboratory in the last months, the current efforts are directed towards development of a rapid and reliable method for screening these new genetic mutations that could find application for diagnosis in the clinical practice.

The second broad goal of this project was to improve the knowledge of miRNAs in MPNs, since preliminary data indicated that these could be abnormally regulated in at least a subset of patients. To this end, I used a multidimensional approach that included cell lines andanimal models, taking advantage of novel molecular tecnhinques, previous experience of the host laboratory as well as tight collaboration with other groups. One accomplishment of this study was the characterization of a “JAK2V617F miRNome”, a list of microRNAs whose activity was deregulated by the expression of JAK2V617F. Thanks to the unique availability of a knock-in (KI) inducible mouse model of the JAK2V617F (kindly concession by Dr. Jean-Luc Villeval group,Inserm U1009, Institut Gustave Roussy, Villejuif, France), I was able to evaluated the microRNA expression profile of purified hematopoietic cells sorted from the bone marrow of $JAK2^{V617F}$ KI and $JAK2^{wt}$ mice. Using very stringent statistical approach, we found that 20 miRNAs were differentially modulated in myeloid GR1+ cells (10 up- and 10 down-regulated) and 23 in erythroid TER119+ cells (11 up- and 12 down-regulated). Among them, nine miRNAs were shared by both erythroid and myeloid cells: five up-regulated (miR-27b, miR-106a, miR-26a, miR-301a, miR-16) and four down-regulated (miR-350, miR-33, miR-150, miR-let-7f). It was very encouraging to find that two of differentially expressed miRNAs (miR-16 and miR-150) have already been described as being deregulated in human MPNs. A second main result of this research line has been the production of a catalog of known miRNAs expressed in SET2 cells, a *JAK2*V617F-mutated cell line established from a patient with leukemic transformation of essential thrombocythemia. We noticed that several among the top 25% expressed miRNAs belonged to 2 clusters, the miR-106b-25 (located on chromosome 7q22.1, which includes miR106b, miR-93, and miR-25) and the miR-17-92 (located on chromosome 13q31.3, which includes miR-17, miR-19b, miR-20a, and miR-92a, miR19a, and miR18b); of these, 3 miRNAs (miR-19b, miR106b and miR-93) were among those found abnormally expressed also in murine model. Considering predicted

targets of highly expressed miRNAs, different signaling (and biochemical) pathways were identified, obtaining interesting clues about possible regulatory circuits that are deregulated in SET2 cells. Recent evidence support the contention that uncontrolled expression of both or either these 2 clusters results in the loss of physiologic control of cell cycle arrest and apoptosis contributing to oncogenesis. Indeed, gene expression profiles in murine cells revealed a list of deregulated target genes involved in pathways such as cell cycle (e.g. *CDC25a* and *DUSP1*), survival and apoptosis (e.g. *PIM-1* and*E2F*), as well as genes having functional relevance for MPN epigenetic regulation (e.g. *EZH2* and *HDAC10*), signal transduction and transcriptional modulation (e.g. *GATA2, MAPK6* and *SMAD3*). In conclusion, these exeriments have contributed to extensively characterize the profile of short RNAs expressed in a human model of MPN, the SET2 cells, and provided proof of concept that modulation of mature miRNA expression may be obtained via inhibition of JAK2. Although these results, represent an interesting start point, we think that they will acquire functional relevance only when validated in primary MPN cells from $JAK2^{V617F}$ mutated patients. We expect that future studies will result in a restricted list of validated "JAK2V617F miRNAs" and putative target genes similar in the murine model, cell lines and human system. To this end, we plan to perform functional studies aimed to confirm a direct link between JAK2 mutational status and those miRNA/mRNA deregulations. This could be achieved either, or both, by silencing JAK2 signaling with small interfering RNA (siRNA/shRNA) specifically directed against the *JAK2*V617F-mutated RNA, or through pharmacologic inhibition of JAK2V617F signaling in primary MPN cells with JAK2 inhibitor (i.e. Ruxolitinib,LY2784544).

