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ABSTRACT

Myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders characterized by an overproduction of differentiated hematopoietic cells all derived from the same progenitor cell that has acquired one or more genetic mutations that give it a proliferative advantage. To this disorders belong polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) and genetic lesions include somatic driver mutations, so called because of their role in driving the myeloproliferative phenotype, are mutually exclusive in MPNs and take place in *JAK2*, *CALR* o *MPL*. The mutational landscape of MPN is much more complex and there are other mutations, with different function that can play a central role in clonal evolution and influence the clinical course. Moreover, exists a not negligible category of patients, not affected by the three mutations drivers and called "triple negative" (TN), for whom known molecular marker are lacking. Thus, the aim of this project is to explore new routes to possible novel prognostic treatments and alternative strategies for MPN patients and circularRNAs (circRNAs), non-coding RNA molecules, could be interesting for this purpose. From an RNA-seq analysis we detected 11 circRNAs disregulated in MPNs. Granulocytes and CD34+ cells were isolated from healthy donors' (n=12 and n=8,

respectively) and from bone marrow or peripheral blood of MPN patients (n=29 and n= 47, respectively) to perform qualitative analysis. We have identified n=6/11 circRNA because backsplicing validation frequencies result more markedly different in MPN compared to controls. Thus we selected one, circPLOD2, that results significantly overexpressed in MPN patients.

We divided patients for driver mutations and focused the interest on JAK2- mutated patients. We evaluated the expression levels of circPLOD2 in patients before and after the treatment with JAK2 inhibitor and we observed a downregulation after the treatment compared to control.
Moreover, CD34+ cells from healthy donors were transfected with the circPLOD2 overexpressing plasmid, and showed a significant increase of the megakaryotic colonies upon circPLOD2 overexpression.
This study showed that circPLOD2 expression is dependent on the JAK-STAT pathway activation. Of interest, enforced circPLOD2 expression significantly boosted differentiation of megakaryocytes. Further studies are needed to understand the role of circular RNAs in the context of myeloproliferative neoplasms, the data presented suggest that circular RNAs can be a useful tool for developing new diagnostic and therapeutic strategies in MPN.

INTRODUCTION

MYELOPROLIFERATIVE NEOPLASMS

Chronic myeloproliferative neoplasms (MPNs) comprise several hematologic diseases that arise from the transformation of a pluripotent stem cell and are characterized by the clonal proliferation of one or more hematopoietic progenitors in the bone marrow and extramedullary sites. The main clinical features of these diseases are the overproduction of mature, functional blood cells and a long clinical course (Campbell et al., 2006). MPNs, first conceptualized in 1951 by William Dameshek, historically included Polycythemia Vera (PV), Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF) and chronic myelogenous leukemia (CML)(Dameshek, 1951).

The association of the Philadelphia (Ph)-chromosome with CML in 1960 by Nowell and Hungerford, and the subsequent recognition of erythroleukemia as a variant of acute myeloid leukemia, distinguished the other three diseases (PV, ET and PMF) as "classic" Ph-negative myeloproliferative disorders. Dameshek was the first, in his seminal editorial published in 1951 in *Blood*, to recognize that these diseases should be classified as a set of phenotypically related "myeloproliferative disorders" characterized by bone marrow proliferation, "*perhaps due to a hitherto undiscovered stimulus*"(Levine et al., 2008).

However, the finding that bone marrow and peripheral blood cells from MPN patients can produce erythroid colonies in vitro in the absence of added growth factors indicated the cell autonomous nature of these diseases (Prchal et al., 1974), and the clonal origin of peripheral blood cells of MPN patients was later proven by analyzing X-chromosome inactivation patterns (Adamson et al., 1976). To underscore the clonal nature of myeloproliferative disorders and their propensity to evolve in Acute Myeloid Leukemia (AML), in 2008 the authors of the World Health Organization (WHO) *Classification of Tumours of Haematopoietic and Lymphoid Tissue* introduced the name "myeloproliferative neoplasms" (MPNs). The 2008 WHO classification for myeloid neoplasms, which incorporated novel information derived from molecular discoveries in *BCR-ABL* negative "classic" myeloproliferative and clonal eosinophilic disorders, included five major entities: Acute Myeloid Leukemia (AML), Myelodisplastic Syndromes (MDS), Myeloproliferative neoplasms (MPN); the category of overlapping Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN) and Myeloid/Lymphoid neoplasms associated with eosinophilia and specific molecular abnormalities. The substitution of the attribute "neoplasm" for "disease" was a relevant, formal recognition that both "classic" and "non-classic" myeloproliferative diseases share a common stem-cell-derived clonal heritage.

