UNIVERSITY OF

MODENA AND REGGIO EMILIA

Degree in Doctor of Philosophy in
Molecular and Regenerative Medicine
XXVI Cycle

"The molecular framework of CD34+ cells from primary myelofibrosis patients unveils disease associated expression alterations"

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April 2014

ABSTRACT

Primary myelofibrosis (PMF), polycythemia vera and essential thrombocythemia are Philadelphia-negative chronic myeloproliferative neoplasms (MPNs) characterized by stem cell-derived clonal proliferation and increased production of mature myeloid cells. PMF is the worst among the MPNs and is associated with abnormal megakaryopoiesis, cytokine overproduction, bone marrow (BM) fibrosis, osteosclerosis, angiogenesis and hepatosplenic hematopoiesis.

Information on molecular abnormalities of MPNs has been scanty until 2005 with the discovery of the somatic gain-of-function mutation of *JAK2*. Since then, many other mutated genes were identified, nevertheless they do not represent the primary mutational event and it is clear that the molecular basis of MPNs have not yet been completely elucidated.

Recently, several new molecular pathogenetic mechanisms were proposed, such as the aberrant expression of coding and non-coding RNAs.

In order to address this issue, in the first phase of this project we investigated the molecular signature of CD34+ cells from PMF patients, performing gene expression profiling of 42 PMF samples and 31 healthy controls.

Interestingly, we found many deregulated genes possibly involved in some pathogenetic steps of PMF such as genes related to BM fibrosis (*MMP9* and *TIMP3*) or regulation of cell migration (*TM4SF1* and *MMP8)* as well as a number of transcription factors and chromatin remodelers implicated in myeloid and megakaryocyte commitment (i.e. *AFF3, MAF* and *IKZF2).* Moreover, PMF samples exhibited increased levels of several mRNAs suitable as biomarkers or as putative molecular targets for diagnostic or prognostic purposes. Then the expression of the most upregulated genes (*LCN2*, *OLFM4*, *ANXA3*, *FGR* and *LEPR*) was validated also in PMF granulocytes, as well as secreted protein (OLFM4 and LCN2*)* levels were assessed on patients' serum. These findings demonstrate that these genes could be considered as disease biomarkers, since they were significantly higher in patients.

Conversely, downregulated genes have been silenced in normal CD34+ cells in order to construct in vitro PMF models and to elucidate the possible role in the

hematopoietic differentiation. Among those genes, we focused on the chromatin remodeler *JARID2,* because chromatin remodeling is a process frequently impaired in MPNs. Our data demonstrated that *JARID2* silencing enforces the hematopoietic differentiation towards the megakaryocytic lineage. Based on these results we suppose that the downregulation of *JARID2* in CD34+ cells from PMF patients could be involved in the abnormal megakaryopoiesis that features this disease.

Finally, since long non-coding RNAs (lncRNAs) are emerging as key regulators of gene expression in normal and cancer cells, we decided to investigate the expression of *ANRIL*, *MEG3* and *WT1-antisense* lncRNAs, previously described as related to hematological malignancies, in CD34+ cells from a different cohort of PMF patients. The results evidenced that the majority of PMF samples displayed a co-upregulation of *WT1* and its antisense RNA compared to controls. These samples also showed an increased *MEG3* expression. In these patients, we found a correlation with *WT1/WT1-as/MEG3* expression levels and high Dynamic International Prognostic Scoring System (DIPPS) plus score and elevated number of circulating CD34+ cells. Moreover, the expression pattern of *CDKN2B/ANRIL* distinguished a group of patients characterized by an upregulation of *CDKN2B*, and among these, a subgroup with downregulated *ANRIL*. Of note, this group of patients exhibited a high grade of BM fibrosis and the presence of *JAK2V617F* mutation. Our results suggest that also a deregulated expression of these lncRNAs could play a role in PMF pathogenesis and progression.

INTRODUCTION

1. Myeloproliferative Neoplasms: history and classification

In 1951, William Dameshek was the first to describe the "myeloproliferative disorders (MPDs)" as a distinct clinicopathologic entity when he gathered together chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) and erythroleukemia (Di Guglielmo Syndrome). Infact he speculated that all these conditions were variable manifestations of proliferative activity of the bone marrow (BM) cells, due to an undiscovered stimulus[1]. Over the years, erythroleukemia and its variants have been recategorized as erythroid leukemia[2], whereas the other four entities were defined as the classic MPDs.

In 1960, CML was associated with the cytogenetic marker Philadelphia (Ph) chromosome that was later shown to harbor the *BCR/ABL* disease-causing genetic mutation[3]. The latter was subsequently shown to represent a reciprocal chromosomal translocation, t(9;22) (q34;q11), which is an acquired somatic mutation that fuses the *ABL* gene from chromosome 9 with the *BCR* gene on chromosome 22[4]. *BCR/ABL* is transcribed into a chimeric 8.5-kb mRNA, and is subsequently translated into a constitutively activated tyrosine kinase that in turns activates a number of different pathways to influence proliferation, survival and differentiation of the neoplastic cell[5].

Since BCR/ABL identification, MPDs lacking Philadelphia chromosome have been classified as BCR/ABL negative or Ph1 negative MPDs[6].

In 2001, the World Health Organization (WHO), in collaboration with the Society for Hematopathology and the European Association of Hematopathology, published a classification of Tumors of the Hematopoietic and Lymphoid Tissues as a part of the third edition of the series, *WHO Classification of Tumors*[2]. In this classification classic MPDs were assigned under the category of chronic myeloproliferative diseases (CMPDs) that includes the four classic MPDs (CML, ET, PV, MF) and, in addition, chronic neutrophilic leukemia, chronic eosinophilic leukemia, hypereosinophilic syndrome, and unclassified CMPD[2] (**Table 1**).

2001 WHO classification of chronic myeloproliferative diseases
Chronic myelogenous leukemia [Ph chromosome, t(9;22)(q34;q11),BCR/ABL-positive]
Chronic neutrophilic leukemia
Chronic eosinophilic leukemia (and the hypereosinophilic syndrome)
Polycythemia vera
Chronic idiopathic myelofibrosis (with extramedullary hematopoiesis)
Essential thrombocythemia
Chronic myeloproliferative disease, unclassifiable

Table 1. 2001 WHO classification of chronic myeloproliferative diseases.

The common feature of CMPDs was effective clonal myeloproliferation (that is peripheral blood (PB) granulocytosis, thrombocytosis or erythrocytosis) that is devoid of dyserythropoiesis, granulocytic dysplasia or monocytosis[7]. Early studies confirmed that CMPDs are clonal hematopoietic stem cell (HSC) disorders which were found to have a shared biology[8,9,10,11].

In 2005, the discovery of a mutation in the JAK2 kinase (*JAK2V617F*)[12,13,14,15], led to a greater understanding of the molecular pathogenesis of BCR-ABL-negative classic MPDs. This mutation is present in more than 90% of patients with PV and nearly one-half of those with ET or PMF[12,13, 14,15].

As a result of this discovery, in 2008 the WHO classification and diagnosis of CMPDs was updated and published as part of the fourth edition of the WHO monograph series[16]. The aim of the revision was to incorporate new scientific and clinical information that has accumulated since the previous edition, in order to refine diagnostic criteria for previously described neoplasms and to introduce newly recognized disease entities[16](**Table 2**).

2008 WHO classification of myeloproliferative neoplasms
Chronic myelogenous leukemia [Ph chromosome, t(9;22)(q34;q11),BCR/ABL-positive]
Chronic neutrophilic leukemia
Chronic eosinophilic leukemia, not otherwise specified
Polycythemia vera
Primary Myelofibrosis
Essential thrombocythemia
Mastocytosis
Myeloproliferative neoplasm, unclassifiable

Table 2 2008 WHO classification of myeloproliferative neoplasms.

CMPDs were reorganized into the Myeloproliferative Neoplasms (MPNs) to underscore that the myeloproliferation is neoplastic and not rective. Two important factors influenced the classification: 1) the use of molecular genetics for diagnostic markers for Ph1-negative MPN and 2) the use of histologic characterization to aid in the identification of MPN subtypes[17]. In any case histology of MPNs may be difficult and no one histologic feauture is sufficient to diagnose an MPN, but rather histology must be interpreted in conjunction with clinical and laboratory data.

Myeloproliferative Neoplasms Philadelphia-1 Negative

According to 2008 WHO classification system for hematopoietic tumors[17], Philadelphia-1 negative MPN is a sub-category of MPNs that includes PV, ET and PMF[7]. These clonal disorders share common feautures[18], such as their origin from multipotent HSC, an overlap in clinical presentation, and in case of PV and ET, the propensity to evolve into post-polycythemic or post-thrombocythemic myelofibrosis (MF) (or less frequently each into the other)[19], and the possibility to transform in acute myeloid leukemia (AML)[20].

1.1. Incidence and epidemiology

Incidence for BCR-ABL1-negative MPNs are estimated at 0.2 to 2.5 per 100000 for ET, 0.4 to 1.5 per 100000 for PMF, and 0.8 to 2.6 per 1000000 for PV[21]. Moreover, because of their relatively smooth clinical course, it is likely that many classic MPN cases actually go undetected or are not reported to registries. Classic MPNs are among the most frequent hematologic neoplasms, usually affecting the adult elderly population. The median age at diagnosis for all three MPNs is usually reported as 60 years, but population based studies report a substantially older age[19].

1.2. Pathogenesis

PV is characterized by an increased proliferation of all three myeloid lineages with consequent overproduction of apparently normal mature red cells, granulocytes, and platelets[10], but the predominant clinical feature is erythrocytosis, defined by an increased red cell mass[22].

The first in vitro abnormality to be observed in PV progenitor cells was that these cells form erythroid colonies in the absence of exogenous Erythropoietin (EPO), a phenomenon not observed in progenitor cells from normal subjects[23]. This observation has been widely used as a diagnostic tool to help distinguish PV from other causes of polycythemia[24,25,26,27].

The presence of erythroid colonies in the absence of exogenous EPO is the hallmark of PV. EPO is the major regulator of mammalian erythropoiesis growth factor. EPO initiates its cellular response by binding to the EPO receptor (EPO-R) expressed on the surface of immature erythroblasts. Following ligand binding, EPO-R activate JAK2 which triggers a signal transduction cascade that leads to the development of early erythroid progenitors into mature erythroblast cells[28].

Since PV is a condition in which the defect is intrinsic to the cells and not driven by an increased EPO production, the hypothesis that PV is caused by a mutation of the *EPO-R* gene was studied first, but even if mutation of the EPO receptor has been reported in families with inherited erythrocytosis, genetic alterations in the *EPO-R* have not been detected in PV[29], and the expression of the EPO receptor and its ability to bind EPO are also normal.

The erythroid progenitors of PV patients are hypersensitive to several growth factors and cytokines, such as stem cell factor (SCF), interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and insulin-like growth factor[31]. Despite these abnormal responses, the total number of receptors and binding affinities for each of these growth factors are normal.

ET involves primarily the megakaryocytic lineage. Platelets are increased in number and often morphologically and functionally abnormal, and, thus, patients are at an increased risk of thrombosis and bleeding[32].

The BM is frequently hypercellular owing to a marked increase in megakaryocytes (MKs), which may be enlarged and hyperlobated, with minimal to no expansion of the granulocytic and erythroid lineages[33]. BM cellularity is normal or slightly increased, with abundance of large, mature-appearing MKs devoid of morphological abnormalities and generally dispersed nuclei[34].

The molecular events underlying the commitment and differentiation of normal and malignant Mks are still poorly understood[35]. It is thought that molecular lesions in critical genes regulate the balance between proliferation/differentiation and apoptosis of MK progenitors. Even though ET is usually considered to be a clonal disease, it has been suggested that some patients do not have a clonal disorder[36]. The concept of 'polyclonal' ET suggests a potential pathogenetic role for thrombopoietin (TPO) or other thrombopoietic cytokines. However, serum TPO levels in ET are usually normal or only slightly elevated[37]. Furthermore, circulating TPO levels in ET are not significantly different from those in reactive thrombocytosis[38].

1.3. Karyotype

By definition, the Philadelphia chromosome (and BCR-ABL1) is absent in PV, ET and PMF. However, other recurrent cytogenetic abnormalities are seen in approximately 33% of patients with PMF[39], 11% in PV[40] and 7% in ET[41]. The types of abnormalities present in these three BCR-ABL1-negative MPNs are similar and include del(20q), del(13q), +8, +9, chromosome 1 abnormalities, and chromosomes 5 and 7 abnormalities. Among these, +9 and del(13q) are relatively specific to MPNs whereas the others are also seen in Myelodysplastic Syndromes

(MDSs)[42]. Chromosomal breakpoint regions seen with del(20q) include q11.2-13.1, with del(13q) q12-22 and with chromosome 1 anomalies q10-25/p10-31.

1.4. Molecular markers

In 2005 to 2007, a series of studies found that a very high frequency of activating mutations in the JAK-STAT pathway is present in MPN patients. These founding discoveries have changed the diagnostic approach to MPNs. The acquired mutation in *JAK2* (*JAK2V617F*) is present in 90% to 95% of patients with PV, 50% to 60% of patients with ET, and 50% to 60% of patients with PMF[12,15,43]. *JAK2* exon 12 mutations are present in *JAK2V617F*-negative PV patients[44]; and mutations activating the TPO receptor MPL in 3% to 5% of patients with ET and 8% to 10% of patients with PMF[45]. Although *JAK2* mutations have been shown to be the phenotypic drivers in MPNs, there is evidence of clonality and mutational events preceding the acquisition of *JAK2V617F*[46,47]. Subsequently, an increasing number of mutations in several genes distinct from *JAK2* have been identified in patients with MPNs. These include mutations in epigenetic modifiers, genes involved in hematopoietic signaling and leukemic progression of MPNs, such as *TET2*[47], *ASXL1*[48], *CBL*[49], *IDH1/2*[50], *LNK*[51] and *EZH2*[52], as well as other genes involved in epigenetic gene regulation and RNA splicing [53,54,55].

Very recently, recurrent mutations in the calreticulin gene (*CALR*) have been reported in in *JAK2/MPL*-unmutated ET and PMF by two next generation sequencing whole exome studies[56,57]. These mutations provide additional diagnostic and prognostic tools in the molecular diagnostic gap in *JAK2/MPL* negative ET/PMF that is now partially addressed by these mutations in the majority of such cases.

1.4.1. JAK2 and MPL

The JAK family comprises 4 kinases (JAK1, 2, and 3 and TYK2) that attach to cytokine receptor cytosolic domains. JAK kinases possess 2 highly homologous domains at the carboxyl terminus: an active kinase domain (JAK homology, JH1) and a catalytically “inactive” pseudokinase domain (JH2). The JH2 domain is a negative regulator of the JH1 kinase activity[58]. At the N-terminus, the JH5-JH7

domains contain a FERM (Band-4.1, ezrin, radixin, and moesin)–like motif, which is involved in the binding to the cytosolic domain of cognate cytokine receptors. *JAK2* is involved in the signaling from "myeloid" cytokine receptors. It binds to the 3 homodimeric "myeloid" receptors ([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), and to IFN-R2. JAK2 is the only JAK capable of mediating the signaling of EPO-R and MPL. Therefore, JAK2 and the 3 "myeloid receptors" form functional units and have been shown to be necessary for the promotion of *JAK2V617F* signaling[59] (**Figure 1**).

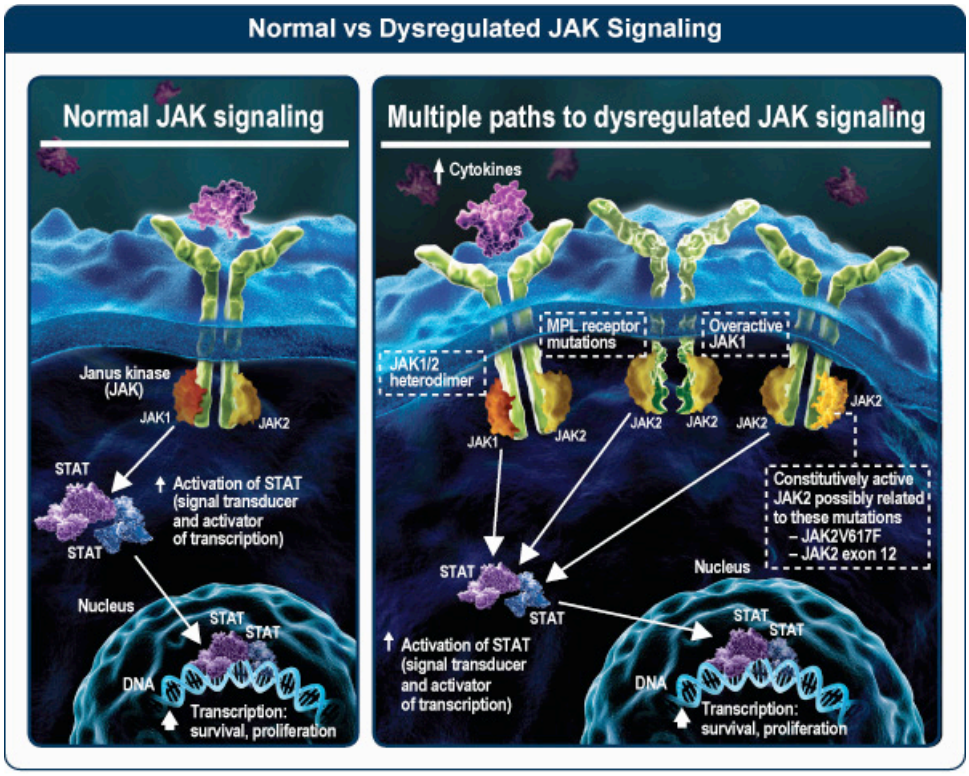

Figure 1. Normal versus pathological JAK signaling.

JAK2V617F mutation

The *JAK2V617F* mutation results from a guanine to thymine change at nucleotide 1849, in exon 14 of the gene. This valine is located at one of the predicted interfaces between JH1 and JH2 domains[60]. Indeed, the *JAK2V617F* mutation has been found in the majority of BCR-ABL-negative MPNs (95% of patients with PV, 50%-60% with ET, and 50%-60% with PMF), as well as in some cases of atypical

MPNs (30%-50% splanchnic vein thrombosis and sideroblastic anemia associated with a thrombocytosis)[61].

Beside its role in the cytokine receptor signaling cascade, *JAK2* has been shown to influence chromatin structure[62,63]. In hematopoietic cells, nuclear *JAK2* phosphorylates histone H3Y41, thereby blocking recruitment of the repressor heterochromatin protein 1 and allowing increased expression of several genes, including the *LMO2* oncogene[64].

Similarly, *JAK2V617F* has been described to interact with and phosphorylate the protein arginine methyltransferase (PRMT5) with a much greater affinity than wild-type *JAK2*[65]. This property seems to be specific 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[65]. This emerging nuclear role of mutated *JAK2* may reveal mutant-specific chromatin effects that may open a novel therapeutic window.

JAK2 exon 12 mutations

In the rare *JAK2V617F*-negative PV, different somatic gain-of function mutations in exon 12 of *JAK2* have been found[44]. 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 more frequently to a secondary MF[66].

Involvement of JAK2 in epigenetic regulation

The transforming effects of activating mutations in *JAK2* have been mostly ascribed to constitutive activation of downstream mitogenic pathways such as the STAT family of transcription factors, MAPK, and AKT. In 2009, Dawson et al made the fascinating observation that JAK2 is also found within the nucleus of both normal and malignan HSCs[64]. At least one functional consequence of this finding is that *JAK2* phosphorylates histone H3 among all core histones. This was

confirmed through evidence of decreased H3 phosphorylation in the presence of at least 2 different JAK2 inhibitors and phosphorylation of H3Y41 only after JAK2 transfection in JAK2-null g2A cells. The investigators then demonstrated that phosphorylation of H3Y41 results in displacement of HP1a and, subsequently, overexpression of *LMO2*, an oncogene with a known role in leukemogenesis. The findings of this study provided evidence that a kinase thought to be restricted to the cytoplasm may regulate gene expression directly by affecting chromatin structure. Evidence for the nuclear localization of JAK2 has since been confirmed by several additional groups and JAK1 has been discovered to be present in the nucleus too[65,67].

Identification of JAK2 within the nucleus led to the question of possible additional nuclear substrates of JAK2 phosphorylation other than histone H3Y41. Previously, study of the physical interactions of JAK2 with other proteins, had focused on the association of JAK2 with cytoplasmic domains of type II cytokine receptors to mediating signals that are triggered by hematopoietic growth factors and activate the STAT5/BCL-XL, PI3K/AKT, and ERK/MAPK pathways. However, as mentioned above, activation of these mitogenic pathways may not completely account for the MPN phenotype[65].

MPL

MPL (named after myeloproliferative leukemia virus oncogene homolog) belongs to the hematopoietin receptor superfamily and enables its ligand, TPO, to facilitate both global hematopoiesis and MKs growth and differentiation[68]. The gene for *MPL* maps to chromosome 1p34 and contains 12 exons[69]. Several gain-of-function mutations of *MPL* have been found in exon 10, which determine the substitution of a tryptophan 515 to a leucine, lysine, asparagine, or alanine[45,70,71]. Amino acid 515 is located in a stretch of 5 amino acids (K/RWQFP) found inside the cytoplasm just after the transmembrane domain. These 5 amino acids play a major role in the cytosolic conformation of MPL and prevent spontaneous activation of the receptor[72,73]. In addition, the *MPLS505N* mutation, initially described in familial ET, was also found in sporadic MPNs[45,71]. These *MPL* mutations have been found in up to 15% of *JAK2V617F*-negative ET or PMF[74]. As is the case of *JAK2V617F*,

MPL515 mutations are early, stem cell-derived events involving both myeloid and lymphoid progenitors[75,76,77], and *MPLW515L* has been shown to transform cell lines in terms of both cytokine-independent growth and TPO hypersensitivity, activate JAK-STAT/ERK/Akt, and induce PMF-like disease in mice that is characterized by a rapid fatal course, marked thrombocytosis, leukocytosis, hepatosplenomegaly and BM fibrosis[45].

Interestingly, some patients display multiple *MPL* mutations and others harbour a minor *JAK2V617F* clone together with a *MPL* mutation[78]. These observations support the secondary nature of such mutations and underscore the complexity of pathogenetic mechanisms in MPNs. Finally, there are information suggesting that *MPL* mutations favour megakaryocytic/myeloid commitment as opposed to the erythroid skewed proliferation/differentiation seen with *JAK2* mutations [75,76].

1.4.2. Genes involved in intracellular signalling

LNK

LNK plays an important role in hematopoiesis by negatively regulating *JAK2* activation through its SH2 domain, thus inhibiting EPO-R and MPL signaling[79,80]. *LNK*-deficient mice have an increased HSCs pool with enhanced self-renewal properties and increased quiescence[81]. This phenotype probably results from increased TPO/MPL signaling[82] because TPO is required for maintaining HSCs quiescence and the HSCs reservoir[83,84]. In addition, LNK-/-mice develop MPN with thrombocytosis, splenomegaly, and fibrosis[85].

As expected from its negative role in *JAK2* signaling, *LNK* is also capable of attenuating the signaling induced by *MPLW515L* or *JAK2V617F*[86]. Loss of *LNK* accelerates the development of MPN induced by JAK2V617F in murine models[87]. In *JAK2V617F* positive patients, *LNK* expression is increased and modulates the myeloproliferative process[88]. More recently, Oh et al[51] identified 2 mutations in *LNK* exon 2, one in a patient with PMF and the other with ET; both MPNs were *JAK2V617F* negative. 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, inhibitory function is in part

maintained. However, other mutations of LNK have been found in leukemic transformation of MPNs at a greater frequency (13%)[89]. Interestingly, some of these mutations appear to be late events involved in disease progression because they were not found in the chronic phase[89]. 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. This finding may suggest that the phenotype of the MPN induced by LNK mutations may depend on different parameters, including the presence of other mutations.

CBL

The Casitas B-cell lymphoma (CBL) family includes 3 homologs: c-CBL, CBL-b, and CBL-c[90]. 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. They are involved in negative regulation of receptor tyrosinekinase (RTK) by competitive blocking of signaling and they induce RTK proteosomal degradation by mediating ubiquitination in endosomes. However, CBL may have numerous targets other than RTK, including JAK2 and cytokine receptors such as MPL[91]. c-CBL is located at 11q.23.3 and is mutated in a variety of myeloid malignancies[92,93].