Thanks to the collection of data deriving from NGS of miRNA in SET2 cells I was also able to contribute to theidentification of a consistent number of novel short RNAs expressed from miRNA loci in SET2 cells. We discovered 78 novel miRNAs expressed from known hairpin precursors. Considering known and new miRNAs together, we took into account expression behavior of pairs of miRNAs derived from 5 and 3′-strands of the same hairpin (miRNA/miRNA*, so-called "sister miRNAs"). Sister miRNAs have different sequences and target different sets of coding RNAs. We showed that for approximately one-half of hairpins both sister miRNAs are expressed concurrently; very likely, both contribute to target repression. Furthermore, we found short expressed RNAs derived from regions of extended hairpins outside known and new miRNAs.[286] These are members of a novel class of miRNA-related RNAs, called moRNAs,recently identified by massive short RNAseq. moRNAs are currently defined as approximately 20-nt-long RNAs that originate predominantly from the 5′-arm of pre-miRNAs, with a biogenesis linked to that of miRNAs but not necessarily interdependent. In the present study, we identified 58 moRNAs expressed from 56 hairpins at moderate to high level. In particular, hsa-5′-moR-103a-2, hsa-5′-moR-106b, hsa-5′-moR-19b-1, and hsa-5′-moR-16-1 were highly expressed in SET2 cells. Moreover, we observed that slightly less than one-half of moRNAs overlapped the nearby expressed mature miRNA of a few nucleotides; in these cases, moRNA and miRNA expression from the same precursor was mutually exclusive. The biologic role of moRNAs is still to be defined: one hypothesis claims that moRNAs might guide RISC to

complementary target mRNAs as miRNAs do. Furthermore, because moRNAs were found to be nuclear enriched, an alternative hypothesis is that moRNAs intervene specifically in nuclear processes as other nuclear short and long RNAs do. We think that the discovery of expressed moRNAs in SET2 cells is intriguing: they can be viewed as a new class of regulators whose qualitative and/or quantitative abnormalities might impact on human MPN. The last main finding of the study regards mature miRNA sequence variation, since it was found that mature miRNAs were not represented by a unique sequence; rather, they were found as mixtures of sequence variants, called isomiRs, that derive mainly from noncanonical processing of hairpin precursors. In particolar, I observed that 60% of miRNAs are associated with 2 to 4 isomiRs, which differ from the most expressed isomiR only for 5′- and 3′-sequence length but align exactly to the hairpin precursor. Interestingly,we found that the sequence variability of isomiRs regards prevalently the 3′-region of mature miRNAs; however, 8% of isomiRs differ from the "classic" miRNA sequence in the 5′-region, possibly impacting on target recognition and/or regulatory activity and strength.[286] Future evaluations of isomiR quality and proportion in different cell types will help to clarify if and how much this miRNA biogenesis feature is cell- and context-specifically regulated, and the putative role in MPN pathogenesis. In conclusion, a better knowledge of the the interplay of known and new miRNAs, isomiRs, and moRNAs might contribute to the abnormal regulation of MPN cellular mechanisms, and it will be challenging to understand whether and how novel JAK2-targeting drugs might affect this complex system of regulators. As a whole, these information could meaningfully complement the emerging information from the mutational profiling of MPN patients, and all together might prospectively contribute to the identification of novel therapeutic targets in these still uncurable hematologic neoplasia.

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1. Guglielmelli P, **Biamonte F**, Spolverini A, Pieri L, Isgrò A, Antonioli E, Pancrazzi A, Bosi A, Barosi G and Vannucchi AM . *Frequency and clinical correlates of JAK2 46/1 (GGCC) haplotype in primary myelofibrosis.* Leukemia 2010, June 3.

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3. Guglielmelli P, **Biamonte F**, Score J, Hidalgo-Curtis C, Cervantes F, Maffioli M, Fanelli T, Ernst T, Winkelman N, Jones AV, Zoi K, Reiter A, Duncombe A, Villani L, Bosi A, Barosi G, Cross NC, Vannucchi AM. *EZH2 mutational status predicts poor survival in myelofibrosis.* Blood. 2011 Nov 10;118(19):5227-34.

4. Vannucchi AM, **Biamonte F**. *Epigenetics and mutations in chronic myeloproliferative neoplasms.* Haematologica. 2011 Oct;96(10):1398-402

5. Bortoluzzi S, Bisognin A, Biasolo M, Guglielmelli P, **Biamonte F**, Norfo R, Manfredini R, Vannucchi AM.*Characterisation and discovery of novel miRNAs and moRNAs in JAK2V617F mutated SET2 cells.* Blood. 2012 Mar 29;119(13):e120-30

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