The three clinical entities share several common characteristics because caused by the clonal proliferation of a pluripotent hematopoietic stem cell, which leads to an increase in the proliferation of different myeloid linages.

The incorporation of clonal markers such as *JAK2V617F* mutation and similar activating, *PDGFRA*, *PDGFRB* or *FGFR1* rearrangements as well the acquisition of new histopathological informationwas consistent with the spirit of this revision (Tefferi et al., 2008; Vardiman et al. 2009). Although the 2008 WHO classification had undoubtedly the merit of promoting a systematic approach to these entities, taking into account clinical, morphological and molecular features, it presented controversial aspects such as the actual reliability of the proposed clinical parameters required for diagnosis, and, particularly, the diagnostic relevance and reproducibility of bone marrow morphology criteria. Moreover, the newly discovered molecular features have provided novel diagnostic and prognostic tools (Barbui et al., 2015; Barbui et al., 2016). As a result, in 2016 the WHO updated the 2008-WHO classification for hematopoietic and lymphoid neoplasms (Table 1) and adressed these issues formally integrating the assessment of the so called "driver mutations" (namely, *JAK2*$_{V617F}$, *MPL*$_{W515K/L}$ and *CALR* mutations) as one of several major criteria in the diagnosis of PV, ET and PMF. This revision regards also the evaluation of bone marrow morphology as another major criterion for the diagnosis of these three major entities of MPNs (Arber et al., 2016).

According to the 2016 WHO criteria, MPN include Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Myelofibrosis, which may be classified as primary (PMF), or secondary to PV and ET (post PV-MF and post ET-MF) (Vardiman et al. 2009). This classification has been updated with the introduction of prefibrotic/early PMF, distinguishable from ET on the basis of bone marrow morphology, having a higher tendency

to develop overt MF and characterized by a reduced overall survival (Arber et al., 2016). All MPN can transform into secondary acute myeloid leukemia, referred to as MPN-blast phase (BP), which is typically refractory to conventional chemotherapy with a poor prognosis (Tefferi et al., 2018). The 15-year risk of leukemia evolution is estimated at 2.1% to 5.3% for ET, 5.5% to 18.7% for PV and more than 20% for PMF whereas fibrotic progression rates in ET and PV, during a similar time interval, are estimated at 4% to 11% and 6% to 14%, respectively (Cerquozzi et al., 2015). MPN have a clinical heterogeneity, with some patients having normal lifespans and others developing disease progression or life-threatening complications. Treatment is mainly focused on reduction of thrombosis risk, control of myeloproliferation, improvement of symptoms, and management of related complications. Many treatments, both standard and experimental, are effective in reducing myeloproliferation, splenomegaly and constitutional symptoms, improving the quality of life in most patients. However, some patients do not respond to treatment, and many others become resistant. Nowadays, the only curative approach is the allogeneic hematopoietic stem cell transplant (allo-HSCT) which has a high mortality risk and is often not feasible for older patients and for those with comorbidities. Clonal proliferation in MPNs is driven by somatic mutations in JAK2, CALR or MPL, but in the last years several reviews focused on familial clustering of these neoplasms in order to identify predisposition allele(s) (Kralovics et al., 2003). This concept was further strengthened when a clear molecular distinction of true familial MPN from other familial syndrome such as familial erythrocytosis and hereditary thrombocythemia became possible

using clonality markers, cellular studies and JAK2 mutation analysis. Currently the GGCC (also known as 46/1) haplotype is the strongest known predisposing factor for MPNs carring JAK2 V617F mutation (Jones et al., 2013). It is known that hereditary factors can affect the pathogenesis of MPN. The analysis of a series of SNPs involving the JAK2 gene in subjects with MPN showed the recurrence of the particular haplotype of the gene, called 46/1. Other genetic variations have been described, like MECOM, TERT, JAK2, HBS1L-MYB, THRB-RARB and NR3C1genes (Tapper et al., 2015).

Table 1: The 2016 World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia. [For the full table referring to the source: Arber DA. et al*., The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia*. Blood. 2016, Vol. 127, n. 20, pp. 2392].