The greatest frequency of mutations is found in chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia. *CBL* has been considered a tumor suppressor gene, in fact, 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[94]. *c-CBL* knockout mice develop a mild MPN with an increase in HSCs. *CBL-b* deficient mice lack a hematologic phenotype[95]. The double knockout leads to a rapidly lethal MPN with leukocytosis and excess of monocytes, a phenotype close to myelomonocytic leukemia[95]. In the chronic phase of classic MPNs, *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[49]. A *c-CBL* mutation has been detected in blasts from a *JAK2V617F*-positive MPN, which became *JAK2V617F* negative during transformation[96]. Actually, *c-CBL* seems to be involved more in progression toward myelofibrosis or AML than in the chronic phase of the disorder, but further studies

are required to establish its precise role[97].

1.4.3. Genes involved in epigenetic modifications,

Outside of *JAK2*, mutations in genes encoding the core members of the polycomb repressive complex 2 (PRC2) and in the polycomb-associated protein *ASXL1* represent the most frequently reported mutations that regulate histone modifications directly in MPN patients.

ASXL1

Gelsi-Boyer et al discovered mutations in *ASXL1* through the identification of a deletion in an MDS patient at the *ASXL1* locus[48]. Further sequencing of *ASXL1* by this group and others led to reports of *ASXL1* mutations in 2% to 5% of patients with PV, 5% to 8% of patients with ET, and 7% to 17% of patients with PMF. *ASXL1* is 1 of 3 mammalian homologs of the additional sex combs gene in *Drosophila*. The genes are named for the fact that deletion in *Drosophila* leads to homeotic transformations. *ASXL1* that have been identified in mammalian hematopoietic cells thus far include physical association with the PRC2 complex[98] and physical interaction with the H2AK119 deubiquitinase enzyme BAP1[99]. *ASXL1* appears to be a critical factor for the function of both BAP1 and PRC2 function in myeloid HSCs. Abdel-Wahab and collegues have recently constructed an N-terminal FLAG-tagged wild-type *ASXL1* (FLAG-ASXL1-WT) as well as N-terminal FLAG-tagged truncated mutants of *ASXL1,* and showed that expression of the mutated form of ASXL1 alone induces MDS in a mouse BM transplantation model[100]. Further work to examine the combined phenotype of *ASXL1* loss with *JAK2* activation may be particularly clarifying given the important prognostic importance of *ASXL1* mutations in PMF[101].

EZH2

After mutations in *ASXL1*, *EZH2*, the catalytic member of the PRC2 complex, was found to be mutated in 2010 in patients with classic MPNs, MDSs, and MPN/MDS overlap disorders. Mutations in *EZH2* occur in 5% to 13% of patients with classic MPNs[52]. Moreover, mutations in *EZH2* predominate in MPN patients with PMF or

post-PV/ET MF and are far less frequent in ET and PV, eventually indicating a role for *EZH2* mutations in fibrotic transformation[52].

Mutations in *EZH2* in myeloid malignancy patients seems to be loss-of-function mutations[102]. Mice with heterozygous deletion of *EED*, a noncatalytic core PRC2 member, have been created and display severe myeloproliferation by 7 months of age, suggesting a pathogenetic role of PRC2 loss in myeloid malignancy[103].

After the description of *EZH2* mutations in MPN patients, several groups have performed candidate gene-sequencing studies of additional PRC2 members in patients with myeloid malignancies. In addition to somatic loss-of-function mutations in *EZH2*, rare additional deletions and putative loss-of-function mutations have been identified in the other core PRC2 members in patients with MDS, including *SUZ12* and *EED* mutations (all at less than 5% frequency)[104], and *JARID2*[105].

JARID2

JARID2/Jumonji is the founding member of the largest family of histone demethylases identified so far[106]. *JARID2* contains a JMJC that is important for catalyze the removal of methyl groups from specific lysine residues of histones through an oxidative reaction that requires iron and alpha-ketoglutarate as cofactors. Jumonji proteins demethylate lysines in histone H3 at position 4,9,27, and 36[107,108]. *JARID2* shares the highest homology with JARID1 proteins, which are are capable of recognizing and removing methyl groups from di-(me2) and trimethylated (me3) histone H3K4[109,110,111]. JARID1 contains at least six functional domains, including two plant homeodomain (PHD) zinc-finger domains that are not present in JARID2. Both proteins contain Jumonji N (JmjN), AT-rich interaction domain (ARID), and a zinc finger, in addition to JmJC domain (**Figure 2**).

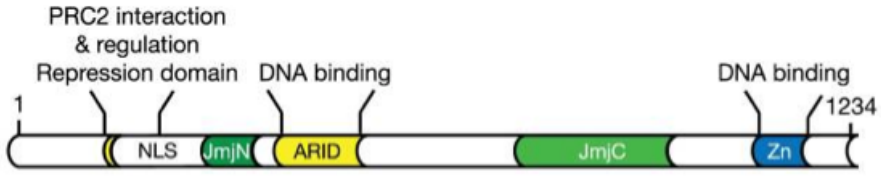

Figure 2. *JARID2* gene structure.

However, *JARID2* contains alterations in the amino acid sequence within the predicted cofactor binding site at the core of the JmJC domain that are predicted to abolish or severely impair the histone demethylase activity of the resulting protein[106], and no demethylating activity for this protein has been demostrated to date. In view of this is surprising that the altered JMJc contained within *JARID2* is highly conserved, a result that suggests that *JARID2* has a function that might be indipendent of histone demethylation. Although there are currently no biochemical data to support an alternative role for *JARID2*, it has been claimed that its ARID and zinc finger domains directly bind DNA[112,113], although with low affinity[114].

JARID2 was identified in 1995 as a regulator of neural development, in gene trap mutagenesis screen in mice[115]. *JARID2* deficiency results in a range of phenotypes, the severity and developmental onset of which are dependent on the genetic background against which chimeras are made and backcrossed. For examples, embryos generated from targeted ES cells[115] displayed defects in neural tube formation, and heart development[116,117,118,119] or hypoplasia of liver (thymus and spleen, together with impaired definitive hematopoiesis[116,120,121]. The role of *JARID2* in the developing embryo is not well understood yet; in mice, the absence of *JARID2* increases proliferation of cardiomyocytes[117], megakaryocytes[122], fibroblasts[123] and cells within the developing brain[119]. In the last years, several studies have provided compelling evidence that *JARID2* is a component of the PRC2 in embryonic stem cells and that *JARID2* and PRC2 bind to a largely overlapping set of target genes. *JARID2* seems to be required for efficient binding of PRC1 and PRC2 to target genes [112,124,125, 126](**Figure 3**).

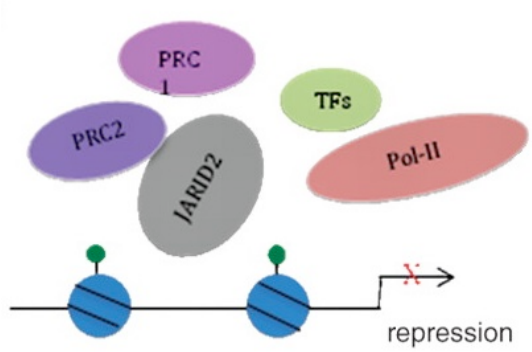

Figure 3. JARID2 interaction with PRCs.

Interestingly, the JARID2 mutant mice embryos die as a result of anemia due to the reduction of common myeloid and erythroid progenitor levels[127].

Notheworthy several studies report that PRC2 complex plays a role in hematopoiesis through epigenetic regulation of proliferative and self renewal capacities of HSCs and this mechanism could involve *JARID2* too[103,128,129].

In 2011, Puda and collegues applied deletion mapping in patients with post-MPN and post-MDS AML and identified frequent lesions of *JARID2*, suggesting that *JARID2* and other PRC2 members represent important tumor suppressors playing a crucial role in the leukemic transformation of chronic myeloid malignancies[105].

1.4.4. Modifiers of DNA cytosine in MPN pathogenesis

TET2

The discovery of *TET2* mutations in patients with myeloid malignancies in 2009 by Delhommeau and co-workers and by Langemeijer et al. was a landmark finding that gave rise to studies on the function of the TET family of proteins in transcriptional regulation and stem cell biology to understand the role of *TET2* mutations in the clinical management of patients with myeloid malignancies[47,130].

Initial studies in patients with MPNs suggested the occurrence of *TET2*-mutant*/JAK2*-mutant and *TET2*-mutant*/JAK2*-wild-type clones, but not *TET2*-wild-type/*JAK2*-mutant clones, suggesting that *TET2* mutations occur as a "pre-JAK2" event[47]. However, subsequent studies have shown the post-*JAK2*V617F acquisition of *TET2* mutations, refuting a paradigm that mutations in *TET2* represent the earliest genetic aberration in MPNs[131]. The current understanding of *TET2* mutations in MPNs patients is that these mutations are loss-of-function mutations that result in decreased 5-hydroxymethylcytosine[132] found in chronic-phase MPNs patients, and may predate the *JAK2V617F* mutation, but at the same time are more frequent at leukemic transformation of MPNs[131].

Perhaps more advanced than the clinical effects of *TET2* mutations in MPN patients has been the in vivo evidence that *TET2* loss results in increased HSCs self-renewal and myeloproliferation.

Currently, 5 different *TET2* knockout mouse models have been created and characterized[133,134,135,136]. In all of these models, *TET2* loss leads to a progressive

enlargement of the HSCs compartment and eventual myeloproliferation in vivo, including splenomegaly, monocytosis, and extramedullary hematopoiesis[134]. In addition, *TET2*-/- mice also display increased stem cell self-renewal and extramedullary hematopoiesis, suggesting that *TET2* haploinsufficiency contributes to hematopoietic transformation in vivo. In addition to *TET2* mutations, *DNMT3A* mutations have also been described in MPNs[53]. Although these mutations are more frequent in AML and post-MPN AML, they also occur in chronic-phase MPN, including in 10% to 15% of PMF patients and 5% to 7% of PV patients.

1.4.5. Mutations affecting modifications of DNA and histones in MPN patients

IDH1/2

Gain-of-function mutations in the genes encoding isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) are now well established genetic events in patients with glioblastoma multiforme and AML. Rare *IDH1/2* mutations were subsequently identified in smaller fraction of patients with MDSs and MPNs[137]. The existing mutational data in MPN patients clearly indicate that *IDH1/2* mutations cluster in patients with blast-phase MPNs and myelofibrosis but are rare in patients with chronic-phase MPNs. From a large study of 1473 MPN patients, only 38 *IDH* mutant patients were identified, with *IDH1/2* mutations being found in 1.9% of PV patients, 0.8% of ET patients, 4.2% of PMF patients, but in 21.6% of patients with blast-phase MPN. Moreover, in blast-phase MPN, the presence of an *IDH* mutation predicted worse survival. In a more recent study of *IDH1/2* mutations in a cohort of 301 patients with PMF, it was shown that *IDH1/2* mutations are associated with decreased overall survival and leukemia-free survival in PMF[138].

It is now established that *IDH1/2* mutations are gain-of-function mutations that result in neomorphic enzymatic activity and the production of 2-hydroxyglutarate by the mutant enzymes[139], and are transforming in classic in vitro experiments[140]. Additional work to clarify the targets of aberrant DNA and histone lysine methylation in malignant HSCs with *IDH1/2* mutations is needed.

1.4.6. CALR

Calreticulin (*CALR*) mutations were recently described in *JAK2* and *MPL* unmutated PMF and ET. Calreticulin is a highly conserved protein, within the endoplasmic reticulum, the protein ensures appropriate folding of newly synthesized glycoproteins and modulates calcium homeostasis[141,142]. Outside the endoplasmic reticulum, calreticulin is also found in intracellular, cell-surface, and extracellular compartments, where it has been implicated in several biologic processes, including proliferation, apoptosis, and immunogenic cell death[143].

Understanding the molecular basis for ET and PMF in patients without *JAK2* mutations has been a major purpose in the field of MPNs. Recently two whole-exome studies by Nangalia[57] and Klampf[56] reported that *CALR*, a previously unrecognized oncogene, is mutated in the vast majority of patients with ET or PMF without *JAK2* or *MPL* mutations[57,144].

The recent exome sequencing study, performed by Nangalia and collegues, identified 1498 mutations in 151 patients, with medians of 6.5, 6.5, and 13.0 mutations per patient in samples of PV, ET, and PMF, respectively. Somatic *CALR* mutations were found in 70 to 84% of samples of MPNs with nonmutated *JAK2*, in 8% of myelodysplasia samples, in occasional samples of other myeloid cancers, and in none of the other solid cancers. A total of 148 *CALR* mutations were identified with 19 distinct variants. Mutations were located in exon 9 and generated a +1 base-pair frameshift, which would produce a mutant protein with a novel C-terminal. Mutant calreticulin was observed in the endoplasmic reticulum but without increased cell-surface or Golgi accumulation. All the *CALR* mutations that were identified in Nangalia and co-workers study are predicted to generate mutant proteins with a novel C-terminal. The extent of the C-terminal alterations vary, but all 19 distinct variants share a loss of a sequence of 27 amino acids with a concomitant gain of a novel peptide consisting of 36 amino acids[57]. These alterations result in the loss of most of the C-terminal acidic domain and the KDEL signal. (The KDEL amino acid sequence [Lys-Asp-Glu-Leu] is present on some resident endoplasmic reticulum proteins and enables retrieval of these proteins from the Golgi apparatus back to the endoplasmic reticulum.) This loss of function increses the possibility of compromised retention or retrieval in the endoplasmic reticulum.

A similar approach was employed by Klampf and collegues[56]. Somatic insertions or deletions in exon 9 of *CALR* were detected in all the six patients who underwent whole-exome sequencing. Resequencing in 1107 samples from patients with MPNs showed that *CALR* mutations were absent in PV. In ET and PMF, *CALR* mutations and *JAK2* and *MPL* mutations were mutually exclusive. Among patients with ET or PMF with nonmutated *JAK*2 or *MPL*, *CALR* mutations were detected in 67% of those with ET and 88% of those with PMF. A total of 36 types of insertions or deletions were identified that all cause a frameshift to the same alternative reading frame and, also in this case, generate a novel C-terminal peptide in the mutant calreticulin. Overexpression of the most frequent *CALR* deletion caused cytokine-independent growth in vitro inducing the activation of signal transducer and activator of transcription 5 (STAT5) by means of a still unknown mechanism.
From a practical point of view *CALR* molecular characterization may become a key component of the clinical management of ET and PMF. Detection of *CALR* mutations in peripheral blood could potentially be used as a diagnostic tool in the same way that tests for *JAK2* mutations have simplified and improved the accuracy of diagnosis of patients with MPNs worldwide[145,146]. Further research is needed to explore these potential uses.

1.5. Diagnosis

Diagnosis of PV,ET or PMF is currently according to the WHO criteria[17]. As reported in **Table 3,** it is clear that these guidelines do not claim that a single histological parameter defines a subgroup, but that the different subtypes of MPN are characterized by specific morphological BM pattens[147]. These patterns are composed of distinctive features and should always be reviewed in close relation to clinical, hematological and molecular-genetic findings to accomplish a consensus based working diagnosis[148].

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

WHO indicates World Health Organization; MPN, myeloproliferative neoplasm; CML, *BCR-ABL1* chronic myelogenous leukemia; PV, polycythemia vera; PMF, primary myelofibrosis; MDS, myelodysplastic syndrome; BM, bone marrow biopsy specimen; Epo, erythropoietin; EEC, endogenous erythroid colonies; LDH, lactate dehydrogenase.
*Small to large megakaryocytes with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei and dense clustering.

Table 3. WHO diagnostic criteria for PV, ET and PMF.

1.6. Clinical manifestations and prognosis

In spite of phenotypic similarity and the same origin from mutated HSC, PV, ET and PMF are three distinct disease entities with regard to clinical manifestations, natural history and outcome in terms of life expectancy.

PV is characterized by initial erythrocytosis, which is in general accompanied by leukocytosis of normal mature granulocytes (or leukocythemia), by thrombocytosis (or thrombocythemia), splenomegaly or hepatomegaly, cyanosis, loss of weight and plethora.

Red cell mass increase in PV may result in blood hyperviscosity. Headaches are frequent, but blurry vision, altered hearing, mucous membrane bleeding, shortness of breath, and malaise are also observed.

Thrombosis, hemorrhage, evolution to post-polycythemic or post-thrombocythemic MF, and AML transformation represent the most clinically relevant issues in the course of classic MPNs[149,150]. Most thrombotic events occur at or in the two years before diagnosis[151].

Arterial thrombosis accounts for 60% to 70% of all cardiovascular events and includes acute myocardial infarction, ischemic stroke, and peripheral arterial occlusion. Recent data indicate that at least 40% of patients with splanchnic vein

thromboses (SVT) not attributable to other causes actually harbor the JAK2V617F mutation; therefore, *JAK2V617F* genotyping represents a first-line test for these conditions[152].

In PV, events involving the venous system, are represented by lower extremity deep venous thrombosis, pulmonary embolism, and SVT (which includes portal vein thrombosis, mesenteric thrombosis, and thrombosis of the hepatic veins causing Budd-Chiari syndrome).

Many patients with ET are asymptomatic when an excess in platelets is discovered by a routine blood count[153,154]. Extramedullary hematopoiesis can occur to a limited degree in the liver or spleen. Splenomegaly is seen in up to 20% to 50% of patients, although the enlargement is mild to moderate and not usually progressive. Hepatomegaly may be seen in 15% to 20% of patients, but significant extramedullary hematopoiesis in the liver is unusual.

Anyway also in ET the predominant clinical feature of symptomatic patients is thrombosis. The involvement of the microcirculatory system 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)[155], 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[156].

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[157]. Conversely, survival of ET patients is reduced by about 2-fold compared with the general population starting from the first decade after diagnosis[158]. Major causes of shortened survival in PV or ET are represented by thrombotic events and transformation to myelofibrosis or AML, which account for 41% and 13% of total deaths among 1,638 PV patients that were included in the observational arm of the ECLAP study[149]. An age of greater than 60 years and leukocytosis were incorporated in a predictive model for survival in ET that discriminated groups of patients with median survivals of 25, 17, and 10 years, respectively[158]. 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 seems appropriate to use this clinical end-point as the criterion for stratifying patients according to their risk [159]. Older age (greater than 60 years) and a previous history of thrombosis are standard risk factors for thrombosis in both PV and ET[160]. In the presence of either of these, a patient is at high-risk, whereas when neither of these is present, the disease is low-risk.

"low-risk" ET patients could be separated into two categories with a respective overall prevalence of thrombosis of 55% and 20% depending on the presence, or not, of an absolute leukocyte count greater than 8.7 10^9/L[161]. Finally, there is also evidence that *JAK2V617F* mutated status in ET[162,163,164], and a high V617F allelic burden in both ET[165], and PV are associated with increased risk of thrombosis. Therefore, both leukocytosis and *JAK2V617F* mutated status represent a powerful, disease-associated, risk factors.

The recently described *CALR* mutations will surely become an important diagnostic marker for MPNs and several groups are already studying the impact of this mutation on risk stratification of patients.

2. Primary Myelofibrosis

Primary Myelofibrosis is characterized by HSC derived clonal myeloproliferation and is associated with reactive BM fibrosis, osteosclerosis, angiogenesis, extramedullary hematopoiesis, and abnormal cytokine expression[166,167].

2.1. Epidemiology

Among classic MPNs, PMF is the least frequent. The yearly calculated incidence for PMF is 0,4 per 100,000 population[168]. The median age at diagnosis is 67 years with no difference in risk between males and females[169]. Approximately 5 and 17% percent of the patients are diagnosed before the age of 40 and 50 years, respectively[170]. The condition is rare in childhood[171]; a familial occurence has been reported in several kindreds forms. The median survival for PMF is 2–5 years [172].

2.2. Pathogenesis

2.2.1.Bone marrow fibrosis

The cause of the excessive BM fibrosis observed in PMF it is still unclear. Platelets, MKs, and monocytes are thought to secrete several cytokines, such as transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), epidermal growth factor, and platelet-derived growth factor (PDGF), which may result in fibroblast proliferation and dysregulation of extracellular matrix formation[173,174]. Patients with PMF have abnormal and elevated neutrophil or eosinophil emperipolesis through MKs. Once in the MK, the neutrophil releases proteolytic enzymes, resulting in the death of both cells[175]. This causes the release of transforming growth factor-β_1 and platelet-derived growth factor from the alpha granules of MKs. PDGF stimulates the proliferation of fibroblasts, mesenchymal, and smooth muscle cells[176] which may interact with TPO (**Figure 4**).

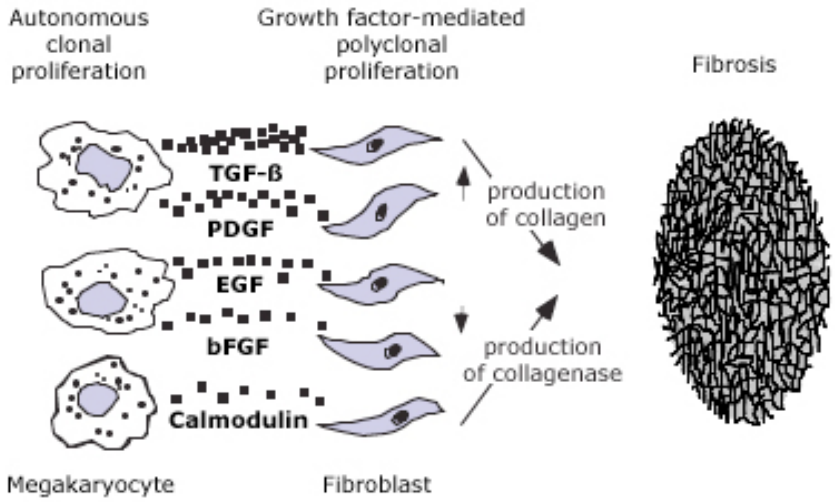

Figure 4. Factors contributing to the pathogenesis of bone marrow fibrosis. Abbreviations: TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

This fibrous reaction with abnormal accumulation of extracellular matrix components is also dependent on matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP)[17]. It has been suggested, based upon a murine model and on BM samples from patients with PMF, that there is a significant degree of entry of hematopoietic cells into MK cytoplasm, a phenomenon called emperipolesis[178]. This is probably brought about by increased expression and abnormal localization of P-selectin by the MKs, leading to engulfment, activation, and damage to the cells, mainly neutrophils and eosinophils, passing through the MK cytoplasm. The result is release of lytic granules from the engulfed cells, progressive destruction of MKs with degradation and lysis of their alpha granules, and release of growth factors, resulting in the marked fibroblast activation and infiltration characteristic of PMF.

PREFIBROTIC STAGE. In the prefibrotic stage, the abnormal PB findings include variable mild anemia, borderline to slight leukocytosis, and frequently elevated platelet count. Leukoerythroblastosis, dacryocytes, atypical platelets, and circulating MKs may be present in low numbers in the blood smear. Basophilia and eosinophilia are found in 10% to 30% of myelofibrosis cases. In the osteomedullary biopsy, the BM in the prefibrotic stage of myelofibrosis is usually hypercellular. There is a prominent, left-shifted, granulocytic proliferation. Reticulin

fibrosis is absent or minimal. Well-marginated focal lymphoid aggregates are seen in one third of patients, and less frequently in patients with other MPNs[179].

In most of cases, as described by Barosi and co-workers, resulted that the frequency of CD34+ cells in PB, CXCR4 expression on CD34+ cells, serum cholesterol and LDH levels were significantly different in pre-myelofibrosis as compared with PMF-fibrotic type[180]. Most of these patients had increase in BM myeloproliferation with MK hyperplasia and dysplasia as the sole biological deviation from normal, and many of them had thrombocytosis as the sole hematologic alteration. This addresses to the idea that the initial imbalance of PMF could be restricted to megakaryopoiesis, and that dysmegakaryopoiesis may occur without any BM fibrosis or hematopoietic progenitor cells mobilization, previously considered specific biological markers of PMF.