WHO myeloid neoplasms and acute leukemia classification
Myeloproliferative neoplasms (MPN)
Chronic myeloid leukemia (CML), BCR-ABL1, Chronic neutrophilic leukemia (CNL) Polycythemia vera (PV) Primary myelofibrosis (PMF) Pre-PMF, prefibrotic/early stage Overt-PMF, overt fibrotic stage Essenial thrombocythemia (ET) Chronic eosinophilic leukemia, not otherwise specified (NOS) MPN, unclassifiable **Mastocytosis**
Myeloid/lymphoid neoplasms with eosinophilia and rearrangement of PDGFRA, PDGFRB, or FGFR1, or with PCM1-JAK2
Myeloid/lymphoid neoplasms with PDGFRA rearrangement Myeloid/lymphoid neoplasms with PDGFRB rearrangement Myeloid/lymphoid neoplasms with FGFR1 rearrangement Provisional entity: Myeloid/lymphoid neoplasms with PCM1-JAK2
Myelodysplastic/myeloproliferative neoplasms (MDS/MPN)
Chronic myelomonocytic leukemia (CMML) Atypical chronic myeloid leukemia (aCML), BCR-ABL1 Juvenile myelomonocytic leukemia (JMML) MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) MDS/MPN, unclassifiable
Myelodysplastic syndromes (MDS)
MDS with MDS with single lineage dysplasia MDS with ring sideroblasts (MDS-RS) MDS with multilineage dysplasia MDS with excess blasts MDS with isolated del(5q) MDS, unclassifiable Provisional entity: Refractory cytopenia of childhood **Myeloid neoplasms with germ line predisposition**
Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities AML with myelodysplasia-related changes Therapy-related myeloid neoplasms AML-NOS Myeloid sarcoma Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage

B-lymphoblastic leukemia/lymphoma
T-lymphoblastic leukemia/lymphoma

Polycythemia Vera

Polycythemia Vera (PV) is the most common myeloproliferative disease, characterized by abnormal expansion of erythroid lineage, variably associated with thrombocytosis and leukocytosis, in absence of a recognizable physiologic stimulation. The first clinical definition of Polycythemia Vera was provided by Vaquez in 1892 when he described it as a condition of "persistent and excessive hypercellularity accompanied by cyanosis" (Vaquez, 1892). The incidence rate of PV is 0.86 cases per 100.000 people, but the true incidence may be higher because of asymptomatic cases. There is a little prevalence in male gender and the median age at diagnosis is 60 years (Stein et al., 2015). The abnormal proliferation of PV is a consequence of JAK2 gain of function gene mutations, with the majority of patients (>95%) harboring the JAK2V617F mutation and an additional 3% of PV patients being positive for mutations in exon 12 of the JAK2 gene (Kralovics et al., 2005; Levine et al., 2005; Scott et al., 2007). Recently other mutations or DNA variants involving 27 genes other than JAK2, CALR or MPL in patients with PV have been reported; the most frequent mutations included TET2, ASXL1, SH2B3,

SRSF2, IDH1/2, LNK (Tefferi et al., 2016; Tefferi et al., 2010). PV is characterized by bone marrow hyperplasia with hyperproliferation of erythroid lineage which is largely independent from erythropoietin (EPO). This is confirmed by the evidence of in-vitro spontaneous formation of erythroid endogenous colonies (EEC) in absence of EPO stimulation (Prchal JF, 1974). The main clinical manifestations of PV are consequences of excessive proliferation of red cells. Erythrocytosis causes augmented blood viscosity that leads to cerebral and peripheral microvascular involvement and related signs and symptoms; water-related pruritus is typical and affects 50% of patients. Constitutional symptoms such as weight loss, night sweats, and fever, are present at diagnosis in <30% of cases. Hepatomegaly, splenomegaly, cyanosis and arterial hypertension are common. The main complications are thrombotic events that can occur also in uncommon sites such as principal abdominal vessels. Increased plasma volume can lead to underestimation or failure to identify the increased erythrocyte mass. Leukocytosis and thrombocytosis are present in more than 50% of cases. During evolution the disease changes, and patients may present evolution in myelofibrosis (post-PV) and more rarely in acute myeloid leukemia. The most frequent cause of death in PV patients are cardiovascular event (41% of deaths). PV diagnosis is based on updated WHO criteria 2016 (Table 2), as a result of new important mutational and clinical discoveries: in order to confirm the diagnosis of PV all three major criteria are required, or the first two major criteria and the minor criterion.

Table 2: The 2016 WHO diagnostic criteria for PV [Arber DA. et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia*. Blood. 2016, Vol. 127, n. 20, pp. 2394].