2.2.2. Osteosclerosis

Bone remodeling results from the equilibrium of two cell types activity: the osteoblasts (the bone-forming cells) and the osteoclasts (the bone-resorbing cells)[181,182]. Osteoblasts derive from mesenchymal progenitors via different regulatory processes including the action of several growth factors such as TGF-β, which could exert stimulatory or inhibitory effects[182,183]. Osteoclasts instead are of hematopoietic origin. They derive from monocytic cells and require M-CSF for proliferation and RANK-L, via its binding to the receptor RANK expressed at the surface of osteoclast progenitors, for differentiation and maturation[184]. Osteoprotegerin (OPG) is a decoy secreted receptor that prevents RANK-L binding to its receptor RANK, thus inhibiting osteoclastogenesis[185]. It has been hypothesized that an increased production of OPG by stromal and endothelial cells contributed to the unbalanced osteoblast production leading to the osteosclerosis frequently associated with MF and to vascular complications[186]. The abnormal trafficking of CD34+ cells and endothelial precursors that features PMF is likely resulting from modification of their adherence to the BM stroma allowing them to escape from this niche into the circulation with homing to the spleen and liver. The pathological process would result from alterations in the cross talk between hematopoietic and stromal cells (**Figure 5**).

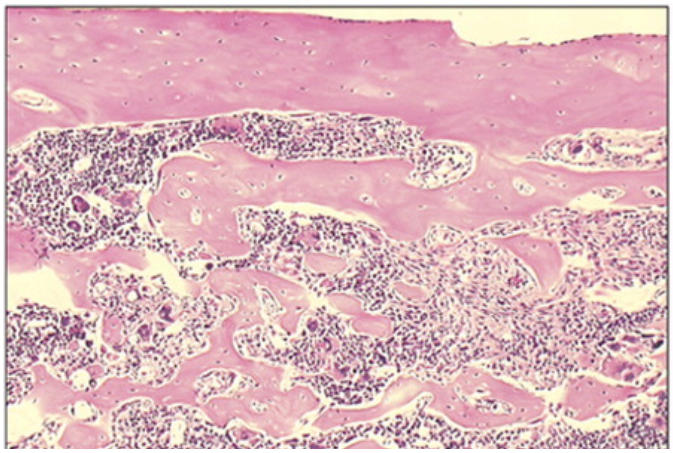

Figure 5. Severe osteosclerosis in bone marrow of PMF patient.

In this process, stromal cells are conditioned by growth factor produced by malignant hematopoietic cells and reciprocally, by acquiring new properties, stromal cells create a pathological microenvironment that takes part in the development and maintenance of the clone, leading to an imbalance that compromises normal hematopoiesis[187].

The critical role of the trio OPG/RANK/RANK-L in bone homeostasis has been demonstrated in genetically manipulated mice. Indeed, OPG overexpression in transgenic mice and RANK or RANK-L knockout mice led to impaired osteoclastogenesis and severe osteosclerosis, whereas administration of soluble RANK-L or OPG knockout mice resulted in enhanced osteoclastogenesis and osteoporosis[188]. Overall, these data strongly suggest a role for the stromal OPG produced by the host microenvironment in the promotion of osteosclerosis. Furthermore, the mechanism of OPG upregulation does not seem to be induced by TGF-β1 overexpression in this mouse model[186]. Indeed, retrospective analysis of the combined TGF-β1 mice and the TPO overexpression mouse model showed no correlation between TGF-β1 levels and OPG levels because, although a baseline level of TGF-β1 was observed in wild-type hosts engrafted with TGF-β1 null donor cells, the same range of upregulation of OPG plasma level was observed compared to the wild-type hosts engrafted with wild-type donor cells, which displayed elevated TGF-β1 plasma levels. It was remarkable that the wild-type hosts engrafted with TGF-β1 null donor cells developed a delayed osteosclerosis compared to the control group, suggesting that another mechanism besides OPG-mediated inhibition of osteoclastogenesis is required, possibly TGF-

β1 mediated stimulation of osteoblast proliferation. Indeed, support for the increased proliferation of osteoblasts and stimulation of bone formation in GATA-1 low mice, possibly mediated by interactions between osteoblasts and mutant MKs, has been provided[189].

2.2.3.Neoangiogenesis

Neoangiogenesis, as a result of the production and release of angiogenic factors, is an important feature of PMF (**Figure 6**); however, neoangiogenesis is also observed in a broad spectrum of tumors and therefore is not specific to myelofibrosis [190,191].

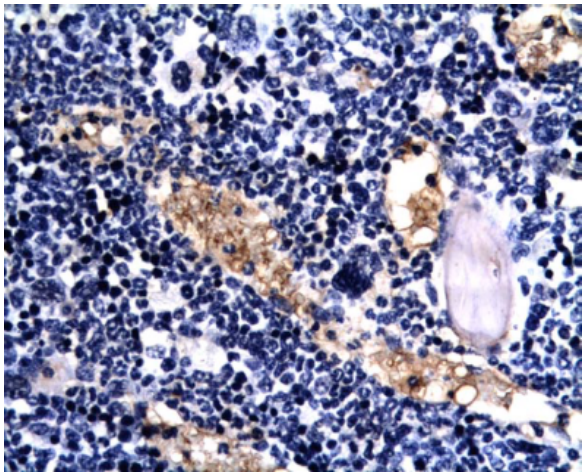

Figure 6. Vessels in bone marrow of PMF patient.

The increase in BM vasculature in PMF has been shown to correlate with increased spleen size and to be an independent risk factor for overall survival[192]. A number of angiogenic growth factors, including bFGF and vascular endothelial growth factor, are thought to be the causative factors for this neoangiogenesis[174]. One study has suggested that the endothelial proliferation and growth of capillary blood vessels in the BM may be the result of upregulations of microvascular TGF-β1 receptor and bFGF overexpression[193]. It appears that neoangiogenesis is caused by angiogenic cytokines, which are likely produced by the abnormal MKs in PMF marrow[193].

2.2.4. Abnormal stem cell trafficking and extramedullary hematopoiesis

PMF is also characterized by increased number of circulating CD34+ HSCs, which is likely caused by abnormal stem cell trafficking. Studies have shown that CD34+ cell mobilization is a caused by proteolytic environment[194].

The elevated levels of neutrophil elastase and MMPs in the plasma of patients with PMF suggest that these proteolytic activities are a product of a malignant clone. However, the elevated plasma levels of neutrophil elastase and MMPs are nonspecific, as these also can be found elevated in PV. Furthermore, vascular cellular adhesion molecule is cleaved by the neutrophil proteases in the BM following HSC or hematopoietic progenitor cell mobilization and is therefore elevated following such mobilization[194].

Xu et al, in their study, demonstrated high levels of soluble vascular cellular adhesion molecule compared with the concentration in the plasma of patients with PV or control patients. These results suggest that the elevation of functional neutrophil elastase activity and the associated cleavage of vascular cellular adhesion molecule are characteristic of patients with PMF and may play a role in the abnormal CD34+ cells mobilization that characterizes PMF as compared with PV[194].

Nevertheless the origin of extramedullary hematopoiesis in PMF remains unclear. The activation of stem cells dormant in the spleen and liver since fetal life, has been hypothesized to be the cause of extramedullary hematopoiesis. According to this hypothesis, PMF would recapitulate ontogenesis by a reversion to fetal distribution of hematopoietic activity, resulting in the expansion of hematopoiesis within the central marrow cavity and the extension of this hematopoietic tissue to the marrow cavities of extramedullary sites. However, there is a fundamental difference in hematopoiesis in the spleens of adult patients with PMF from what occurs in fetal life. Although there is extensive hematopoiesis in the spleens of patients with PMF, the fetal spleen contributes only marginally to hematopoiesis and contains numerous late erythroid precursors but few early erythroid or granulocytic cells[195].

2.2.5. Abnormal megakaryocytopoiesis

Assessment of megakaryocytic histotopography, such as their arrangement within th BM space, and detection of specific nuclear abnormalities are the keys to the PMF diagnosis[196]. Typically, an extensive clustering of MKs with loose to dense groupings with abnormal localization towards the endosteal borders is observed but there are also striking abnormalities in MKs morphology and maturation[196,197,198,199]. MKs present high degree of cellular pleomorphism with variations in size that range from small to giant forms. Abnormal nuclear folding and an aberration of the nuclear cytoplasmatic ratio created by large, bulbous and hyperchromatic cloud like nuclei are frequent[198]. Overall MKs in PMF are characterized by a higher degree of cytological atypia than other MPNs[200] (**Figure 7**).

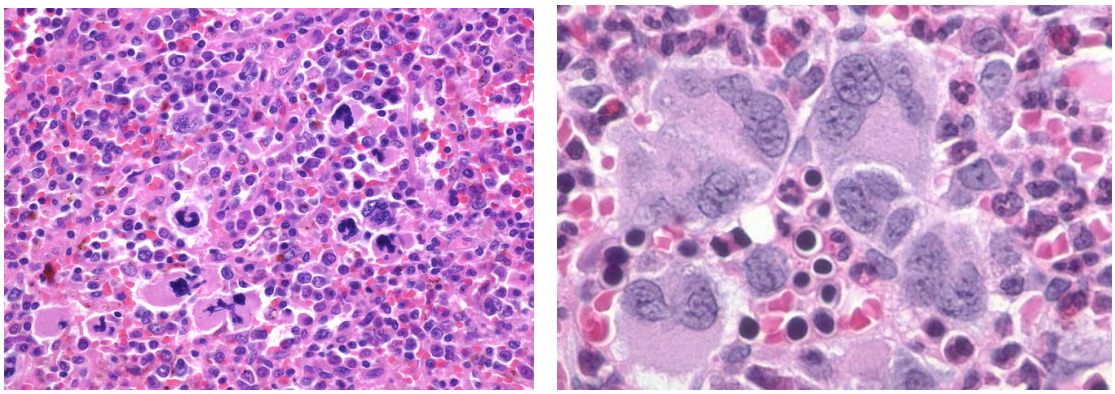

Figure 7. PMF megakaryocytes morphology at different magnifications.

In prefibrotic stage MKs proliferation is characterized by abnormal growth and paratrabecular clustering[201].

MKs are immature and exhibit marked pleomorphism ranging from giant MKs to atypical micromegakaryocytes. Defective nuclear-cytoplasmic differentiation, dense or coarse chromatin, with bulky, clumsy, and irregular-looking (‘‘cloud-like’’ or ‘‘balloon-shaped’’) lobulations of the megakaryocytic nuclei are characteristic findings[202].

The megakaryopoiesis is the most prominent hallmark and key feature to diagnose prefibrotic myelofibrosis. Thus MK dysplasia is one of the most important feature discriminating prefibrotic/early stage PMF from ET[198].

2.3. Clinical manifestation

Approximately 30% of patients with PMF are asymptomatic at presentation, and the diagnosis is suggested by abnormal blood findings or incidentally discovered splenomegaly[203].

The most common presenting complaint in primary myelofibrosis is that of severe fatigue, occuring in 50 to 70% of patients[169]. Symptoms due to an enlarged spleen have been described in 25 to 50% of patients, while a smaller number note weight loss and 5 to 20% present other signs of a hypermetabolic state such as low-grade fever, bone pain and night sweats. Enlargement of the spleen and liver are due to the marked extramedullary hematopoiesis associated with PMF. Pulmonary hypertension has been detected in patients with PMF; while often is asymptomatic, it has been associated with reduced overall survival[204,205,206]. The incidence of arterial and venous thrombotic events in PMF is 2 per 100 patients per year[207]. In a retrospective analysis of 205 patients with PMF, 13.2% had experienced a throbotic event at or prior to their diagnosis, and 10.7% developed post-diagnosis thrombosis at a median follow up of 31 months[208].

Splenomegaly, often marked, is the hallmark of PMF[173]. The spleen may be so large that its lower border is below the pelvic brim and its right border extends across the midline (**Figure 8**).

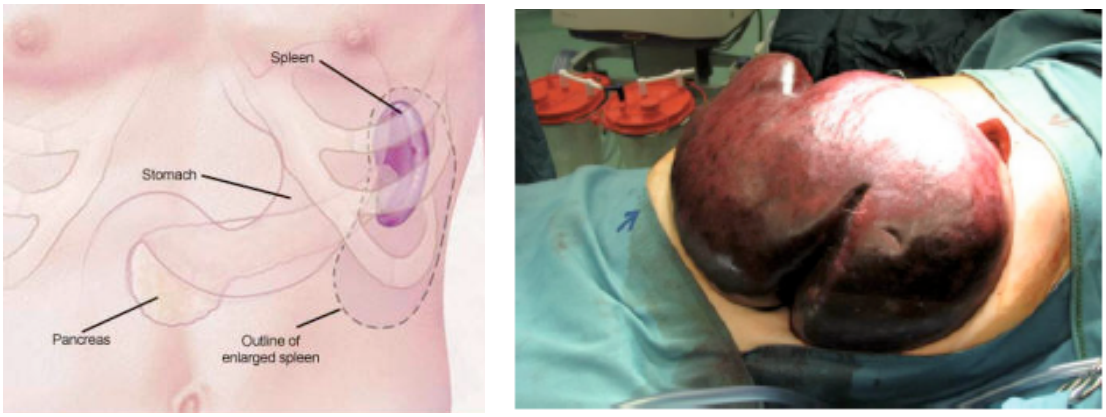

Figure 8. Left: Normal versus enlarged spleen anatomycal site. Right: splenectomy on a PMF patient.

Symptoms due to splenic disease often figure prominently in PMF. Patients may perceive a dragging sensation in the left upper abdomen, and the spleen may compress the patient's stomach leading to early satiety. Severe left upper

quadrant pain, with or without left shoulder, may result from multiple and/or recurrent episodes of splenic infarction or perisplenitis.

Palpable hepatomegaly is present in 40 to 70% percents of patients. Portal hypertension may develop as a result of increased splanchnic flow due to splenomegaly and/or intrahepatic obstruction associated with extramedullary hematopoiesis[209].

Portal vein throbosis is a recognized complication of PMF and other MPNs[209] and may precede the clinical onset of the disease[210].

Foci of extramedullary hematopoiesis may occur in almost any organ[211]. Organ involvement may present as splenomegaly, hepatomegaly, lymphadenopathy, pleural, pericardial or abdominal effusion, or involvement of the gastrointestinal or genitourinary tracts or lung[212,213,214].

2.4. Diagnosis

Diagnosis of PMF is based on 2008 WHO revised diagnostic criteria and enlist a composite assessment of clinical and laboratoy findings[215]. PB leukoerythroblastosis is a typical but not invariable feauture of PMF; prefibrotic PMF might not display overt leukoerythroblastosis[148]. BM fibrosis in PMF is usually associated with *JAK2V617F*, trisomy 9, or deletion[39]. The presence of these genetic markers, strongly support a diagnosis of PMF, in the presence of a myeloid neoplasms associated with BM fibrosis. PMF should be distinguished from other closely related neoplasms. Prefibrotic PMF can mimic ET in presentation and careful morphologic examination is necessary for distinguishing the two. Anemia, palpable splenomegaly, and raised LDH levels are additional diagnostic criteria.

Thiele and co-workers disrupted the dogma of BM fibrosis being an intrinsic and necessary stigma of PMF, and they first proposed a new category of patients characterized by absence of relevant reticulin fibrosis in BM with dual megakaryocytic and granulocytic myeloproliferation associated with characteristic MK dysplasia[201,202]. This variant, called prefibrotic myelofibrosis (pre-MF), has been included as a prodromic phase of PMF into the WHO classification of MPNs since 2001[217], and the criteria for the diagnosis were further outlined in 2008 [215] .

Now, pre-MF has joined the ranks of the diagnostic categories used in the practice of most of the hematopathologists worldwide, even though there is still a number of unresolved issues concerning its diagnostic reproducibility[219,220], and molecular and biological identity[221].

2.5. Prognosis

Robust prognostic modeling in PMF started with the development of the International Prognostic Scoring System (IPSS) in 2009[172]. The IPSS for PMF is applicable to patients being evaluated at time of initial diagnosis and uses five indipendent predictors of inferior survival: age > 65 years, hemoglobin <10g/dL, leukocyte count >253 10^9/L, circulating blasts 1%, and presence of constitutional symptoms. The presence of 0,1,2, and 3 adverse factors defines low, intermediate-1, intermediate-2, and high risk disease. The corresponding median survivals were 11.3, 7.9, 4, and 2.3 years.

The International Working Group for MPNs Research and Treatment (IWG-MRT) subsequently developed a dynamic prognostic model (Dynamic International Prognostic Scoring System, DIPSS) that utilizes the same prognostic variables used in IPSS but can be applied at any time during the disease course[223].

More recently, IPSS- and DIPSS-independent risk factors for survival in PMF were identified and included unfavorable karyotype (i.e. complex karyotype or abnormalities such as i(17q) inv(3)) [224,225] and platelet count <100 x 10^9/L)[226]. Accordingly, DIPSS was modified into DIPSS-plus by incorporating these three additional risk factors[227]. The four DIPSS-plus risk categories based on the 8 aformentioned risk factors are low (no risk factors), intermediate-1 (1 risk factor), intermediate-2 (two or 3 risk factors), and high (four or more risk factors) with respective median survivals of 15.4, 6.5, 2.9, and 1.3 years[227].

Data suggest inferior survival in PMF associated with nullizygosity for JAK2 46/1 haplotype[228], low JAK2V617F allele burden[229,230], presence of *IDH*[137], and *EZH2* mutations. The recently identified CALR mutations will surely become an important diagnostic marker for myeloproliferative neoplasms. Genotyping for *CALR* mutations represents a novel useful tool for establishing a clonal myeloproliferative

disorder in *JAK2* and *MPL* wild-type patients that may have prognostic and therapeutic relevance.

2.6. Therapy

2.6.1.Conventional therapy

Low-risk[227] patients with PMF can be observed without any therapeutic intervention. High or intermediate-2 risk patients should be considered for investigational drug therapy or allo-stem-cell transplantation (allo-SCT). Management of intermediate-1 risk patients should be individualized and might include observation, conventional drug therapy, or participation in investigational drug trials.

Anemia and symptomatic splenomegaly are the main indications for treatment in PMF. Anemia is treated with androgens, prednisone, danazol thalidomide, or lenalidomide[231]. Response rates to prednisone, androgen preparations, or danazol are nearly 20% and response durations average about 1 to 2 years.

Thalidomide and lenalidomide anemia response rate is approximately 20% with single-agent thalidomide therapy[232], whereas the addition of prednisone to low-dose thalidomide appeared to attenuate thalidomide-associated adverse effects and increase the response rate[233]. However, the usual adverse effect of peripheral neuropathy remains unaltered. Single-agent lenalidomide therapy was associated with a 22% anemia response rate, but thrombocytopenia or neutropenia was seen in one third of the patients[234]. Severe myelosuppression was also the main issue with combined lenalidomide and prednisone therapy, and the anemia response rates in two recent studies were 19%[235] and 30%[236]. Both thalidomide and lenalidomide improve thrombocytopenia and splenomegaly in approximately 10% of patients[232].

The drug of choice for symptomatic splenomegaly in PMF is hydroxyurea. Hydroxyurea refractory patients are often managed by splenectomy since the value of other conventional drugs in this regard is limited[237]. Other indications for splenectomy include symptomatic portal hypertension and frequent red blood cell transfusions. The perioperative mortality of splenectomy in PMF is between 5% and 10%. Postsplenectomy complications occur in approximately 50% of the

patients and include bleeding, thrombosis, hepatomegaly, extreme thrombocytosis, leukocytosis, and an increase in circulating blasts[237].

Transplant

The choice of allo-SCT as a treatment modality, should be carefully considered for the risks involved. In one of the largest studies of allo-SCT in PMF[238], 5-year disease-free survival (DFS) and treatment-related mortality (TRM) were 33% and 35% for matched related, and 27% and 50% for unrelated transplants, respectively. Of note, outcome did not appear to be favorably affected by reduced intensity conditioning (RIC). In another RIC transplant study, 5-year DFS was estimated at 51%; chronic graft-versus-host disease (cGVHD) occurred in 49% of the patients and relapse (29%) was predicted by high-risk disease and prior splenectomy[239]. In the earlier study [238], the respective cGVHD and relapse rates for matched related transplants were 40% and 32% and history of splenectomy did not affect outcome.

2.6.2. Investigational drug therapy

Several experimental drugs are currently being evaluated in PMF, post-PV/ET MF, and other related MPNs[240]. So far, pomalidomide, JAK2 inhibitor ATP mimetics, and mammalian target of rapamycin (mTOR) inhibitors have shown the most promising results[241,242].

Pomalidomide

Pomalidomide is a second generation immunomodulatory drug and in a Phase-2 randomized study, 25% of patients with anemia responded to this drug used alone or in combination with prednisone[241]. In a subsequent Phase-2 study of single agent pomalidomide[243], anemia response was documented only in the presence of *JAK2*V617F (24% vs. 0%) and predicted by the presence of pomalidomide-induced basophilia (38% vs. 6%) or absence of marked splenomegaly (38% vs. 11%). Platelet response was seen in 58% of patients but the drug had limited activity in reducing spleen size[243]. Drug-associated neuropathy or myelosuppression was infrequent but possible.

JAK2 inhibitor ATP mimetics

JAK2 inhibitor ATP mimetics that are currently in clinical trials include ruxolitinib (INCB018424), SAR302503 (TG101348), CYT387, lestaurtinib (CEP-701), SB1518, AZD1480, BMS911543, LY2784544, and XL019. Results of these studies so far suggest substantial differences among these drugs in their toxicity and efficacy profiles, some of which might be linked to their variable in vitro activity against other JAK and non-JAK kinase targets.

Ruxolitinib is a JAK1/JAK2 inhibitor. The drug was evaluated in 153 patients with PMF or post-PV/ET MF, in a Phase-1/2 study[244]. Dose limiting toxicity (DLT) was thrombocytopenia and the maximum tolerated dose (MTD) was either 25 mg twice-daily or 100 mg once-daily. Adverse events included thrombocytopenia, anemia, and a "cytokine rebound reaction" upon drug discontinuation, characterized by acute relapse of symptoms and splenomegaly[245]. Non-hematologic adverse events were infrequent. Grade 3/4 thrombocytopenia or anemia (in transfusion-independent patients at baseline) respectively occurred in 39% and 43% of patients receiving the drug at 25 or 10 mg twice daily. Among all evaluable patients, 44% experienced ≥50% decrease in palpable spleen size. Improvement in constitutional symptoms (fatigue, pruritus, abdominal discomfort, early satiety, night sweats, and exercise tolerance) and weight gain were seen in the majority of patients. Four (14%) of 28 transfusion-dependent patients became transfusion-independent. The drug's effect on *JAK2V617F* allele burden or BM pathology was negligible but a major reduction in proinflammatory cytokines (e.g., IL-1RA, IL-6, TNF-a, MIP-1b) was documented and coincided with improvement in constitutional symptoms.

Two randomized studies comparing ruxolitinib with either placebo or best available therapy have now been published[246]. In the COMFORT-1 trial that compared the drug with placebo (*n*=309), the spleen response rate was approximately 42% for ruxolitinib versus <1% for placebo. In addition, about 46% of patients experienced substantial improvement in their constitutional symptoms. However, the benefit of the drug was antagonized by ruxolitinib-associated anemia (31% vs. 13.9%) and thrombocytopenia (34.2% vs. 9.3%). In the COMFORT-2 trial that compared the drug with "best available therapy" (*n*=219), the spleen response was 28.5% with

ruxolitinib vs. 0% otherwise but the drug was detrimental in terms of thrombocytopenia (44.5% vs. 9.6%), anemia (40.4% vs. 12.3%), and diarrhea (24.0% vs. 11.0%). The long-term outcome of ruxolitinib therapy in myelofibrosis was recently reported and disclosed a very high treatment discontinuation rate (92% after a median time of 9.2 months) and the occurrence of severe withdrawal symptoms during ruxolitinib treatment discontinuation ("ruxolitinib withdrawal syndrome") characterized by acute relapse of disease symptoms, accelerated splenomegaly, worsening of cytopenias, and occasional hemodynamic decompensation, including a septic shock-like syndrome[245].