2016 WHO PV criteria
Major criteria
1. Hemoglobin >16.5 g/dL in men; Hemoglobin >16.0 g/dL in women or, Hematocrit >49% in men; Hematocrit >48% in women or, increased red cell mass (RCM)*   2. BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)   3. Presence of *JAK2*^{V617F} or *JAK2* exon 12 mutation
Minor criteria
Subnormal serum erythropoietin level

*More than 25% above mean normal predicted value.

Essential Thrombocythemia

Essential Thrombocythemia (ET) is characterized by abnormal megakaryocytic proliferation with consequent increased peripheral platelets. The first report is from 1934 by Dr. Emil Epstein and Dr. Alfred Goedel (Epstein et al. 1934). The incidence rate varies between 1.0-2.5 cases per 100.000 people and it is more common in females. It is more frequent between the fifth and seventh decades of life, although it may be present at any age.

ET is triggered by several mutations causing hyperactive cytokine signaling. The most common mutations is *JAK2V617F* (50-60% of cases), followed by *CALR* (15-35% of cases) and *MPL* (4% of cases) mutations. The recent targeted study mentioned above identified *SH2B3, SF3B1, U2AF1, TP53, IDH2* and *EZH2* as adverse mutations (Tefferi et al., 2017; Tefferi et al., 2016). If none of the most common mutations are found essential thrombocytosis is largely a diagnosis of exclusion because isolated thrombocytosis may be the first manifestation of polycythemia vera, primary myelofibrosis or myelodysplastic disorders (Spivak et al., 2017). Other possible causes of reactive thrombocytosis have different prognosis and treatment. Thromboembolic symptoms are the most severe manifestation in this pathology, especially when they occur in central nervous system arteries or in the cardiovascular system. Other characteristic symptoms bound with thrombosis in ET are: erythromelalgia, deep vein thrombosis, with high risk of pulmonary embolism, and splanchnic veins thrombosis. Hemorrhagic events are less common than thromboembolic symptoms. Acquired form of von Willebrand disease induced by high platelet count in ET has been also reported: this makes worse prognosis in these patients.

In addition to thrombo-hemorrhagic symptoms other possible complications that must be considered are myelofibrotic transformation (post-ET) and leukemic transformation (Passamonti et al., 2008, Wolanskyi et al., 2006). WHO developed

new criteria for ET diagnosis in 2016 (Table 3). Diagnosis of ET requires meeting all four major criteria or the first three major criteria and the minor criterion.

Table 3: The 2016 WHO diagnostic criteria for ET [Arber DA. et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood. 2016, Vol. 127, n. 20, pp. 2395].

2016 WHO ET criteria
Major criteria
1. Platelet count ≥ 450 × 10^9/L 2. BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers 3. Not meeting WHO criteria for *BCR-ABL*[11] *CML, PV, PMF,* myelodysplastic syndromes, or other myeloid neoplasms 4. Presence of *JAK2, CALR*,or *MPL* mutation
Minor criteria
Presence of a clonal marker or absence of evidence for reactive thrombocytosis

Morphological features of megakaryocytes in ET are very important especially in patients with suspected ET but that are negative for somatic mutations. Marrow aspiration or biopsy show large, hyperlobulated and mature appearing megakaryocytes, without cells adhesion (this is an important difference vis-à-vis myelofibrosis which features megakaryocytes organized in clusters) or dysplastic features (also typical of MF).

Primary Myelofibrosis

Primary Myelofibrosis (PMF) is a clonal disorder of multipotent hematopoietic progenitor cell of unknown etiology characterized by marrow fibrosis and osteosclerosis, extramedullary hematopoiesis, splenomegaly, anemia, neutrophilia and thrombocytosis or thrombocytopenia and leukopenia, immature granulocytes and increased CD34+ cells, erythroblasts and teardrop-shaped red cells in the blood, and constitutional symptoms due to inflammatory cytokine production. The first description of PMF dates back to 1879, when Heuck firstly reported about myelofibrosis in a paper titled "*Two cases of leukemia and peculiar blood bone marrow findings*" (Heuck et al., 1879). Over the years, PMF has also been referred to by a variety of others terms, such as agnogenic myeloid metaplasia, myelosclerosis, idiopathic myeloid metaplasia and idiopathic myelofibrosis (Tefferi et al., 2007). The current designation PMF reflects our greater understanding of the origin of this disorder, and differentiates a *de novo* disease from myelofibrosis which is preceded by a history of PV or ET (post PV/ET myelofibrosis).