TG101348, a selective JAK2 inhibitor, was evaluated in 59 patients with PMF or post-PV/ET MF, in a Phase-1/2 study[247]. The DLT was a reversible and asymptomatic increase in serum amylase/lipase and the MTD was 680 mg/day. Grade 3 or 4 adverse events were all reversible and dose-dependent and included nausea (3%), vomiting (3%), diarrhea (10%), asymptomatic mild increases in serum lipase (27%), transaminases (27%) or creatinine (24%), thrombocytopenia (24%), and anemia (35%). By 6 or 12 months of treatment, 39% and 47% of patients, respectively, experienced a ≥50% decrease in palpable spleen size. In addition, the majority of patients with early satiety, fatigue, night sweats, cough, or pruritus reported a durable resolution of their symptoms. Almost all patients with thrombocytosis and the majority with leukocytosis had normalization of their counts. Among 23 patients with a baseline *JAK2V617F* allele burden of >20%, 9 (39%) had ≥50% decrease in allele burden. Effect on bone marrow pathology was limited. In general, response was not affected by the presence of *JAK2V617F*.

Ruxolitinib is the only JAK inhibitor to complete phase III trials, and has been approved by the U.S. Food and Drug Administration for the treatment of intermediate or high-risk PMF, and more recently by Health Canada and the European Commission, for the treatment of myelofibrosis-related splenomegaly or symptoms on the basis of results from the phase III COMFORT-I and -II studies, in which ruxolitinib therapy resulted in pronounced reductions in splenomegaly, as well as improvements in disease-related symptoms compared with both placebo

and best available therapy[248,249]. A follow-up in both the phase I/II study and the COMFORT I study reported an overall survival advantage in the ruxolitinib arm[246].

mTOR inhibitors. JAK-STAT activation leads to Akt/mTOR activation as well and it is therefore reasonable to evaluate the therapeutic activity of Akt and mTOR inhibitors. In a Phase 1/2 study involving the mTOR inhibitor everolimus including 39 MF patients[250], the commonest toxicity was Grades 1–2 stomatitis. A >50% reduction in splenomegaly occurred in 20% of the patients evaluated and the constitutional symptoms response was 69%; 80% experienced complete resolution of pruritus. Drug effect on cytosis or anemia was modest and on *JAK2V617F* burden negligible.

3. Long non-coding RNAs

Tiling resolution genomic microarrays[251,252,253], whole genome and transcriptome sequencing technologies[254] have shown that thousands of the transcription products of the human genome are non-coding RNAs (ncRNAs). Among these ncRNAs, little is known about the long non-coding RNAs (lncRNAs) that were originally discovered through the sequencing of full-length mouse cDNA libraries[255.]

3.1. Defining long non-coding RNAs

LncRNAs are defined as endogenous RNAs consisting of more than 200 nucleotides and lacking an open reading frame of significant lenght[256]. LncRNAs are generally transcribed by RNA polymerase II, and can account for nearly 60% of all non-ribosomal and non-mitochondrial RNAs in human cells[257]. These lncRNAs have a 5' terminal methylguanosine cap and are usually spliced and polyadenylated. Alternate pathways also contribute to the generation of known lncRNAs, which include a poorly characterized contingent of non-polyadenylated lncRNAs likely expressed from RNA polymerease III promoters[256] and lncRNAs that are excised during splicing and small nucleolar RNA production[258].

It is difficult to calculate an exact number of lncRNAs, with current lncRNA catalogs ranging between 5000 and 15000 transcripts[259,260]. Moreover there is a little overlap between these differnet long non-coding RNAs catalogs, and this may merely represent a lower bound, with many lncRNAs yet to be annotated[261]. Whereas the number of known human protein-coding genes has remained stable over recent years, the number of known lncRNAs continues to increase, and lncRNAs may eventually rival protein-coding genes in number and diversity[261].

LncRNAs can be intergenic, intronic, antisense, and can overlap with protein-coding genes or other ncRNAs[262]. Alternative splicing generates many lncRNAs isoforms and can merge gene structures by incorporating both coding and non-coding exons in a single transcripts. In addition, lncRNAs expression is finely regulated in a tissue or developmental-stage manner[263].

3.2. Long non-coding RNAs functions

Given their unexpected abundance, lncRNAs were initially thought to be spurious transcriptional noise resulting from low RNA polymerase fidelity[264].

For the vast majority of recently discovered lncRNAs, the cellular function need to be elucidated.

Few lncRNAs have been well-characterized, but several of these have been reported to affect gene expression through a variety of mechanisms: 1) repression by chromatin modification, in which chromatin remodeling complexes are recruited to specific gene loci, 2) by enhancing or inhibiting the transcription of target genes[265,266] 3) post-transcriptional processing, including RNA splicing, editing, transport, translation and degradation[267,268,269].

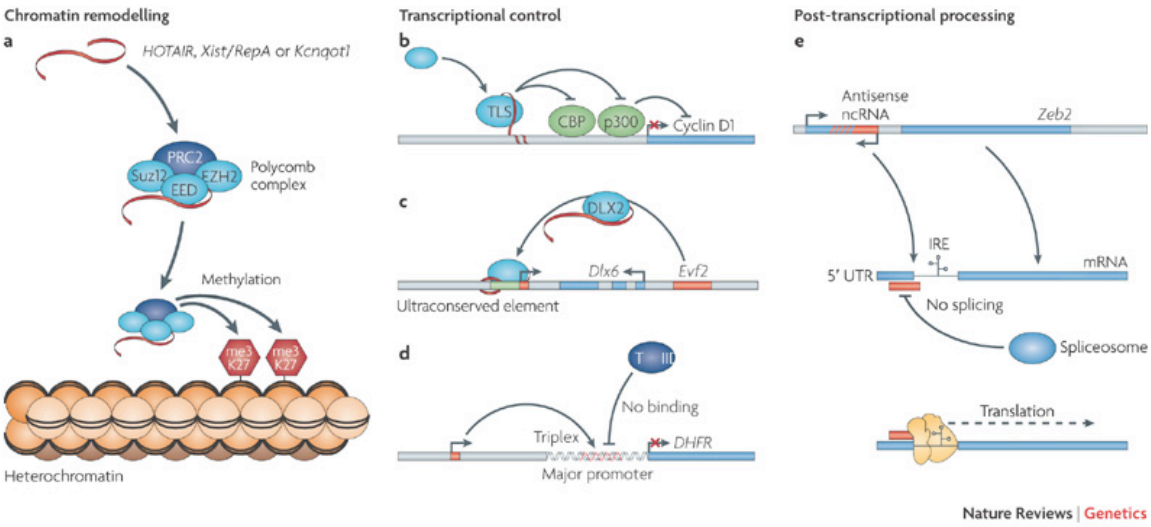

Figure 9. LncRNAs known mechanisms of action.

Deregulation of these lncRNAs can also play key roles in malignant transformation and cancer cell behavior. In fact, by functioning as oncogenes or tumor suppressor genes, lncRNAs can activate cellular pathways that lead to either tumorigenesis or tumor suppression[270,271].

3.3. Long non-coding RNAs in epigenetics

Although the several functions of lncRNAs are only beginning to be uncovered, their potential ability to interact with and modulate the activity of chromatin regulatory

complexes may allow lncRNAs to affect gene expression on a genome-wide scale[272]. The initial links between imprinting and X chromosome inactivation, the two major epigenetic gene silencing phenomena in mammals, and lncRNAs, were made through the discovery of the *H19* and *Xist* RNAs, respectively. *H19* is an imprinted and maternally expressed lncRNA that is spliced, polyadenylated, and exported into the cytoplasm where it accumulates to very high levels[273]. The function of the *H19* RNA is still unclear, although seems to be involved in growth regulation. Recently, it was found that the *H19* RNA is host to an exonic microRNA, miR-675, which, as a result, is also imprinted and maternally expressed[274]. Although the role of *H19* needs further investigation, it is interesting to ask how an RNA that is spliced and exported from the nucleus evades destruction by the nonsense mediated decay pathway, which normally surveys RNAs and ensures that only those with extensive open reading frames reach the cytoplasm intact for translation. Indeed, key components of the nuclear mRNA degradation and nonsense mediated decay pathways may regulate the levels of *H19* RNA during embryonic stem cell differentiation[275].

The *Xist* RNA, which is crucial for X chromosome inactivation in mammals[276], shares some similarities with *H19* in that it is also spliced and polyadenylated, and its stability is regulated by the same nonsense mediated decay pathway as *H19*. However, this is probably where the similarities end, because Xist evades export into the cytoplasm and instead is associated as an RNA domain or compartment with the X chromosome that it inactivates[277]. This “coating” of the chromatin region that is silenced gave the first model of how lncRNAs might be involved in stable epigenetic gene silencing in cis. Indeed, it is now thought that the *Xist* RNA establishes a specialized nuclear compartment devoid of Pol II, into which most of the chromatin of the future inactive X chromosome becomes localized during inactivation[278].

Several subsequent results, combined with the observation that thousands of protein-coding genes present antisense transcripts, have enforced the idea that antisense lncRNAs generally control the expression of their cognate protein-coding genes through epigenetic regulations[279,280]. This model has profound implication for our understanding of diasease, particularly cancer since dysregulation of lncRNA regulating the expression of a tumor suppressor or oncogene, and not the protein-coding sequence itself, may be one of the hits that leads to oncogenesis[281].

3.4. Long non-coding RNAs in hematopoiesis

Despite the increasing number of studies on lncRNAs expression and their involvement in solid tumor formation, lncRNAs have not been extensively characterized in malignant hematopoiesis[282]. Only in february 2014 Paralkar and co-workers published a study based on the use of deep sequencing of polyA+ RNA to examine lncRNAs expression in purified murine megakaryocyte-erythroid precursors (MEPs), MK and erythroblasts, as well as in human erythroblasts, defining hundreds of lncRNAs unique to each cell type[283].

In any case a few hematopoietic lncRNAs have been studied closely. In example lincRNA-EPS is a mouse nuclear lncRNA that is upregulated during terminal erythropoiesis and represses Pycard, a pro-apoptotic gene. RNAi knockdown of LincRNA-EPS in erythroblasts de-represses Pycard, causing apoptosis[284]. The lncRNA EGO regulates eosinophil granule protein expression, and HOTAIRM1, a lncRNA in the HOXA cluster, is upregulated during myeloid development and is required for normal induction of certain HOXA and myeloid differentiation genes[285].

Recent reports have identified aberrant expression of several lncRNAs, such as *WT1-as*[286]*, MEG3*[287], and *CDKN2B-as* (*ANRIL*)[288], in hematological malignancies, thus these lncRNAs could be involved in the development of hematological neoplasms and their abnormal expression could be used as potential disease marker.

The *WT1* gene on human chromosome 11p13 encodes the antisense transcript (*WT1-as*) that spans the *WT1* exon 1 and continues upstream of *WT1*[289]*. WT1-as* and *WT1* have been shown to be coexpressed, and thus have the potential to form *WT1-as* RNA: *WT1* RNA duplexes that may affect *WT1* transcription[286]. Furthermore, altered *WT1-as* expression has been associated with aberrantly spliced products in AML cells, therefore it is possibly implicated in the development of leukemia[286,290,291]*.* As previously reported by our group, high levels of *WT1* mRNA in PMF are associated with some disease characteristics, such as an elevated number of circulating CD34+ cells and an high disease severity score [292]. However, no correlation between *WT1-as* expression in PMF cells and patients' clinical features have been identified.

MEG3 is an imprinted gene located on human chromosome 14q32, which was first identified in 2000 by Miyoshi *et al.*[293]*.* The mouse ortholog *GTL2/MEG3* has been identified as Polycomb Repressive Complex 2 (PRC2) cofactor. An aberrant methylation status of the *MEG3* promoter has been found in hematopoietic

malignancies such as Multiple Myeloma[294], MDSs[295], and AML[296]

Finally, *ANRIL* is located on human chromosome 9p21.3, is transcribed in antisense orientation, and is part of the *CDKN2B-ARF-CDKN2A* locus, which has an important role in cell cycle control, cell senescence, stem cell renewal, and apoptosis. Aberrant expression of *CDKN2B* and single nucleotide polymorphisms (SNPs) within *CDKN2B* have been associated with susceptibility to a range of human diseases, including cancer [297,298]. Moreover, Iacobucci *et. al*. found a significant association between *ANRIL* SNPs and acute lymphoblastic leukemia phenoptype[299]. In addition, PRC1 and PRC2 interact with *ANRIL* to form heterochromatin, which leads to *ANRIL* repression[300].

STUDY DESIGN

MPNs include a spectrum of disorders that originate from deregulated clonal proliferation of hematopoietic stem cell and are associated with overproduction of mature blood cells. These hematologic neoplasms comprise three clinicopathologic entities: PV, ET and PMF, with a propensity to evolve to acute myeloid leukemia[17].

PMF is the worst among MPNs and is characterized by a shortened life expectancy, myeloproliferation and in particular hyperplastic megakaryopoiesis, neoangiogenesis, progressive BM fibrosis and osteosclerosis. Patients frequently present splenomegaly caused by extramedullary hematopoiesis due to stem cells mobilization[150].

Information on molecular abnormalities of MPNs has been scanty until 2005 and 2006 with the identification of somatic gain of function mutations of *JAK2*[12,15] and *MPL*[45]. These mutations lead to a constitutive activation of the JAK-STAT pathway independently from cytokines stimulation. Since then, many other mutated genes have been found, included mutations in epigenetic modifiers, but with very low frequency, and the disease initiating event it is not completely clear yet. Noteworthy two striking studies recently published identified *CALR* mutation in the majority of *JAK2* wild type patients[56,57] and probably willl help us to better define the pathogenesis of these complex diseases.

Nevertheless there is still a number of patients that doesn't harbour any of the described mutations and our current understanding of the molecular pathogenesis of the disease is not completely defined yet.

Recently, several new molecular pathogenetic mechanisms were proposed, such as the aberrant expression of coding and non-coding RNAs.

Thus, in this project we decided to define the molecular framework of CD34+ cells from PMF patients by means of transcriptome analysis, to identify other molecular abnormalities held by these patients, including pathogenetic mechanisms and new disease markers suitable as biomarker and eventually relevant to target therapy approaches. Circulating CD34+ cells from PMF patients belong to the malignant clone[301] and circulate in high frequency in the PB allowing us to study a cell

population representative of the disease molecular aberrations, obviating for difficulties in collecting BM aspirates because of dry tap due to fibrosis.

In the first part of our study, we performed gene expression profiling (GEP) of CD34+ cells from 42 PMF patients. As a control, we used CD34+ cells of 15 BM and 16 PB from healthy donors.

GEP was carried out using the Affymetrix HG-U219 chip and data were processed with Partek Genomic Suite Software. Differentially expressed genes were selected as positive to t-test with a Westfall and Young's correction, with at least a 2 fold increase or decrease in PMF versus controls (**Figure 10**).

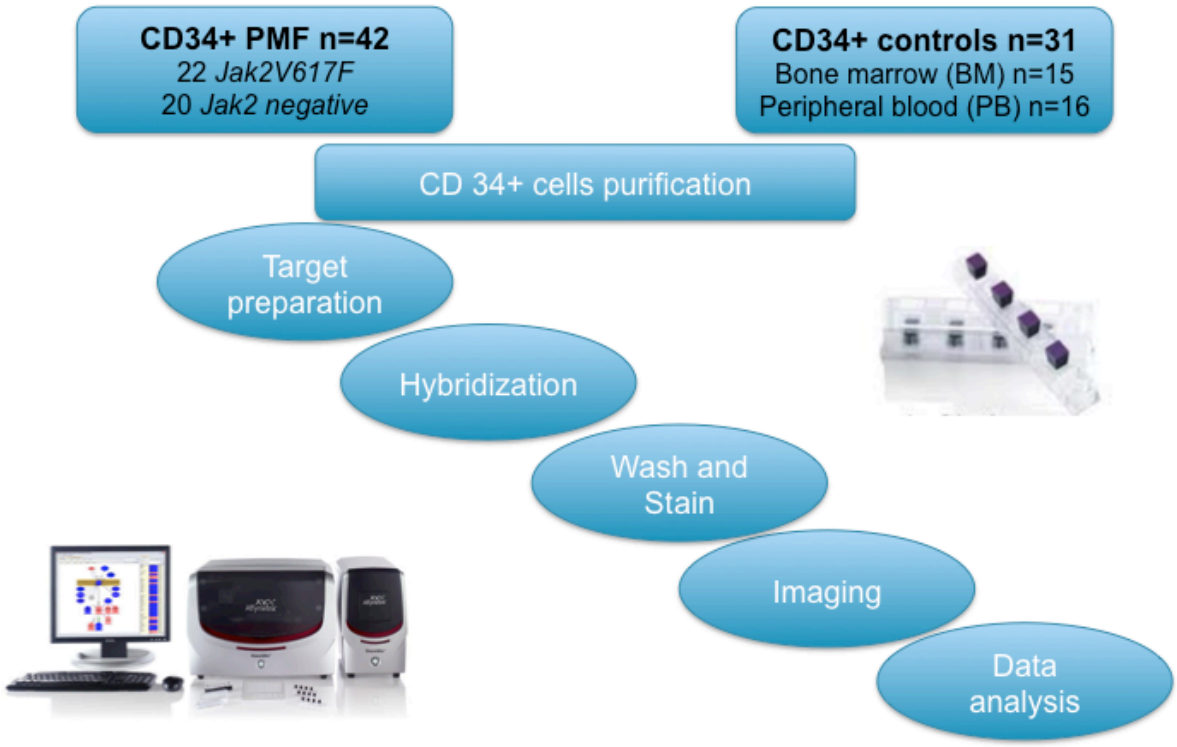

Figure 10. Gene expression profiling study workflow.

In order to validate array data, we designed a TaqMan low density array containing 63+GAPDH TaqMan gene expression assays. Selection of genes was based on either the highest absolute fold change contrast and/or their putative role in PMF pathogenesis. TaqMan assays were carried out in an independent cohort of CD34+ cells from 10 PMF patients and 8 healthy subjects (**Figure 11**).

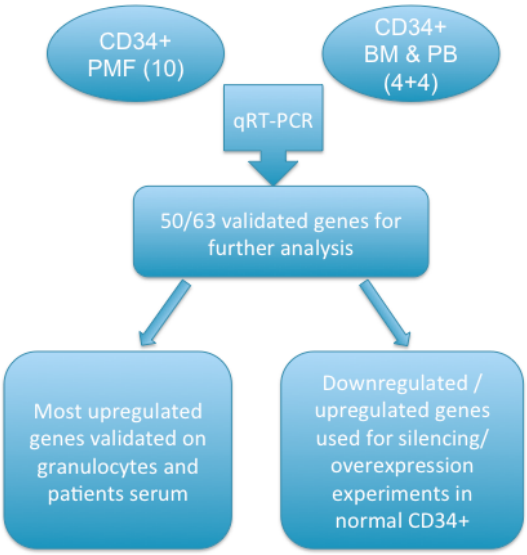

Figure 11. Validation of differentially expressed genes by means of qRT-PCR and subsequent experiments.

Among the validated genes by means of quantitative reverse transcription polymerase chain reaction (qRT-PCR), we selected a set of 7 genes out of the most up-regulated ones to validate their expression in PMF granulocytes (n=32) compared to healthy controls (n=12), since granulocytes represent a more appropriate source for clinical purposes. Moreover, given that two of most upregulated genes encode for two secreted proteins, we assessed their protein levels also in the serum of the PMF patients and healthy donors, by means of ELISA assay.

Subsequently, promising validated upregulated or downregulated genes have been used for overexpression or silencing experiments in vitro in normal CD34+ cells.

In particular we focused on the chromatin remodeler *JARID2*, because chromatin remodeling is a process frequently impaired in MPNs. *JARID2* is member of the jumonji family of TF genes that belong to the PRC2. Interestingly Puda and

collegues demonstrated that *JARID2* is frequently deleted in leukemic transformation of chronic myeloid malignancies, although its role has not been defined yet[105]. Thus we decided to investigate the biological effects exerted by *JARID2* downregulation in hematopoietic differentiation by means of RNA interference. After CD34+ cells purification with immunomagnetic selection, we performed silencing experiments using Nucleofection Amaxa technology. This technology is suitable for the transfection of siRNA oligonucleotides into hematopoietic cells, hard-to-transfect with the common procedures. To exclude non-specific effects caused by interfering RNA (RNAi) nucleofection, a sample transfected with a non-targeting siRNA (NegCTR) was always included. After confirming *JARID2* silencing by means of quantitative Real Time PCR, we performed assays to evaluate cell proliferation analyzing cell cycle by means of citofluorimetric analysis, then we studied effects on differentiation analyzing the immunophenotype by flow cytometric analysis, setting up clonogenic assays in semisolid cultures and evaluating cell morphology by means of May-Grunwald-Giemsa–stained cytospins. In addition, to better characterize changes in gene expression induced by *JARID2* gene silencing, we performed mRNA profiling in NegCTR and *JARID2*-siRNA CD34+ cells, using the Affymetrix HG-U219 Array. Microarray analysis was performed on total RNA derived from three independent experiments at 24h after the last nucleofection (**Figure 12**).

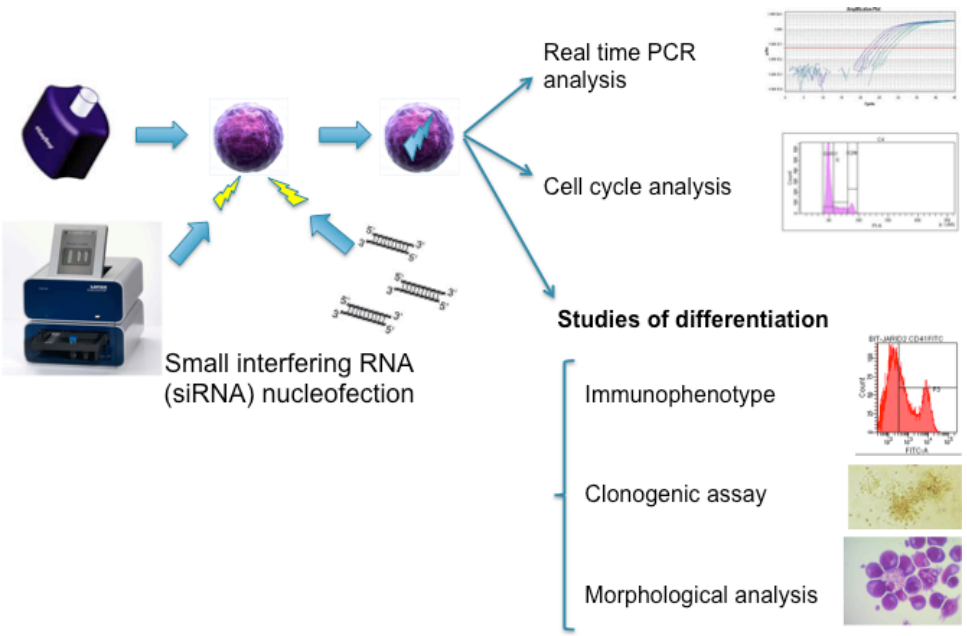

Figure 12. Silencing experiments on normal CD34+ cells workflow.

Finally, since non coding RNAs have been proposed as involved in the development of disease, we decided to investigate lncRNAs expression in CD34+ cells from 26 PMF patients and from 16 healthy subjects.

Despite the increasing number of studies on lncRNAs expression and potential involvement in solid tumor formation, little is known regarding lncRNAs role in malignant hematopoiesis and there are no studies describing the expression profiles of human lncRNAs in CD34+ cells from PMF.

After a screening of a specific lncRNAs database (http://www.lncrnadb.org) containing comprehensive annotations of eukaryotic lncRNAs[302], we identified some lncRNAs that could be involved in hematological diseases.

Given the deregulation of *WT1-as*, *MEG3* and *ANRIL* lncRNAs in hematopoietic malignancies and their involvement in epigenetic mechanisms, which can be altered in MPNs[303], we investigated whether their expression was deregulated in PMF.

Since perturbations in antisense RNAs can alter expression of sense genes[280], we also analyzed expression of the *ANRIL* and *WT1-as* coding genes, namely *CDKN2B* and *WT1*, to evaluate correlations between each sense-antisense couple. Conversely a coding RNA for the *MEG3* locus has not been identified.

Thus we analyzed the expression patterns of these lncRNAs in CD34+ cells purified from 26 PMF patients and 16 healthy control subjects by qRT-PCR analysis with specific non coding assays.

Next, we determined whether expression of the three lncRNAs and their coding counterparts correlated with clinical characteristics of PMF patients using specific statystical test such as the χ^2 Fisher exact test (2 × 2 table), or the χ^2 test for trend (larger contingency table) to compare variables among patient groups which were classified according to their lncRNA expression trends. The Mann-Whitney *U* test (2 groups) or the Kruskal-Wallis test along with the Dunn method for multiple comparisons of continuous variables among groups, and Cox regression models were used for the multivariate analysis (**Figure 13**).

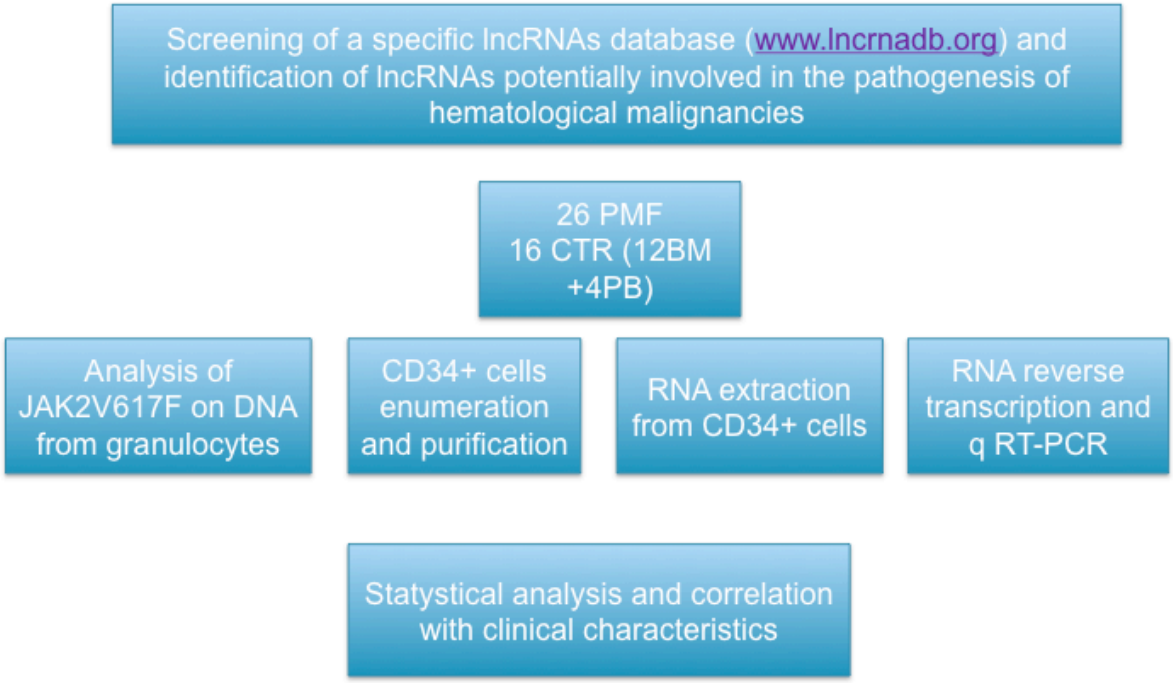

Figure 13. LncRNAs expression study workflow.

MATERIALS AND METHODS

1. Patients and samples

Forty-two patients with a diagnosis of PMF in a typical fibrotic stage of the disease according to the World Health Organization [16] were included in the array data set to perform gene expression study. Their characteristics are reported in **Table 4**.

Table 4. Clinical characteristics of patients with PMF included in GEP study.

VARIABLES	
N. of PMF	42
Follow-up (months); median (range)	33 (4-275)
Age in years; median (range)	61 (30-86)
Males; n (%)	29 (69)
Hemoglobin, g/dL; median (range)	10.9 (6.1-16.3)
Leukocytes, x 10^9/L; median (range)	11.7 (2.5-64.0)
Platelets, x 10^9/L; median (range)	285 (44-1199)
PB CD34+, x 10^6/l; median (range)	178 (1.4-9039)
IPSS risk group; n (%)* Low Intermediate-1 Intermediate-2 High	 6 (18.2) 8 (24.2) 10 (30.3) 9 (27.3)
DIPSS-plus risk group; n (%)* Low Intermediate- 1 Intermediate- 2 High	 0 4 (15.4) 11 (42.3) 11 (42.3)
Constitutional symptoms; n (%)	26 (61.9)
Circulating blasts ≥1%; n (%)	14 (33.3)
Palpable spleen; n (%)†	28 (66.7)
Transfusion dependance; n (%)	11 (26.2)
Abnormal Karyotype*; n (%)	16 (59.3)
Unfav. Karyotype*‡; n (%)	3 (11.1)
JAK2V617F mutation; n (%)	23 (54.8)
Progression to acute leukemia; n (%)	8 (19.0)
Dead; n (%)	14 (33.3)

Abbreviations: PMF, primary myelofibrosis; PB, peripheral blood; IPSS, international prognostic scoring system; DIPSS, dynamic international scoring system.

* evaluated on available data.

† palpable spleen: palpable at 10 cm from left costal margin.

‡complex karyotype or single or 2 abnormalities including 8,_7/7q, i(17q),5/5q, 12p, inv(3), or 11q23.

In addition, 16 PB samples, 15 BM samples, and 16 cord blood (CB) samples were collected from normal donors.

For what concern the study of lncRNAs expression it included 26 PMF patients from an independent cohort and 16 healthy subjects, also in this case the diagnosis of PMF was made according to criteria determined by the World Health Organization [17]. Their clinical characteristic are reported in **Table 5.**

Characteristics	
N. of PMF patients	26
Age, years dx	
Median	62
Range	38-86
Hemoglobin g/dl	
Median	10,6
Range	8.2-16.3
WBC count, x 10e9/l	
Median	8.25
Range	5.68-28.6
Platelet count, x 10e9/l	
Median	235.5
Range	25-666
PB CD34+, x 10e6/l	
Median	130
Range	10-7,335
Red cell transfusion need	12 (46.2%)
Splenomegaly	
Not palpable	1 (3.8%)
11-15 cm	7 (26.9%)
15-20 cm	8 (30.8%)
>20 cm	10 (38.5%)
Constitutional Symptoms	20 (76.9%)
Circulating Blasts >1%	18 (69.2%)
DIPSS plus score	
Intermediate 1	5 (19.2%)
Intermediate 2	8 (30.8%)
High	13 (50%)
JAK2V617F mutation	15 (57.7%)

Table 5. Clinical characteristics of patients with PMF included in lncRNAs expression study.

Abbreviations: PMF, primary myelofibrosis; dx, diagnosis; WBC, white blood cell; PB, peripheral blood. Splenomegaly was measured in cm from the left costal margin. Constituional symptoms include night sweats, fever, weight loss.

2. Ethics Committee approval

All subjects provided informed written consent, and the study was performed under the local Institutional Review Board's approved protocol (Florence: approval date: April 22, 2011, approval file number # 2011/0014777; Pavia: approval date: February 24, 2011, file number #174; Bergamo: approval date: November 4, 2010, file number # 43558). The study was conducted in accordance with the Declaration of Helsinki.

3. CD34+ cell purification

In order to obtain GEP, CD34+ cells were purified from 30–50 mL of PB collected from PMF patients, from 150 mL of PB from G-CSF untreated healthy donors, or from 5 mL of BM aspirates, all obtained in preservative-free heparin. Mononuclear cells were separated over a Ficoll–Paque gradient (Lympholyte; Cederlane Labs) and processed through two sequential steps of immunomagnetic CD34+ selection (Miltenyi Biotec; Bergisch Gladbach, Germany). Purity of the isolated CD34+ cell population was evaluated by flow cytometry after labeling with PE-HPCA2 anti-CD34 monoclonal antibody (BD Biosciences) and was always >95%. CD34+ cells (3×10^5) were immediately lysed in Qiazol (Qiagen; Valencia, CA).

Umbilical CB samples from normal donors, used for *in vitro* experiments, were collected after normal deliveries, according to the institutional guidelines for discarded material. Mononuclear cells were isolated by Ficoll-Hypaque (Lympholyte, Cederlane Labs) gradient separation, washed twice with phosphate-buffered saline, and then CD34+ cells were purified by immunomagnetic sorting (EasySep Human CD34 Positive Selection kit, Stem Cell Technologies). The purity of CD34+ cells, assessed by flow cytometry, was always > 95%[304].

4. RNA extraction and microarray data analysis

GEP was performed on the RNA isolated from CD34+ cells of 42 PMF patients and 31 healthy donors (n=15 BM, n=16 PB), whereas for lncRNAs expression study RNA was isolated from CD34+ cells of 26 patients.

Total cellular RNA, was isolated from CD34+ cells using the miRNeasy mini RNA isolation kit (Qiagen) following the manufacturer's recommendations. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the purity and integrity of RNA samples by using an Agilent 2100 Bioanalyzer (Agilent Technologies; Waldbrunn, Germany). NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE) was used to evaluate the RNA sample concentration, while 260/280 nm and 260/230 nm ratios were used to assess the purity of RNA.

cDNA synthesis, as well as biotin-labeled target synthesis, was performed using the GeneAtlas 3´ IVT Express Kit according to the standard protocol supplied by Affymetrix. The HG-U219 Array Strip (Affymetrix; Santa Clara, CA) hybridization, staining, and scanning were performed by using the GeneAtlas Platform.

The probe level data were normalized and converted into expression values using the robust multiarray average (RMA) procedure[305]. Quality control assessment was performed using different Bioconductor packages such as R-AffyQC Report, R-Affy-PLM, R-RNA Degradation Plot, and QC procedures included in the Partek GS. 6.6 Software Package (http://www.partek.com).

Before analysis, a variance filter was applied to remove flat genes with low variation in expression over the samples. An exploratory principal component analysis (PCA) was performed using the PCA module implemented in Partek GS™.

Differentially expressed genes (DEGs) were then selected using a supervised approach with the ANOVA module included in Partek GS package. In particular, we selected all the probe sets with a fold change contrast ≥2 for DEGs, in the pairwise comparison of PMF versus controls, and a false discovery rate (FDR) (q-value) < .05.

5. Electroporation of CD34+ cells

The electroporation program of CD34+ cells was based on a previously published protocol[306], which was optimized to be performed on the 4D-Nucleofector™ System (Lonza). Briefly, each sample was electroporated three times once every 24 hours (h) with a mix of three Silencer Select small interfering RNAs (siRNAs) targeting human *JARID2* (**Table 6**) (Life Technologies), starting from the day after CD34+ cell purification.

RefSeq Accession Number	siRNA ID	Sense siRNA Sequence	Antisense siRNA Sequence
NM_004973	s7657	GGUUUCUAAGGUAAACGGAtt	UCCGUUUACCUUAGAAACCtg
NM_004973	s7655	GAAGAACGGGUGGUACGUAtt	UACGUACCACCCGUUCUUCtg
NM_004973	s7656	GGUGGUACAAGAGAACGAAtt	UUCGUUCUCUUGUACCACCat

Table 6. Silenced Select siRNAs employed in JARID2 silencing experiments.

For each electroporation, 4×10^5 CD34+ cells were resuspended in 100 µL of P3 Primary Cell Solution (Lonza), containing 3 µg of siRNA mix, and pulsed with the program DS112. To exclude non-specific effects caused by RNAi nucleofection, a sample transfected with a non-targeting siRNA (NegCTR; Silencer Select Negative #2 Control siRNA; Life Technologies) was included. Cells were analyzed 24 h and 48 h after the last nucleofection for both cell viability and *JARID2* expression.

6. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

cDNA was reverse-transcribed from total RNA (100 ng per sample) using the High Capacity cDNA Archive Kit (Life technologies; Carlsbad, CA, USA;), TaqMan PCR was carried out in triplicate using either Custom TaqMan Array 384-well Cards, TaqMan gene expression assays and TaqMan specific non-coding assays (all reagents from Life Technologies) by using an AB 7900HT Fast Real-Time PCR System (Applied Biosystems). Gene expression relative quantification (RQ) was achieved using the comparative cycle threshold (CT) method using GAPDH as the housekeeping gene. GAPDH was confirmed to be an effective housekeeping gene

for qPCR analysis because it demonstrated stable expression across the sample cohort. To normalize the data, ΔΔCT was calculated for each sample using the mean of its ΔCT values subtracted from the mean ΔCT value measured in the entire population of healthy subjects, considered as a calibrator; the RQ value was expressed as $2^{-\Delta\Delta CT}$. To make the RQ value symmetric for up- and down-regulated genes, fold change (FC) was used in the Tables for ease of interpretation (for RQ > 1, FC = RQ; for RQ < 1 FC = −1/RQ).

For what concerns lncRNAs analysis ΔΔCTs were calculated for each sample using the median ΔΔCTs measured among the entire population of subjects (PMF patients and healthy donors) for calibration. Thus, the relative quantity (RQ) value (=2^-ΔΔCT) represents the mRNA/lncRNA expression ratio for each sample relative to the median expression of that genes in all subjects.

7. Enzyme-linked immunosorbent assay (ELISA)

Serum levels of LCN2 (NGAL) and OLFM4 secreted proteins in PMF patients and healthy donors were evaluated by ELISA using two commercial kits (NGAL rapid ELISA kit; BioPorto Diagnostics; Gentofte, Denmark, and OLFM4 ELISA kit; Antibodies-online GmbH; Aachen, Germany; according to the manufacturer's instructions. The results are expressed as mean concentration (ng/mL) with each sample assayed in triplicate.

8. CD34+ cell-culture conditions

After immunomagnetic separation, CD34+ cells were seeded in 24-well plates at 5×10^5/mL in Iscove's-modified Dulbecco medium (IMDM; Euroclone) containing 20% human serum (Bio-Whittaker), stem cell factor (SCF; 50 ng/mL), Fms-like tyrosine kinase 3 ligand (Flt3L; 50 ng/mL), thrombopoietin (TPO; 20 ng/mL), interleukin-6 (IL-6; 10 ng/mL), and interleukin-3 (IL-3; 10 ng/mL; all from Miltenyi).

After each transfection, CD34+ cells were transferred into pre-warmed fresh medium in 24-well plates (Euroclone) and maintained in the same culture conditions as described above.

For liquid culture differentiation assays, CD34+ cells were plated (5×10^5/mL) in IMDM with the addition of 20% BIT 9500 serum substitute (bovine serum albumin, insulin, and transferrin; StemCell Technologies) 24 h after the last nucleofection, in order to set up a multilineage cell culture (SCF, 50 ng/mL; Flt3L, 50 ng/mL; TPO, 20 ng/mL; IL-3, 10 ng/mL; IL-6, 10 ng/mL; all cytokines from Miltenyi) and MK unilineage culture (TPO, 100 ng/mL)[304].

9. Methylcellulose and collagen clonogenic assays

The methylcellulose assay was carried out by plating CD34+ cells in MethoCultTM GF H4434 (StemCell Technologies Inc.; Vancouver), as previously described [307]. MK colony forming units (CFU-MK) were assayed in collagen-based medium, using a commercial MK assay detection kit (MegaCult-C; StemCell Technologies Inc.) as previously reported[307].

10. Morphological and immunophenotypic analysis

Differentiation of CD34+ cells was monitored by morphological analysis of May–Grunwald–Giemsa-stained cytospins and by flow cytometric analysis of CD34, CD14, CD66b, CD163, Glycophorin A (GPA), and CD41 surface antigen expression at day 0, 3, 5, 8, 10, and 12 after the last nucleofection. Images were captured by using an Ax10scopeA1 microscope equipped with an AxioCam ERc 5S Digital Camera and Axion software 4.8 (all Carl Zeiss MicroImaging Inc.; Thornwood, NY, USA). The images were then processed with Adobe Photoshop 7.0 software.

The following monoclonal antibodies (MoAbs) were used for flow cytometric analysis: phycoerythrin (PE)-conjugated mouse anti-human CD14 MoAb, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 MoAb, FITC-conjugated mouse anti-human CD66b MoAb (all from Miltenyi Biotech; Auburn, CA, USA), FITC-conjugated mouse anti-human CD41 MoAb, and PE-conjugated mouse anti-human GPA MoAb (all from Dako; Milano, Italia). After staining, cells were analyzed by using a BD FACSCanto II (BD Biosciences; San

Jose, CA USA). At least 10,000 events were counted for each sample to ensure statistical relevance.

11. Statistical analysis

SPSS software (StaSoft; Tulsa) was used for statistical analysis of clinical correlation. Comparison between groups was performed by the Student's *t*-test, and the chosen level of significance was $P < .05$ with a two-sided test.

The statistics used for data analysis in silencing assays were based on two-tailed Student's *t*-tests for averages comparison in paired samples. Data were analyzed by using Microsoft Excel (Microsoft Office, 2008 release) and are reported as mean ± standard error of the mean (SEM). $P < .05$ was considered significant.

For what concerns long non coding RNAs data normalized QQCTs values were analyzed using Microsoft Excel 2008 software. The $\chi 2$, Fisher exact test (2×2 table), or the $\chi 2$ test for trend (larger contingency table) were used to compare variables among patient groups, which were classified according to their lncRNA expression trends. The analysis of continuous variables among groups was performed using the Mann-Whitney *U* test (2 groups) or the Kruskal-Wallis test along with the Dunn method for multiple comparisons. Cox regression models were used for the multivariate analysis. *P* values < .05 were considered statistically significant, and all *p*-values were 2-tailed. Data were processed using SPSS Version 17.0 software (StatSoft).

12. Analysis of *JAK2V617F*

Analysis of the *JAK2* mutation was performed by allele specific PCR with 75 ng of DNA purified from granulocytes as described by Baxter *et al* in 2005[13].

RESULTS

1. Gene expression profile of CD34+ cells from PMF patients

In order to investigate the molecular framework of CD34+ cells from PMF patients and point out an abnormal gene expression regulation in PMF, we performed mRNA expression profiling in CD34+ cells from 42 PMF patients (n=23 *JAK2V617F*-positive, n=19 wild-type *JAK2*) and 31 healthy donors (n=15 from the BM and n=16 from the PB) by means of Affymetrix HG-U219 arrays.

After data pre-processing, to explore the relationships between samples, we performed a PCA. **Figure 14** shows that the PMF samples are clustered together and were clearly separated from both the BM and PB control samples.

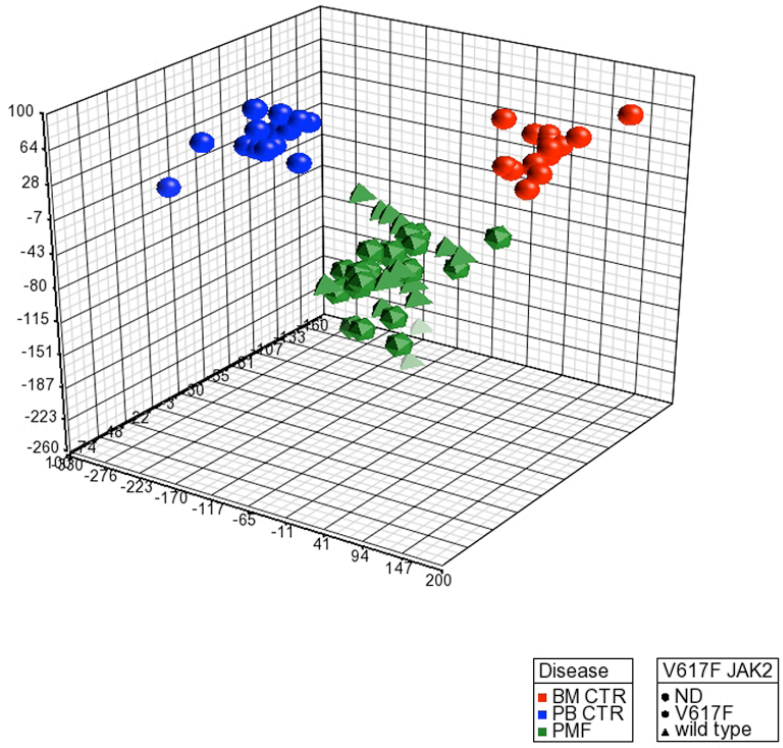

Figure 14. PCA graph of global gene expression data; BM control samples are shown as red spheres; PB control samples are shown as blue spheres; PMF samples are shown in green. PMF *JAK2* wild-type samples are shown as pyramids, while PMF *JAK2V617F* samples are shown as prisms.

Data mining with another unsupervised grouping method, hierarchical clustering analysis, confirms the relationship uncovered by PCA as well (**Figure 15**). Of note,

none of the unsupervised analysis performed was able to ungroup *JAK2V617F* and *JAK2* wild-type patients.

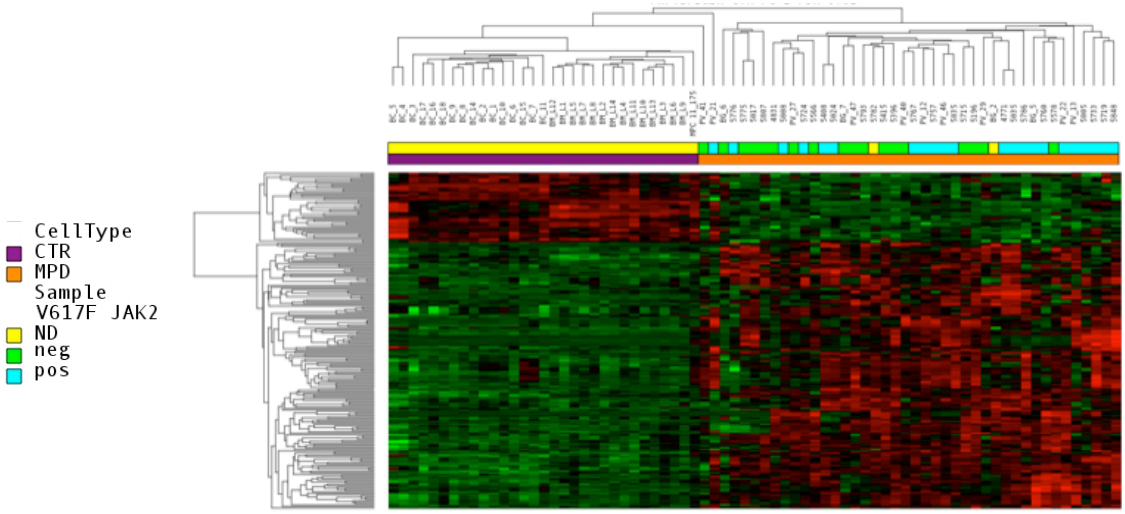

Figure 15. Unsupervised hierarchical clustering analysis. Upregulated genes are shown in red and downregulated genes are shown in green. PMF samples are clearly separated from healthy donors.

Next, using an analysis of variance (ANOVA)-based supervised approach for comparing PMF samples with both BM and PB controls, we finally identified 718 differentially expressed genes (DEG) in PMF CD34+ cells compared to both controls.