PMF is the least common and most aggressive myeloproliferative neoplasm, occurring in the majority of cases after the fifth decade of life, with a peak of incidence at 65-70 years old; however, several studies report it can occur also in childhood and adolescence (Cervantes et al., 2001). Clinically the manifestations are similar in adult and younger patients, but normally the frequency of indolent

cases is higher in pediatric age. The estimated incidence of PMF is between 0.1 and 1.0 per 100.000 per year, with a higher prevalence in males than in females (Moulard et al., 2014).

The key mutational abnormalities associated with PMF are: *JAK2* mutations, especially V617F, that occur in almost 60% of PMF patients; *CALR* mutation, either 52- bp deletion and 5-bp insertion, in 20% of cases; and *MPL* in 7% of cases. Other somatic mutations have also been detected such as *TET2, ASXL1, DNMT3A, EZH2*, and *IDH1/2, SRSF2*, implicated in epigenetic regulation, and *TP53* and *CBL* (Lundberg et al., 2014). Additional adverse mutations identified in PMF include *CBL, KIT, RUNX1, SH2B3* and *CEBPA* (Tefferi et al., 2016). The clonality of this disorder has been extensively observed in several studies in murine models. CD34+ cells play a key role in peripheral blood migration and neoplastic megakaryocytopoiesis of PMF. The migration of CD34+ cells is probably a consequence of an epigenetic alteration due to the hypermethylation of the CXCR4 promoter. This modification induces reduced expression of CXCR4 on CD34+ cells and enhances their migration into the blood (Bogani et al., 2008). In addition, a study about the molecular profiling of CD34+ cells in PMF demonstrated that these cells have a higher expression of some genes, namely *CD9, GAS2, DLK1, CDH1, WT1, NFE2, HMGA2*, and *CXCR4*, that is abnormal for this kind of cells. These genes are indeed involved in the pathogenesis and in the clinical features of PMF (Guglielmelli et al., 2007).

Another characteristic of PMF is the enhanced bone marrow angiogenesis and blood flow, probably connected with an increasing of circulating endothelial cell progenitors (Massa et al., 2005). Finally, we have to consider the different features of hematopoiesis dysfunction. Neoplastic proliferation is the main bone marrow alteration especially in granulocytic and megakaryocytic lineages, resulting in panmyelosis but few circulating mature blood cells, especially granulocytes and platelets. Likely, this is related to an exaggerated apoptosis of very early precursors. Anemia is a frequent finding in PMF as a result of insufficient erythropoiesis, short erythrocyte survival and splenic sequestration, as a consequence of hypersplenism. However, it is important to exclude other possible causes of bone marrow fibrosis.

Many patients are asymptomatic at the time of diagnosis, but some of them refer fatigue as a principal complaint in association with night sweats, fever, weight loss and bone pain. Inflammatory cytokines, like IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, TNF receptor II (TNFRII), and C-reactive protein show significantly higher concentration and play a role in the constitutional symptoms (Tefferi et al., 2008). In most PMF cases it is possible to palpate a splenic enlargement. Dragging sensation, early satiety, left hypochondrial pain or left shoulder pain are consequences of splenomegaly and splenic infarction, respectively.

Patients can show organ failure due to extramedullary hematopoiesis, and portal hypertension and varices, due to an enhanced hepatic blood flow, hepatic vein

thrombosis or decreased hepatic vascular compliance (Jacobs et al., 1985). Thrombotic risk is elevated in PMF patients, but not as much as in PV or ET. However almost 10% of cases developed a thrombotic event in the first four years from the diagnosis. The two main risk factors for thromboembolic events in PMF patients are elevated leukocyte count and age, but not platelet count (Buxhofer-Ausch et al., 2012).

The increasing marrow failure with transfusion-dependent anemia and increasing organomegaly due to extramedullary hematopoiesis is one of the most common complications in PMF. Leukemic transformation could also occur and has dismal prognosis because of the poor response of the treatment.

The 2016 revision of WHO classification of myeloproliferative neoplasms defines two stages of primary myelofibrosis (PMF): prefibrotic/early (pre-PMF) (Table 4) and overt fibrotic (overt-PMF) (Table 5) phase. In comparison with overt PMF, patients with pre-PMF are more frequently female, younger, and have higher hemoglobin. By contrast, leukocytosis and blasts >1% in peripheral blood are more frequent in overt-PMF as well as LDH level, clinical features (especially splenomegaly) and unfavorable karyotype. Diagnosis of pre-PMF as well as for overt-PMF requires meeting all three major criteria and at least one minor criterion. According to the International Prognostic Scoring System (IPSS), pre-PMF patients may be included in lower-category risk in comparison to patients with overt- PMF,

more frequently classified in higher-category risk as demonstrated by Guglielmelli et al. (2017).