Array data confirmed the abnormal expression of many genes (i.e., *WT1*, *NFE2*, *CXCR4*, *CD9*) previously identified as deregulated in PMF CD34+ cells by our group in a different cohort of PMF patients[292]. Moreover, PMF samples displayed increased levels of several putative cancer markers, such as *ANGPT1*, *CEACAM8*, and *CP*, previously reported to be associated with poor prognosis in hematological and solid neoplasms, as well as genes associated with BM fibrosis (*LEPR*, *MMP9*, and *TIMP3*) and aberrant cellular migration (*TM4SF1*, *RHOB*, *ARHGAP18*, and *MMP8*). Furthermore, PMF samples showed a deregulated expression pattern of a number of transcription factors and chromatin remodelers involved in myeloid and MK commitment, either downregulated (i.e., *JARID2*, *RUNX2*, *KLF3*, and *AFF3*) or upregulated (i.e., *FHL2*, *MAF*, and *IKZF2)*. A selected list of DEGs chosen for their biological significance is presented in **Table**

7. The fold change contrast in PMF samples compared to healthy controls is also reported.

Gene Symbol	Gene Ontology	Notes	FC
ANGPT1	Secreted protein	Overexpressed in AML, CML, MDSs[308]	3,1
ANXA3	Cytoplasmatic protein	Negative prognostic factor for prostate cancer[309]	10,4
ARHGAP18	Cytoplasmatic protein	Involved in cell spreading and motility[310]	3,0
CD9	Membrane protein	Involved in platelet activation and aggregation[311]; involved in BM remodeling in PMF[312]	2,2
CEACAM8	Membrane protein	Overexpressed in imatinib resistant CML cells[313]	5,5
CP	Enzyme	Overexpressed in AML[314]	2,6
DEFA1	Secreted protein	Overexpressed in imatinib resistant CML cells[315]; biomarker for diagnosis of CRCA[316]	60,2
FGR	Kinase	Involved in cell migration[317]	1,9
FHL2	Transcription factor	Promotes myeloid proliferation; overexpressed in AML[318]	2,7
IDH1	Cytoplasmatic protein	Mutated in MPNs[50]	2,4
IFI27	Membrane protein	Involved in defense and immunity[319]	2,4
IFIH1	Cytoplasmatic protein	Involved in defense and immunity[320]	2,3
IKZF2	Transcription factor	Overexpressed in in Hodgkin Lymphoma and ALL[321]	3,6
ITGB3	Membrane protein	Involved in platelet activation and aggregation[322]	2,5
LCN2	Secreted protein	Expression induced by BCR-ABL protein[323]; negative prognostic factor for breast cancer[324]	7,0
LEPR	Membrane protein	Overexpressed in AML and PMF; involved in fibrosis	9,7
MAF	Transcription	Negative prognostic factor for MM [325]	8,0

	factor		
MEF2C	Transcription factor	Involved in MKs differentiation [326]	2,3
MMP9	Extracellular matrix protein	Involved in the development of fibrosis[176]	3,8
MYC	Transcription factor	Involved in MKs differentiation, cancer marker[327]	2,8
NFE2	Transcription factor	Involved in MKs differentiation[328] overexpressed in PMF[292]	2,0
OLFM4	Secreted protein	Negative prognostic factor for colorectal, breast and lung cancer[329]	3,5
PF4	Secreted protein	Involved in platelet activation and aggregation[330]	3,4
PIM1	Transcription factor	Overexpressed in PMF[292]	2,6
RHOB	Cytoplasmatic protein	Involved in cell spreading and motility[331]	3,7
TIMP3	Extracellular matrix protein	Involved in the development of fibrosis[177]	4,0
TM4SF1	Membrane protein	Involved in cell spreading and motility[332]	4,4
VWF	Secreted protein	Highly expressed by early MKs, involved in platelet adhesion[333]	4,4
WT1	Transcription factor	Negative prognostic factor in AML[334]; associated with high severity score in PMF[292]	2,0
AFF3	Transcription factor	Fusion with MLL gene in ALL[335] and with RUNX1 gene[336]	-2,2
ARHGEF7	Cytoplasmatic protein	Involved in cell migration, attachment and cell spreading[337]	-2,2
ARID4A	Nuclear protein	Involved in chromatin remodeling; K/O mice develop myelofibrosis[338]	-2,3
BRWD1	Nuclear protein	Involved in chromatin remodeling[339]	-1,8
CDC42	Cytoplasmatic protein	Cdc42-deficient mice developed a fatal myeloproliferative disorder[340]	-4,9
CEBPD	Transcription factor	Myeloid commitment regulator[341]	-2,2
CEBPG	Transcription	Myeloid commitment regulator[341]	-2,5

	factor		
CXCR4	Membrane protein	Involved in bone marrow homing[342]	-2,5
EIF2AK3	Kinase	The ablation in tumor cells resultes in accumulation of ROS[343]	-3,0
FOXO1	Transcription factor	Involved in OXS response[344]; negative prognostic factor for AML[345]	-2,3
HMGB3	Nuclear protein	Involved in chromatin remodeling; required for the proper balance between HSC self-renewal and	-2,5
IRF4	Transcription factor	Downregulated in CML[347]	-2,7
IRF8	Transcription factor	Downregulated in CML[347]	-6,4
JARID2	Nuclear protein	Involved in chromatin remodeling[125] and in AML progression[105]	-2,5
KLF3	Transcription factor	Downregulated in AML; K/O mice display abnormalities in hematopoiesis[348]	-3,6
MAFF	Transcription factor	Myeloid commitment regulator[349]	-2,0
MEF2D	Transcription factor	Involved in myogenic differentiation; fusion with DAZAP1 gene in ALL[350]	-2,7
MLL5	Transcription factor	Frequently deleted in human myeloid malignancies[351]	-2,7
MXD1	Transcription factor	Involved in regulation of cell proliferation; antagonizes MYC gene[352]	-3,4
NR4A3	Nuclear protein	Involved in chromatin remodeling; hypoallelic mice display MDS/MPNs feautures[353]	-2,4
NUP98	Transcription factor	Involved in fusions with different partner genes in patients with hematopoietic malignancies[354]	-2,5
PHC3	Nuclear protein	Component of polycomb repressive complex[355]	-2,1
PURB	Nuclear protein	Deleted in MDS and AML[356]	-2,5
RUNX2	Transcription factor	Involved in hematopoietic and osteogenic lineages differentiation[357]	-2,1
SF3B1	Nuclear protein	Mutated in MDS and in MDS/MPNs[358]	-2,0
SMAD7	Transcription	Involved in fibrosis[359] downregulated in MDSs[360]	-7,2

	factor		
TCF4	Transcription factor	Myeloid commitment regulator[361]	-2,4
TLE4	Transcription factor	Deleted in AML[362]	-2,2
TP53INP1	Nuclear protein	Loss of expression in several cancers; inactivation correlates with increased cell	-2,5

Table 7. DEGs selected by biological significance

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; MDSs, myelodysplastic syndromes; BM, bone marrow; PMF, primary myelofibrosis; ALL, acute lymphoblastic leukemia; CRCA, colorectal cancer; MPNs, myeloproliferative neoplasms; MM, multiple myeloma; MKs, megakaryocytes; K/O, knock/out; ROS, reactive oxygen species; OXS, oxidative stress; HSC, hematopoietic stem cell; MDS/MPNs, myelodysplastic/myeloproliferative neoplasms

In order to validate the array data, we designed a TaqMan low-density array containing 63+*GAPDH* TaqMan gene expression assays. The selection of genes was based on either the highest absolute fold change contrast and/or their putative pathogenetic role in PMF. TaqMan assays were carried out in an independent cohort of CD34+ cells from 10 PMF patients and 8 healthy subjects (n=4 BM, n=4 PB) and allowed the validation of the expression of 50 out of 63 genes (79.4%) (**Table 8**).

IDH1	2,859498651	0,0246
NFE2	2,828565996	0,000201
TIMP3	2,273046937	0,0381
ANGPT1	2,050691438	0,00523
IKZF2	2,006254815	0,0112
NLK	1,899432857	0,00031
ETS1	1,730562802	0,0352
CD9	1,729284735	0,048
WT1	1,634469034	0,046
STAT4	1,508229055	0,042
RUNX1	-1,540916484	0,0236
JARID2	-1,687376857	0,00385
ENAH	2,937389921	0,00949

ARHGEF7	-1,719343517	0,0155
SF3B1	-1,773990652	0,00949
EZR	-1,830096959	0,00949
PHC3	-1,858593381	0,00949
HMGB3	-1,890570914	0,00307
MLL5	-1,901225952	0,0109
PURB	-2,043843204	0,0246
FOXO1	-2,070367747	0,00949
EIF2AK3	-2,076933681	0,00883
BRWD1	-2,220413423	0,00307
AFF3	-2,236224773	0,0122
SPTBN1	-2,432094523	0,00031
ARID4A	-2,482995561	0,00949
MEF2D	-2,506266935	0,000259
TP53INP1	-2,544673086	0,0195
KLF3	-2,630388738	0,0236
BCL6	-2,989893989	0,0406
CXCR4	-3,062339265	0,00318
MXD1	-3,171338643	0,00158
TLE4	-3,481183902	0,00116
RUNX2	-3,59589105	0,00031
FOSL2	-4,040814286	0,000426
ZNF295	-4,065351806	0,0053
CDC42	-7,242772449	0,00949
NR4A3	-8,926140019	0,00307
IRF4	-12,36971377	0,00318
SMAD7	-24,52797221	0,00256

Table 8. Relative quantity and false discovery rate of genes validated by means of q-RT-PCR**.**

The hierarchical clustering shown in **Figure 16,** highlightes that also in the q-RT-PCR validation data-set, PMF samples are gathered together and the pattern of expression of differentially expressed genes validated by means of q-RT-PCR is quite different in PMF compared to healthy controls.

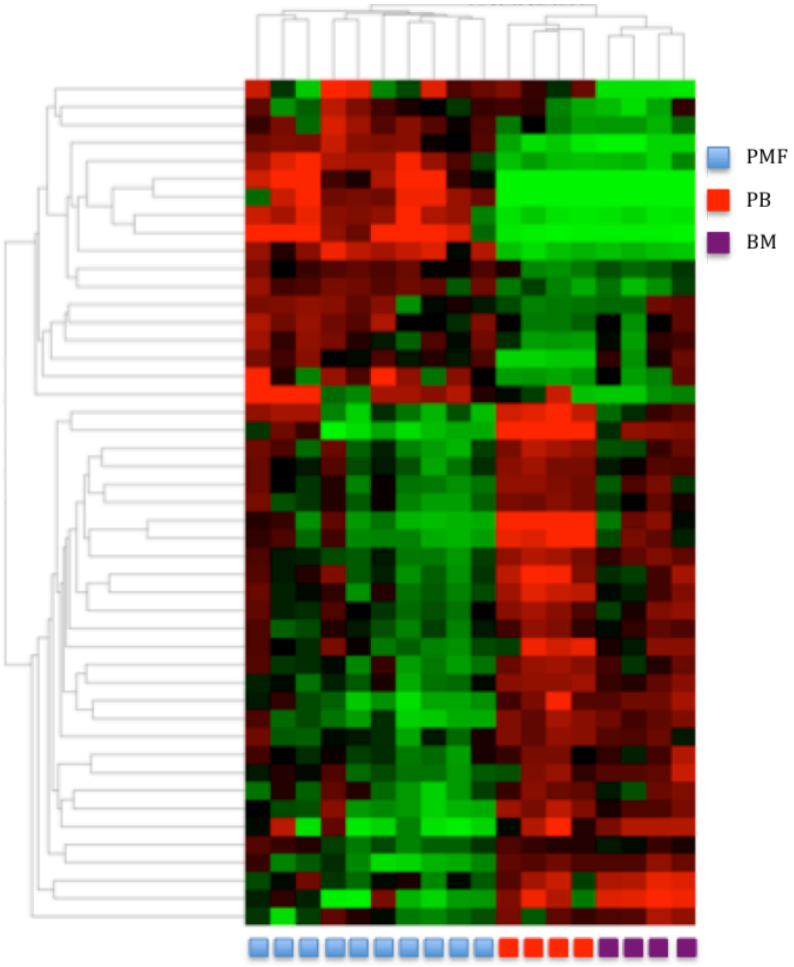

Figure 16 Hierarchical clustering of genes validated by means of q-RT-PCR. Upregulated genes are shown in red and downregulated genes are shown in green.

2. Validation of a gene set on granulocytes and serum from PMF patients

Among the 50 validated genes described above, we selected a set of 7 genes (*OLFM4*, *LCN2*, *LEPR*, *FGR*, *ANXA3*, *CEACAM8*, and *DEF1A*) out of those most upregulated to validate their expression in granulocytes, which represent a more convenient source for diagnostic/prognostic purposes. Using qRT-PCR, we observed that *OLFM4*, *LCN2*, *LEPR*, *FGR*, and *ANXA3* mRNA levels were significantly increased in PMF granulocytes (n=32) compared to healthy donors (n=12) (**Figure 17A**), whereas *CEACAM8* and *DEF1A* expression was not statistically modulated between the two groups (data not shown).

Since *OLFM4* and *LCN2* genes encode for two secreted proteins, we assessed OLFM4 and LCN2 protein levels in the serum of the same PMF patients (n=32) and healthy controls (n=8) by means of an ELISA. As shown in **Figure 17B**, the levels of OLFM4 and LCN2 secreted proteins were significantly higher in PMF patients than in healthy donors. Of note, the median concentration of OLFM4 serum protein was 5-fold higher in PMF patients than in controls.

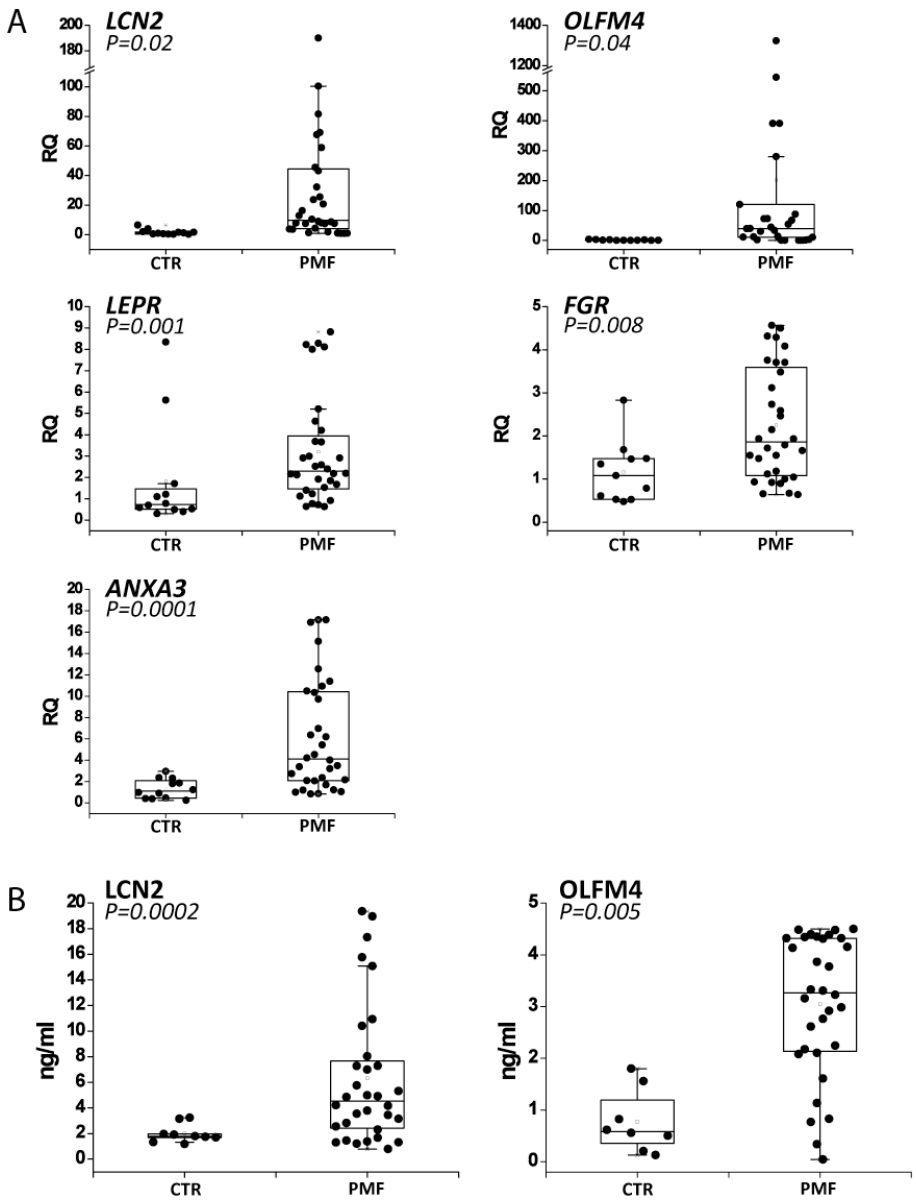

Figure 17. Validation of a gene set on granulocytes and plasma from PMF patients. (**A**) Expression of selected genes in granulocytes from PMF patients and healthy donors. Gene expression levels were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) starting from granulocyte total RNA and were expressed as relative quantity (RQ). Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, and the bars show the range of values. Data are representative of 32 PMF and 12 control (CTR) samples. (**B**) The serum levels of two secreted proteins (LCN2 and OLFM4) in PMF patients and healthy donors. Protein levels were measured by enzyme-linked immunosorbent assay (ELISA) and were expressed as ng/mL. Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, and the bars show the range of values. Data are representative of 30 PMF and 8 CTR samples. *, $P < .05$ vs. CTR.

3. Optimization of CD34+ cells electroporation

Synthetic siRNAs and mimic miRNAs were transfected in human CB-derived CD34+hematopoietic progenitor cells by using the Amaxa® 4D-Nucleofector® instrument. Nucleofection conditions were optimized by comparing different nucleofection protocols. In detail, seven different nucleofection programs were first compared by transfecting equal amounts of cells (300,000 cells/sample) with an Alexa488-conjugated siRNA (2.5µg/sample) in 100 µL of Nucleocuvettes®. The transfection efficiency was evaluated by flow cytometric detection of the Alexa488-postive cell fraction 4 h post-transfection; cell viability was detected by conducting a Trypan Blue exclusion assay; and flow cytometric analysis of the cell cycle distribution was evaluated with PI staining 24 h post-transfection. Based on these results, the best three nucleofection programs in terms of transfection efficiency and cell viability (EO-100, ED-120, DS-112) were further tested for three nucleofection cycles, one nucleofection every 24 h. For this purpose, 500,000 CD34+cells/sample were transfected in 100 µL Nucleocuvettes® with an siRNA targeting the CD34 antigen transcript (2.5 µg/sample). Transfection efficiency was evaluated by flow cytometric detection of the CD34-postive cell fraction while cell viability was detected byTrypan Blue exclusion assay and flow cytometric analysis of cell cycle distribution by PI staining 24 hours post-transfection. Based on this comparison, the Nucleofection program DS-112 was selected for the lower cytotoxicity (5.3% ± 0,57%; median ± SD) sub-G1 fraction and the higher transfection efficiency, evaluated by flow cytometric detection of the siRNA-targeted CD34 antigen downregulation (52.3% CD34+ cells in non-targeting siRNA-transfected cells and 13.3% CD34+ cells in CD34-targeting siRNA-trasfected cells for the DS-112 program).

4. Functional validation of *JARID2* in normal CD34+ cells

For what concerns downregulated genes we focused our attention on the chromatin remodeler *JARID2*

Since the contribution of *JARID2* to PMF pathogenesis has never been investigated, we performed RNAi-mediated gene silencing experiments on normal

CD34+ cells purified from CB. As described above, we optimized the CD34+ cell nucleofection protocol for the Amaxa 4D-Nucleofector™ System technology. CD34+ cells were transfected with a mixture of three Silencer Select siRNAs targeting *JARID2* mRNA (**Table 6**) and with a non-targeting siRNA as a negative control (NegCTR). The expression level of *JARID2* in control samples and *JARID2*-siRNA cells was assessed by qRT-PCR at 24 h (relative quantity [RQ] ± SEM, 0.2±0.036, $P < .001$) and 48h (RQ±SEM, 0.32±0.026, $P < .001$) after the last nucleofection.

Flow cytometric analysis of the hematopoietic stem cell marker CD34 highlighted a significant increase in the percentage of CD34+ cells in *JARID2* silenced sample compared to NegCTR until 8 days after last nucleofection (**Figure 18**).

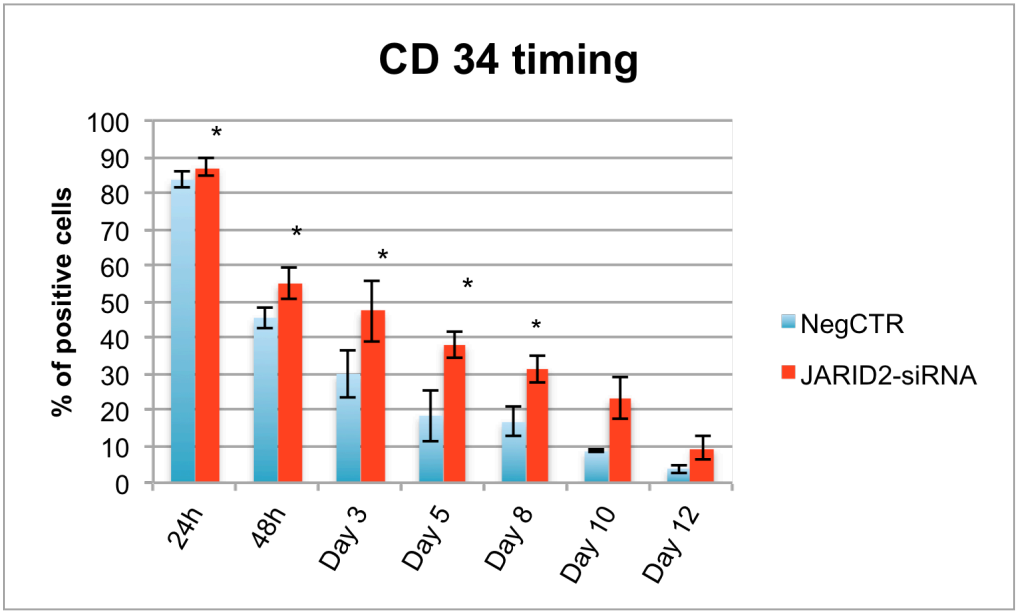

Figure 18. Effect of JARID2 silencing in normal CD34+ cells on differentiation. Results of the statistical analysis on the percentage of positive cells for the CD34 marker performed by flow cytometry at day 1, 2, 3, 5, 8, 10, and 12 after the last nucleofection on human serum culture. Values are reported as mean ± standard error of the mean (SEM). *, $P < .05$ versus NegCTR. The results come from five independent experiments.

Flow cytometric analysis of granulocytic, mono-macrophagic, and erythrocyte differentiation markers did not highlight any significant modulation between *JARID2*-siRNA CD34+ cells and the NegCTR sample (data not shown). The methylcellulose assay indicated an increase in the clonogenic efficiency of

JARID2-siRNA CD34+ cells versus the NegCTR sample that is nearly the 30 percent, whereas there was no significant difference in the percentage of erythroid and myeloid colonies (**Figure 19**).

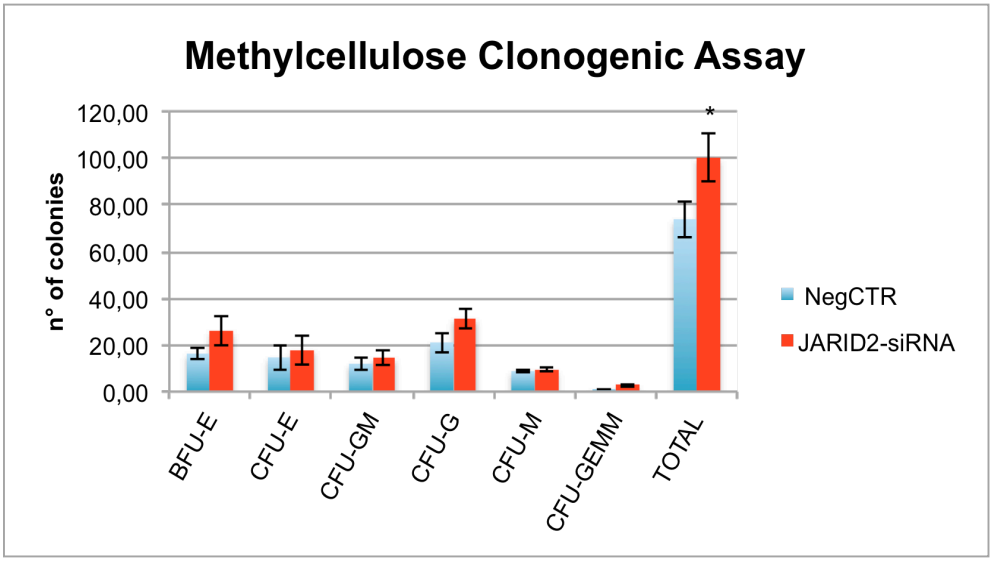

Figure 19. Results of the statistical analysis of collagen-based clonogenic assay. The cells were plated 24 h after the last nucleofection and scored after 14 days. Values are reported as mean ± standard error of the mean (SEM). *, *P* < .05 versus NegCTR. The results come from five independent experiments.

Flow cytometric analysis of the CD41 MK marker performed on serum-free multilineage culture at day 8, 10, and 12 showed that *JARID2* inhibition induces a significant increase in the MK fraction compared to the NegCTR sample (**Figure 20**).

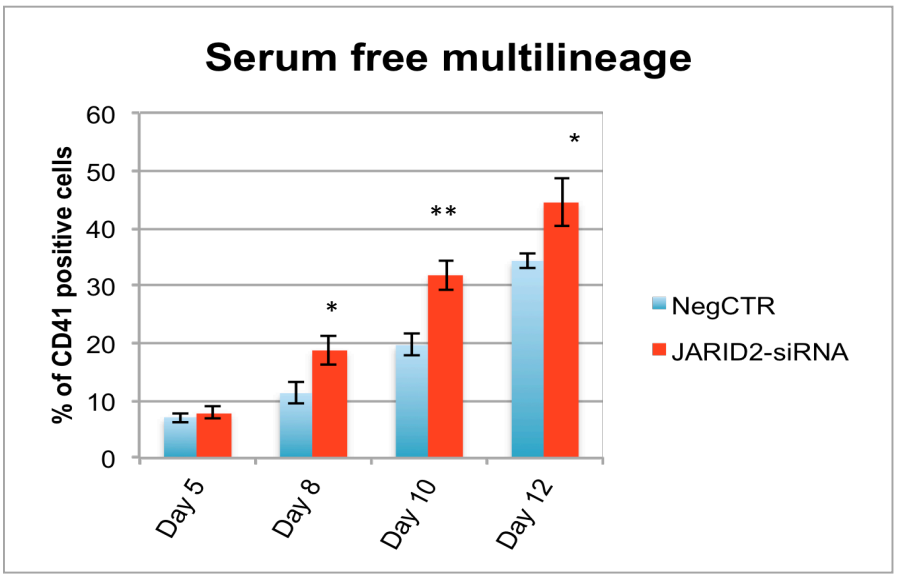

Figure 20. Effect of JARID2 silencing in normal CD34+ cells on differentiation. Results of the statistical analysis on the percentage of positive cells for the CD41 marker performed by flow cytometry at day 5, 8, 10, and 12 after the last nucleofection on serum-free multilineage culture. Values are reported as mean ± standard error of the mean (SEM). **, $P < .01$ versus NegCTR; *, $P < .05$ versus NegCTR. The results come from five independent experiments.

Unilineage MK differentiation culture experiments further confirmed these results (**Figure 21**).

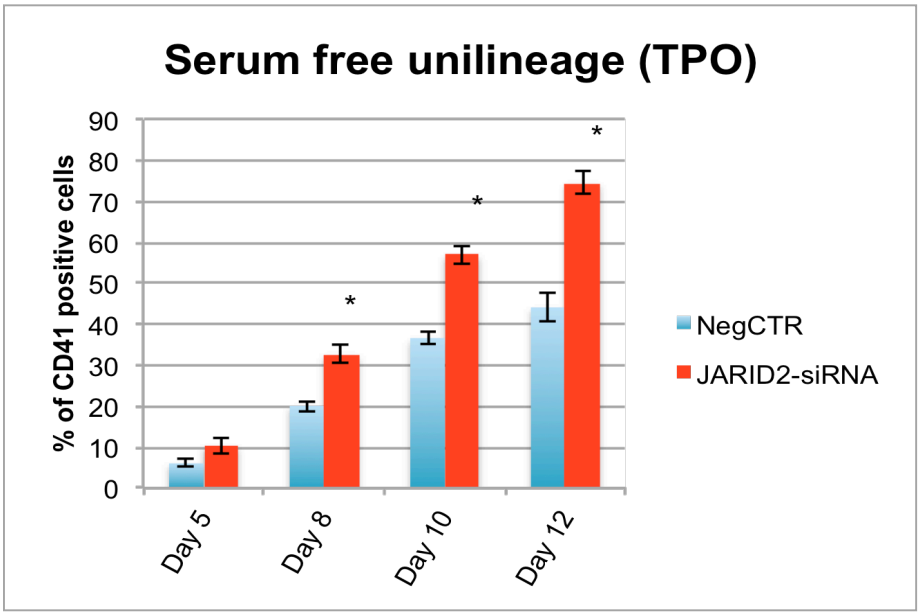

Figure 21. Effect of JARID2 silencing in normal CD34+ cells on differentiation. Results of the statistical analysis on the percentage of positive cells for the CD41 marker performed by flow cytometry at day 5, 8, 10, and 12 after the last nucleofection on serum-free unilineage culture. Values are reported as mean ± standard error of the mean (SEM). **, $P < .01$ versus NegCTR; *, $P < .05$ versus NegCTR. The results come from five independent experiments.

Next, we examined the effect of *JARID2* silencing on MK commitment by plating NegCTR and *JARID2*-siRNA CD34+ cells in a collagen-based serum-free semisolid culture medium that supports the growth of MK progenitors *in vitro*. The results, reported in **Figure 22**, demonstrated that *JARID2* silencing induces a remarkable increase in colony-forming unit-megakaryocytes (CFU-MK) and a strong decrease of non-megakaryocyte colonies (CFU non-MK) compared to the NegCTR sample.

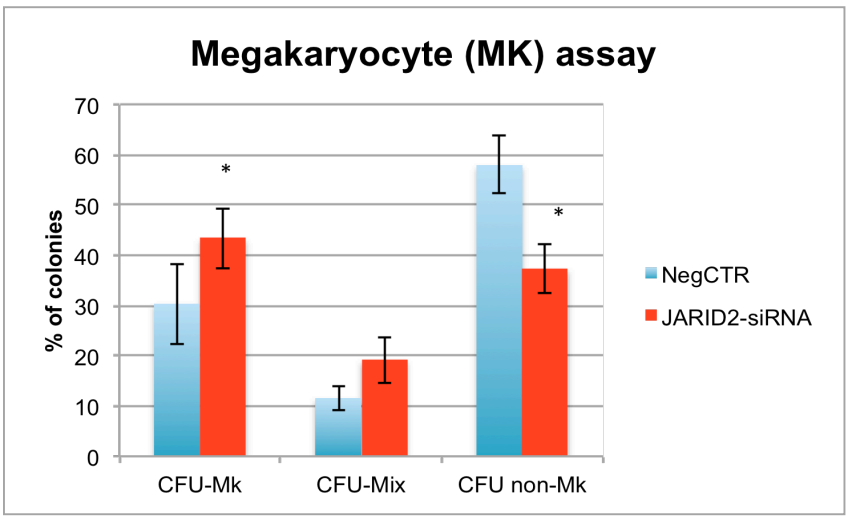

Figure 22. Results of the statistical analysis of collagen-based clonogenic assay. The cells were plated 24 h after the last nucleofection and scored after 12 days. Values are reported as mean ± standard error of the mean (SEM). *, $P < .05$ versus NegCTR. The results come from five independent experiments.

Moreover, morphological evaluation of May–Grünwald–Giemsa-stained (MGG-stained) cytospins of thrombopoietin-treated cells at day 8 and 10 after the last nucleofection confirmed the results obtained by collagen based assay. As shown in figure X *JARID2*-siRNA cells clearly displayed a considerable enrichment in MK precursors at different stages of maturation compared to NegCTR (**Figure 23**).

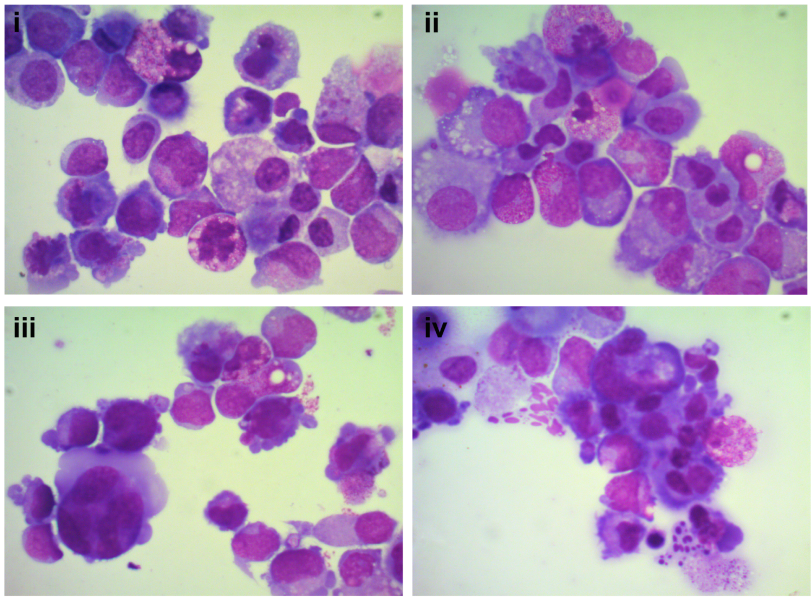

Figure 23. Morphological analysis of negative control (NegCTR) (**i–ii**) and *JARID2*- short interfering RNA (*JARID2*-siRNA) (**iii–iv**) samples after May–Grünwald–Giemsa staining at day 8 and 10 of MK unilineage culture after the last nucleofection in a representative experiment. Magnification, ×1000.

In addition, to better characterize the changes in gene expression induced by *JARID2* gene silencing, we performed mRNA profiling in NegCTR and *JARID2*-siRNA CD34+ cells. We investigated the changes in gene expression in CD34+ cells upon *JARID2* silencing. In particular, microarray analysis was performed on the total RNA derived from three independent experiments at 24 h after the last nucleofection. The analysis of the microarray data showed that several genes involved in the immune response, such as *CD74*, *GBP4*, and *IL2RA*, were increased in *JARID2*-siRNA CD34+ cells. Interestingly, we also found the upregulation of *ERG*, a member of the *ETS* transcription factor gene family required for normal megakaryopoiesis [365]. Among the downregulated genes, the antiproliferative factor *TOB1*, which is reported as tumor suppressor, was detected [366],. *JARID2* silenced CD34+ cells showed the reduction of the small GTPase Rab10 that has been found to be related with altered VWF secretion, whereas the tetraspanin CD63 has been implicated in the regulation of membrane protein trafficking, leukocyte recruitment, and adhesion processes, a complete list of the differentially expressed genes is reported in **Figure 24.**

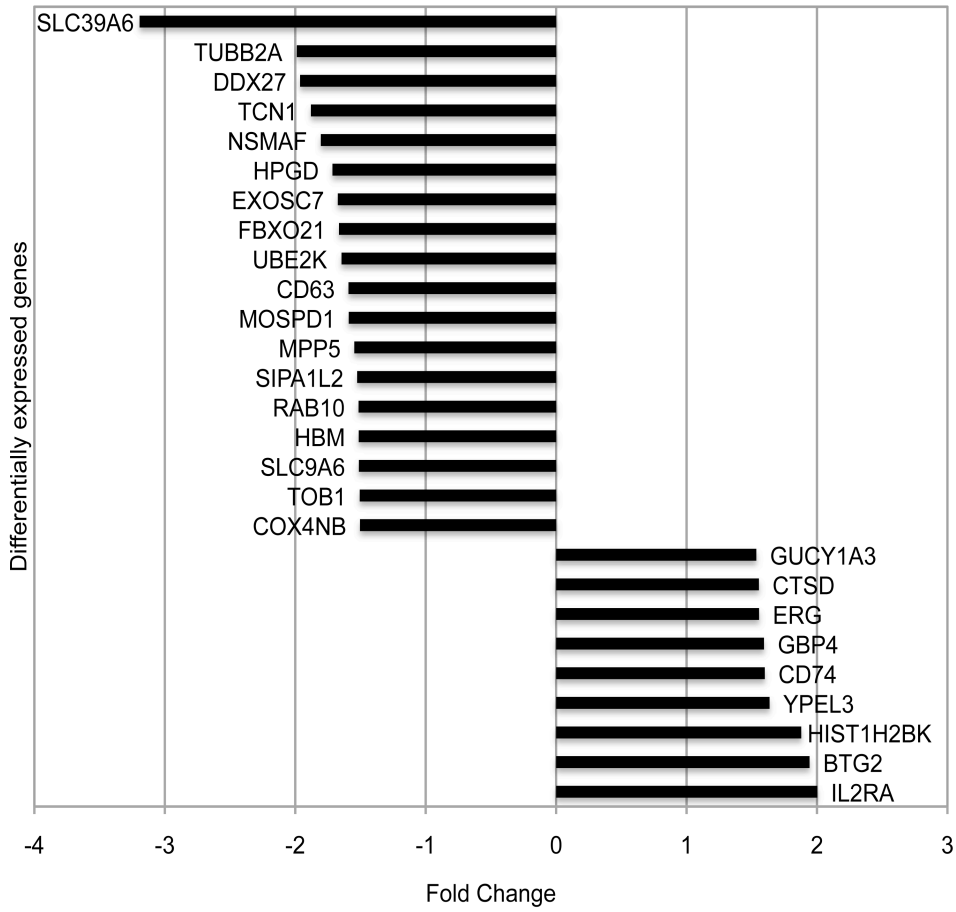

Figure 24. Differentially expressed genes in the *JARID2*-siRNA sample versus NegCTR.

5. Expression of lncRNAs in CD34+ cells from PMF patients

After a screening of an lncRNAs database (http://www.lncrnadb.org) containing comprehensive annotations of eukaryotic lncRNAs[302], we identified some lncRNAs that could be involved in hematological diseases. We found that *WT1* sense and antisense transcripts, *MEG3* lncRNA and *CDKN2B* sense and antisense transcripts could be involved in the pathogenesis of hematological malignancies[286,282], thus we analyzed their expression patterns in CD34+ cells purified from 26 PMF patients and 16 healthy control subjects by qRT-PCR analysis. Our data show that the control samples uniformly presented a coordinated downregulation of *WT1* mRNA and *WT1-as* transcripts, whereas 19 of the 26 PMF samples displayed coordinated upregulation of *WT1* mRNA and *WT1-as* transcripts. In contrast, the 7 remaining PMF samples displayed scattered

results and downregulation of *WT1-as* (**Figure 25A**). A very similar pattern of expression was obtained for *MEG3,* which was downregulated in the control samples and in 9 PMF samples, but was upregulated in the remaining 17 PMF samples (**Figure 25B**). Noteworthy, 89.5% of the patients with increased and coordinated expression of *WT1* mRNA and *WT1-as* displayed a concurrent upregulation of *MEG3* (*p* = .0001, **Figure 25C**).

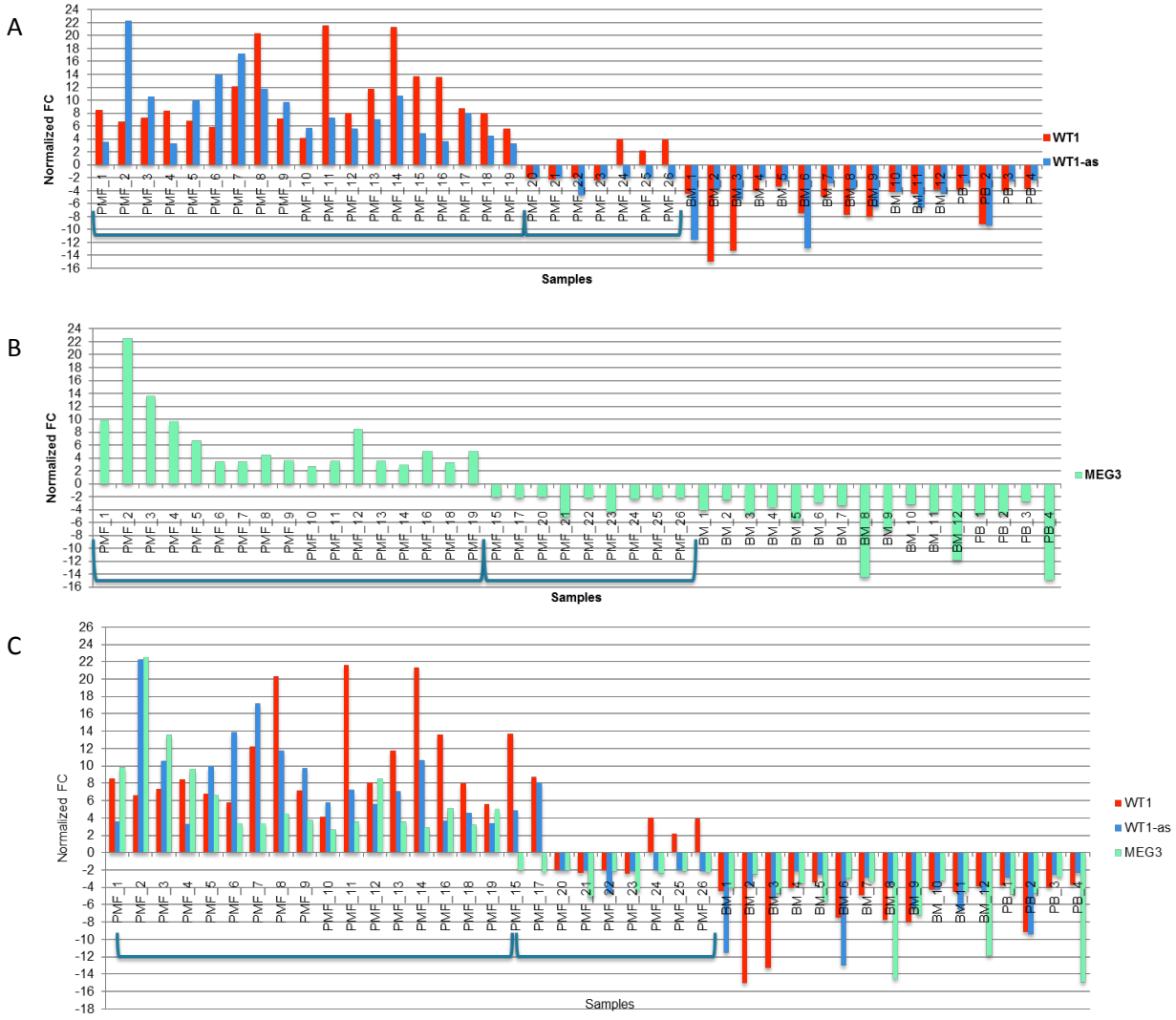

Figure 25. Expression of *WT1*, *WT1-as*, and *MEG3* in CD34+ cells from PMF patients**.** (**A**) *WT1* and *WT1-as* expression levels in PMF and control samples (**B**) *MEG3* expression levels in PMF and control samples. (**C**). Comparison between *WT1*, *WT1-as* and *MEG3* expression levels in PMF and control samples. PMF, primary myelofibrosis; PB, peripheral blood; BM, bone marrow; FC: fold change.

As for *CDKN2B* and *ANRIL,* we found two patterns of regulation. *CDKN2B* was

upregulated in a group of 17 PMF samples, whereas it was downregulated in a second group of 9 PMF samples (**Figure 26**). An inverse correlation was found for the expression of coding (*CDKN2B*) and noncoding (*ANRIL*) RNA in all the BM control samples, but only in a minority of the PMF patient and PB control samples.

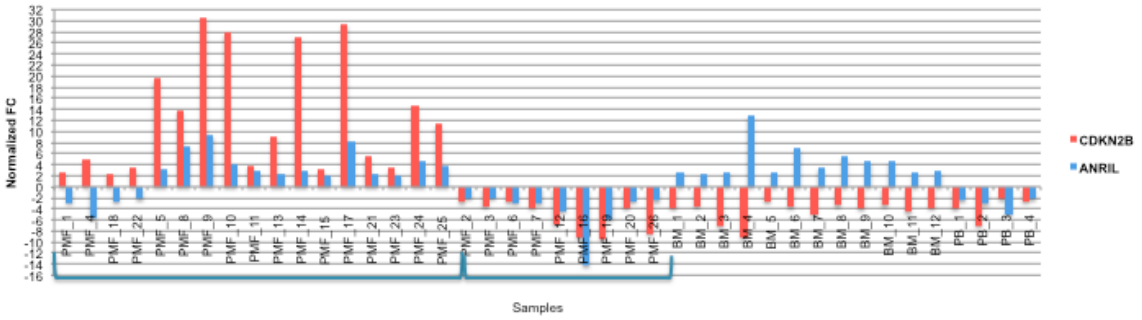

Figure 26. Expression of *CDKN2B* and *CDKN2B-AS (ANRIL*) in CD34+ cells from PMF patients**.** PMF, primary myelofibrosis; PB, peripheral blood; BM, bone marrow; FC: Fold Change.

Statistical analysis did not show a significant correlation among patients with increased *CDKN2B* expression and those with concomitant upregulation of *WT1*, *WT1-as* and *MEG3*.

6. Clinical characteristics correlate with lncRNA expression

Next, we determined whether expression of the three lncRNAs and their coding counterparts correlated with clinical characteristics of PMF patients. We compared the following categories of patients: 1.) Patients showing coordinated upregulation of *WT1* and *WT1-as* versus those who did not present this correlation (19 vs. 7 patients), 2.) Patients showing increased expression of *MEG3* compared with patients presenting *MEG3* downregulation (17 vs. 9), 3.) Patients showing *CDKN2B* upregulation compared with patients showing *CDKN2B* downregulation (17 vs 9). We determined whether patients exhibiting the above expression patterns clustered preferentially within different risk categories of the Dynamic International Prognostic Scoring System (DIPSS) plus[227]. This scoring system includes the following variables: age, leucocytosis, constitutional symptoms, anemia, PB blasts, thrombocytopenia, necessity for red blood transfusion, and unfavorable karyotype.

Figure 27 shows the distribution of patients displaying different *WT1*/*WT1-as* expression patterns in the distinct risk categories of the DIPSS-plus scoring system. 62% of patients with concurrent upregulation of *WT1* and *WT1-as* clustered together in the high-risk category, 31.3% were in the intermediate-2 risk category, and only 6.3% were in the intermediate-1 risk category (**Figure 27A**). Conversely, patients showing downregulation and discordant upregulation were exclusively distributed in the lowest risk categories (57.1% and 42.9% in intermediate-1 and intermediate-2, respectively; p = .006, **Figure 27B**). Patients with high *WT1* and *WT1-as* expression more frequently presented blast counts >1% (83.3% vs. 42.9%, p = .043, data not shown) and hemoglobin (Hb) levels less than 10g/dl (66.7% vs. 14.3%, p = .019, **Figure 27C**). Furthermore, a higher percentage of these patients were transfusion dependent (61.1% vs. 0%, p = .006, data not shown). Finally, as shown in **Figure 27D**, these patients displayed a trend towards having more circulating CD34+ cells (p = .05)**.**

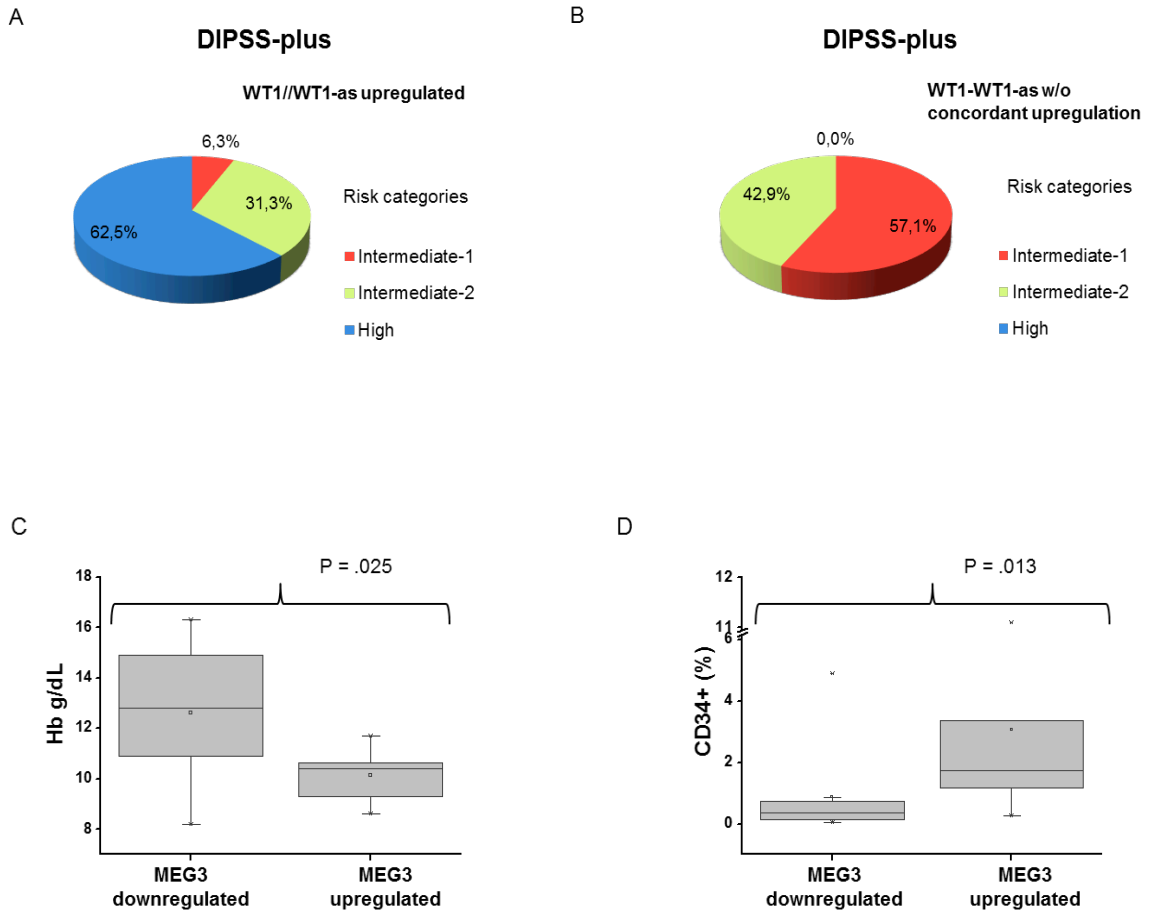

Figure 27. Correlation between clinical features of PMF patients and *WT1*/*WT1-as* expression. (**A**) According to the DIPSS-plus scoring system, 62.5% of PMF patients displaying concordant upregulation of *WT1* and *WT1-as* clustered in the high-risk category, 31.3% in intermediate-2, and 6.3% in intermediate-1. (**B**) According to the DIPSS-plus scoring system, 0% of PMF patients displaying discordant regulation of *WT1* and *WT1-as* clustered in the high-risk category, 42.9% in intermediate-2, and 57.1% in intermediate-1 (p = .006)**.** (**C**) Box-plot representation of Hb levels of PMF patients with high levels of *WT1* sense and antisense RNA vs. patients without this coordinated upregulation (p = .019). (**D**) Box-plot representation of the percentage of CD34+ cells in the PB of PMF patients with high levels of *WT1* sense and antisense RNA compared with samples without this coordinated upregulation (p = .05). PMF, primary myelofibrosis; Hb, hemoglobin; PB, peripheral blood.

No differences in age, sex, platelet count, splenomegaly, white blood cells (WBC) count, or occurrence of constitutional symptoms between the two groups of patients were found.

A similar DIPSS distribution was found for *MEG3* expression (**Figure 28**). 64.3% of patients with upregulated *MEG3* were in the high risk category, 28.6% in intermediate-2, and 7.1% in intermediate-1 (**Figure 28A**), whereas 11.8% of

patients with downregulated *MEG3* were in the high risk category, 44.4% in intermediate-2, and 44.4% in intermediate-1 (p = .024, **Figure 28B**). Patients with increased *MEG3* expression were also characterized by blast counts >1% (77.8% vs. 44.4%, p = .021, data not shown), Hb levels less than 10g/dl (66.8% vs. 22.2%, p = .025, **Figure 28C**), with the consequent need for blood transfusions (62.5% vs. 11.1%, p = .016, data not shown), and higher percentages of circulating CD34+ cells (p = .013, **Figure 28D**).

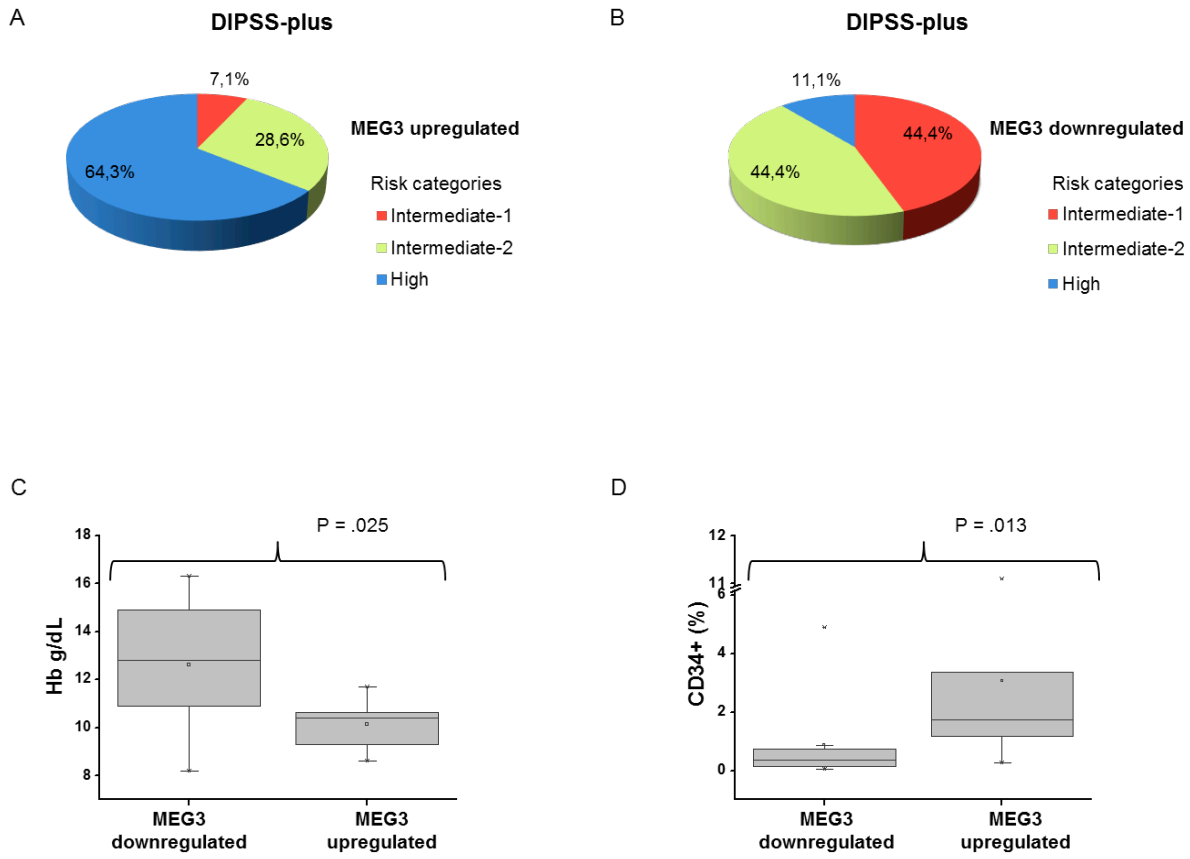

Figure 28. Correlation between clinical features of PMF patients and *MEG3* expression. (**A**) According to the DIPSS-plus scoring system, 64.3% of PMF patients displaying upregulation of *MEG3* clustered in the high-risk category, 28.6% in intermediate-2, and 7.1% in intermediate-1. (**B**) According to the DIPSS-plus scoring system, 11.1% of PMF patients displaying downregulation of *MEG3* clustered in the high-risk category, 44.4% in intermediate-2, and 44.4% in intermediate-1. (**C**) Box-plot representation of Hb levels of PMF patients with and without *MEG3* upregulation (p = .025). (**D**) Box-plot representation of the percentage of CD34+ cells in the PB of PMF patients with and without *MEG3* upregulation (p = .013). PMF, primary myelofibrosis; Hb, hemoglobin; PB, peripheral blood.

No differences in age, sex, platelet count, splenomegaly, WBC count, or occurrence of constitutional symptoms between the two groups of patients were found.

Regarding the group characterized by upregulated *CDKN2B* expression, we found a correlation between expression and the grade of BM fibrosis using the current grading classification scheme[198], in which grade 0 corresponds to normal BM. As depicted in **Figure 29A**, the majority (87.5%) of samples with upregulated *CDKN2B* clustered in grades 2 and 3, whereas only 4.4% of samples showing *CDKN2B* downregulation clustered in grades 2 and 3 (p = .042 **Figure 29B**). Finally, a direct correlation was found between *CDKN2B* upregulation and the *JAK2V617F* mutation. 81.3% of patients with upregulated *CDKN2B* were *JAK2V617F* positive as compared to 22.2% with downregulated *CDKN2B* (p = .004) (**Figure 29C**). No differences in age, sex, platelet count, splenomegaly, WBC, count or occurrence of constitutional symptoms between the two groups of patients were found.

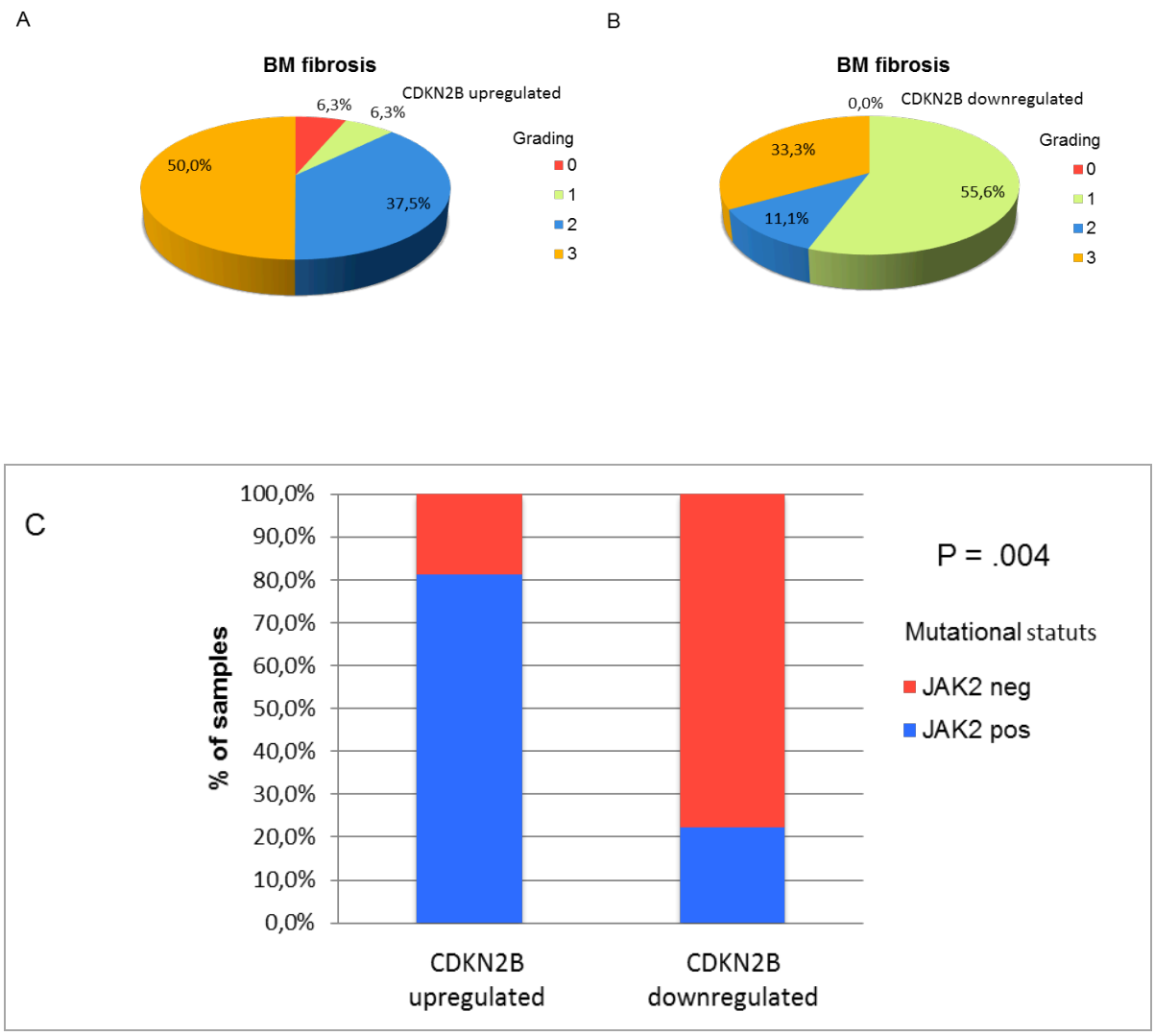

Figure 29. Correlation between clinical features of PMF patients and *CDKN2B* expression. (**A**) According to the BM fibrosis grading scale, 50% of PMF patients with upregulated *CDKN2B* were grade 3, 37.5% were grade 2, and 12.6% were grades 1 and 0. (**B**) According to the BM fibrosis grading scale, 33.3% of PMF patients without upregulated *CDKN2B* were grade 3, 11.1% grade 2, and 55.6% were grades 1 and 0. (**C**) Correlation of the *JAK2* mutational status with *CDKN2B* regulation (p = .004). Upregulation of *CDKN2B* correlated with the presence of the *JAK2 V617F* mutation. PMF, primary myelofibrosis; BM, bone marrow.

DISCUSSION

In this study, we aimed at characterizing the role of genes and long non-coding RNAs in PMF pathogenesis. To this end, first we employed GEP of PMF CD34+ cells, identifying several differentially expressed genes potentially involved in PMF pathogenesis and eventually suitable for diagnostic and prognostic purposes and hopefully for targeted therapy in PMF. Moreover we demonstrated that the silencing of *JARID2*, in normal CD34+ cells, determines the expansion of the megakaryocytic lineage. In parallel, the assessment of the expression levels of several DEGs in PMF granulocytes highlighted a number of possible disease markers, which might eventually become relevant for target therapy approaches, such as membrane protein- and kinase-coding genes.

In the second part of this study we analyzed the expression of selected lncRNAs in PMF CD34+ cells and we were able to correlate different expression levels with clinical characteristics of disease, suggesting that also lncRNAs could play key roles in PMF pathogenesis and could be used as new diagnostic biomarkers with potential prognostic implications.

Since MPNs are considered to arise from the hematopoietic stem cell compartment, understanding of the pathogenetic molecular mechanisms should best be assessed by studying CD34+ cells. Here, we provide the results of an extensive study that profiled gene expression in CD34+ cell sample from 42 PMF patients.

GEP analysis showed that PMF circulating CD34+ cells present a different expression pattern compared to BM and unmobilized PB CD34+ cells; of note, PCA was unable to separate PMF patients according to *JAK2* mutational status (**Figures 14**). Differential expression analysis enabled the identification of several deregulated mRNAs suitable as biomarkers or as putative molecular targets for diagnostic or prognostic purposes. Therefore, the most upregulated genes were monitored on PMF granulocytes because they could represent a more suitable cell source for clinical praxis, whereas secreted protein levels were assessed in the patients' sera. We identified a set of five genes (i.e., *LCN2*, *OLFM4*, *ANXA3*, *FGR*, and *LEPR*) (**Figure 17**) whose expression levels are aberrant in PMF granulocytes

as well as in CD34+ cells. Evidence in PMF of a higher mRNA expression of the leptin receptor (*LEPR*), previously reported in AML[367], as well as of Src kinase *FGR*[368], could also be useful to drive the future design of targeted drugs. Of note, LEPR and Src kinase inhibitors are already being used in preclinical or clinical trials[369]. In addition, we demonstrated that OLFM4 and LCN2 secreted protein levels could be considered as PMF biomarkers, since they were significantly high in patients' sera compared to those of healthy controls. Strikingly, more than 80% of the PMF patients presented with higher OLFM4 protein levels compared to all the evaluated controls.

The present study has provided information about the molecular mechanisms underlying PMF pathogenesis. Indeed, data analysis clearly showed that several genes involved in adhesion or migration processes (*TM4SF1*, *RHOB*, *ARHGAP18*, and *MMP8*) as well as fibrogenic potential (*LEPR*, *MMP9,* and *TIMP3*) are deregulated in PMF CD34+ cells. Interestingly, regulators of megakaryocytic commitment were also upregulated (i.e., *NFE-2*, *MEF2C*). Furthermore, we found an increased expression of genes with oncogenic potential, i.e., *CEACAM8*, *ANGPT1* (**Table 7**), chromatin remodeler genes such as *PHC3* and *HMGB3*. We also observed that low expression of different hematopoietic transcription factors as well as leukemia suppressors (i.e., *CEBP*, *FOXO1*, *MLL5*). It's interesting th altered expression of genes like *CDC42* and *NR4A3*, whose downregulation leads to myeloproliferative disorders in murine models[340,353], as well as *HMGB3*, which codes for a regulator of the self-renewal/differentiation balance in murine hematopoietic stem cells[346]. Moreover, we observed the downregulation of *JARID2*, a chromatin remodeler that is a member of the Jumonji family of transcription factors belonging to the polycomb repressive complex 2 (PRC2)[124]. Of note, Puda and colleagues demonstrated that *JARID2* is frequently deleted in leukemic transformation of chronic myeloid malignancies[105], although its role has not yet been defined. Thus, we decided to investigate the effect of *JARID2* downregulation in hematopoiesis by means of RNAi-mediated silencing in human normal CD34+ cells. A first interesting data that we could observe in human serum liquide culture is the significant increase in the percentage of CD34+ cells in *JARID2*-sirna culture compared to NegCTR until 8 days after the last nucleofection (**Figure 18**). Silencing effects of *JARID2* have been evaluated also by means of

methylcellulose clonogenic assay, here *JARID2*-silenced sample displayed a significant increase in the absolute number of total colonies compared to NegCTR, with an increased of clonogenic capacity that is about the 30 percent. These data are particularly intriguing since altered balance between self-renewal and differentiation in MPNs hematopoietic cells have been described and defect intrinsic to individual HSCs with the consequent skewed of their progeny toward proliferation are also reported by Kent and collegues. However characterising the mechanisms that link MPNs mutations with clonal expansions that eventually lead to development of MPNs is still necessary.

On the other hand, our findings support the contributing role of *JARID2* deficiency in the expansion of the MK lineage as reveald by the experiments performed both in liquid (**Figure 20,21, and 23**) and in semisolid culture (**Figure 22**). Microarray data analysis performed in CD34+ cells upon *JARID2* silencing further confirmed the above mentioned data since we found the upregulation of ERG that is a fundamental transcription factor gene required for normal megakaryopoiesis (**Figure 24**). These data suggest that the altered expression of *JARID2* could explain the MK hyperplasia observed in bone marrow biopsies of PMF patients[17] and the high proliferative potential of MKs derived from PMF CD34+ cells reported in studies *in vitro*[370].

Then, since long non-coding RNAs are emerging as new regulator of gene expression, in the second part of this project we decided to investigate also their expression in CD34+ cells from PMF patients.

In recent years, high-throughput sequencing and expression technologies have found that a large proportion of the human genome is transcribed in ncRNAs[251,252,253,371]. Interest in lncRNAs has grown, and several studies have shown that lncRNAs play important roles in cellular processes and are associated with a broad range of diseases[270,271,372]. Anyway the number of well-characterized lncRNAs is small, but several have been found to affect gene expression through chromatin modification[373,374,375], transcriptional regulation[265,266], and post-transcriptional processing[267,269].

Deregulation of lncRNAs can also play key roles in malignant transformation and cancer cell behavior by activating cellular pathways that lead to tumorigenesis[270,271,372]. Despite the increasing number of studies on lncRNAs

expression and lncRNAs involvement in solid tumor formation, little is known regarding their role in malignant haematopoiesis[282]. After extensive screening of the lncRNA database (lncRNAdb), a database containing comprehensive annotations of eukaryotic lncRNAs (http://www.lncrnadb.org)[302], we selected *ANRIL*, *MEG3*, and *WT1-as* lncRNAs that have been recently identified as being involved in hematological malignancies[286,287,288] and in chromatin modification, which is known to be altered in PMF[303].

This is the first study describing the altered lncRNAs expression in PMF and correlating lncRNAs expression with PMF clinical characteristics.

In this work, we used qRT-PCR to measure *WT1-as*, *MEG3*, and *ANRIL* expression in CD34+ cells from 26 PMF patients and 16 healthy control subjects. Previously results described by Dallosso[286] and Gugliemelli[292] indicated that *WT1* mRNA was not detectable in normal BM, whereas increased levels of *WT1* mRNA were found in PMF samples. Here we showed that both *WT1* and *WT1-as* were expressed at high levels in the majority (73%) of PMF samples, but not in healthy control samples (**Figure 25A**) suggesting that a direct interaction between *WT1* antisense and sense transcripts could be necessary for *WT1* function in PMF CD34+ cells.

Regarding *MEG3* expression, 17 PMF samples showed a significant upregulation compared to healthy control samples, whereas 9 samples were downregulated (**Figure 25B**). Although *MEG3* expression is decreased or absent in several tumor types[376,377,378], high levels have been found in the K562 myelogenous leukemia cell line, and variable levels were detected in AML samples[287]. It is noteworthy that, in our study, 90% of patients with coordinated upregulation of *WT1* and *WT1-as* also displayed concurrent expression of *MEG3* (**Figure 25C**).

In addition, we demonstrated that BM of healthy control subjects uniformly exhibited an inverse correlation between *CDKN2B* and *ANRIL* expression, in which *CDKN2B* was downregulated and *ANRIL* was upregulated. These data are consistent with Teofili *et al.*[379], who reported a lack of *CDKN2B* expression in normal G0/G1- arrested CD34+ cells. In contrast, *CDKN2B* and *ANRIL* expression patterns were heterogenous among PMF samples, and these samples were divided into two groups based on whether CDKN2B was positively or negatively regulated (**Figure 26**). Although transcriptional silencing of *CDKN2B* by promoter

hypermethylation has been one of the most frequent molecular abnormalities reported for MDS[380], Herman *et. al.* reported that *CDKN2B* promoter methylation is rarely observed in myeloproliferative neoplasms such as CML[381]*.* Kotake *et al.* demonstrated that expression of the *CDKN2B* gene cluster is silenced by PRCs during normal cell growth and activated by oncogenic insult and by aging[288]; this is consistent with the downregulated *CDKN2B* expression that we found in our healthy control subjects. In the second part of this study, we determined whether expression patterns of *WT1-as*, *MEG3* and *ANRIL* in CD34+ cells from PMF patients correlated with their clinical features. We found that a large percentage of patients with concordant upregulation of *WT1* sense and antisense transcripts were exclusively classified into the intermediate-2 and high risk categories of the DIPSS-plus scoring system (**Figure 27A**), presented low Hb levels (**Figure 27C**), a higher percentage of circulating CD34+ cells (**Figure 27D**) and blast counts > 1% (data not shown). As we previously reported, high *WT1* gene expression levels in CD34+ cells from PMF patients were found to be associated with disease activity, such as the number of CD34+ cells in the PB or the disease severity score[292]. These data indicate that in addition to high levels of *WT1*, high levels of the *WT1* antisense transcript could identify PMF patients with more active disease. Similarly, patients with higher levels of *MEG3* expression cluster in higher risk DIPSS-plus scoring system categories (**Figure 28A**), suffer from anemia (**Figure 28C**), have higher levels of circulating CD34+ cells (**Figure 28D**), and blast counts >1% (data not shown). These data suggest that *MEG3* upregulation can also be used to identify patients with more severe disease. Interestingly, 90% of patients with increased *WT1* expression display concurrent *MEG3* expression (**Figure 25C**) and share the same clinical variables associated with poor outcome.

Finally, we observed a correlation between *CDKN2B* upregulation and the grade of BM fibrosis (**Figure 29A**). Further, 81.2% of patients with upregulated *CDKN2B* expression harbor the *JAK2V617F* mutation (**Figure 29C**). Given that chromosome 9p instability has been associated with MPNs[42] and that *CDKN2B*, *ANRIL* and *JAK2* genes are located on human chromosome 9p, this association is particularly interesting.

To our knowledge, this is the first study describing the expression profiles of human lncRNAs in CD34+ cells from PMF patients. Our data demonstrated

aberrant expression of *WT1-as*, *MEG3* and *ANRIL* lncRNAs in PMF CD34+ cells compared to healthy control cells. We were also able to correlate lncRNA expression patterns with PMF clinical features. In conclusion, our results suggest that *WT1-as*, *MEG3* and *ANRIL* could play key roles in PMF pathogenesis and could be used as new PMF diagnostic biomarkers with potential prognostic implications.

Taken together, results obtained for both coding and non coding RNAs elucidate some of the pathogenetic characteristics of PMF and shed lights on multiple alteration of CD34+ cells molecular signature.

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