Table 4: The 2016 WHO diagnostic criteria for pre-PMF [Arber DA. et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia*. Blood. 2016, Vol. 127, n. 20, pp. 2395].

2016 WHO pre-PMF criteria
Major criteria
1. Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade 1, accompanied by increased age-adjusted BM cellularuty, ranulocytic proliferation, and often decreased erythropoiesis. 2. Not meeting WHO criteria for *BCR-ABL*[11] *CML, PV, ET,* myelodysplastic syndromes, or other myeloid neoplasms 3. Presence of *JAK2, CALR* or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of minor reactive BM reticulin fibrosis.
Minor criteria
Presence of at least 1 of the following, confirmed in 2 consecutive determinations: a. Anemia not attributed to a comorbid condition b. Leukocytosis ≥ 11 x 10^9/L c. Palpable splenomegaly d. LDH increased to above upper normal limit of institutional reference range

Table 5: The 2016 WHO diagnostic criteria for overt-PMF [Arber DA. et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood. 2016, Vol. 127, n. 20, pp. 2396].

2016 WHO overt-PMF criteria
Major criteria
1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grade 2 or 3. 2. Not meeting WHO criteria for *BCR-ABL*[11] *CML, PV, ET,* myelodysplastic syndromes, or other myeloid neoplasms 3. Presence of *JAK2, CALR* or *MPL* mutation or in the absence of these mutations, presence of another clonal marker, or absence of reactive myelofibrosis.
Minor criteria
Presence of at least 1 of the following, confirmed in 2 consecutive determinations: a. Anemia not attributed to a comorbid condition b. Leukocytosis ≥ 11 x 10^9/L c. Palpable splenomegaly d. LDH increased to above upper normal limit of institutional reference range e. Leukoerythoblastosis

Molecular basis of Myeloproliferative Neoplasms

In the last decade, our understanding of the molecular aspects of Philadelphia-negative MPN has been dramatically revolutionized by the genetic discoveries in

2005 with the identification of the JAK2V617F mutation (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005; Baxter et al., 2005). Since then, several somatic mutation have been described, and their pathogenetic role is currently under investigation, but non of them appear to garner the disease specificity or pathogenetic relevance otherwise displayed by the Philadelphia chromosome in CML. Overall, it is now clear that among the numerous genetic culprits that can affect the neoplastic clone, three mutations (JAK2V617F, MPLW515K/L and CALR mutations) play a crucial role in driving the disease phenotype and affecting the clinical course of MPNs, so that they have been recently re-defined as "driver mutations" (Tefferi et al., 2015). JAK2, MPL and CALR mutations are considered phenotypic driver since the expression of the mutated gene in cell lines caused cytokine independent or hypersensitive growth, as known to occur in primary cells from MPN patients, and in animal models phenotypes closely resembling a myeloproliferative disease were observed in transgenic or conditional animals (Li et al., 2011). The subclonal mutations usually occur in hematopoietic cell subclones of variable size, often but not invariably together with one of the phenotypic driver mutations, and may either antedate or follow the acquisition of phenotypic driver mutations (Vainchenker et al., 2011). The order by which these mutations appear in the mutational phylogenesis of MPN contributes to the disease phenotype, while the significance is unclear if any, is far from being appreciated (Ortmann et al., 2015; Nangalia et al., 2015). Since these mutations are commonly represented also

in myelodysplastic syndromes, other myeloid neoplasia and acute leukemias, they have no specific diagnostic value, while, on the other hand, they contribute remarkably to the prognosis of patients with PMF.

Driver mutations in MPNs

JAK2 MUTATIONS. JAK2 is the most common myeloproliferative neoplasm driver gene. A member of the Janus tyrosine kinase family, JAK2 is responsible for signal transduction by the erythropoietin (EPO), thrombopoietin (TPO), granulocyte macrophage colony stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) receptors in hematopoietic cells, as well as for signal transduction by many cytokine receptors, such as interleukin IL-3, IL-5, and IL-6 receptors (Verma et al. et, 2003). The cytoplasmic domains of these receptors are docking sites for Janus Kinases (JAK). JAK2 is one of the four JAKs, it has a dual kinase structure and contain an N-terminal FERM domain, a Src-homology like domain (SH2), a pseudokinase domain (JH2), and a C-term kinase domain (JH1). The FERM and SH2 domains anchor JAK2 to cytokine receptors, JH2 regulates kinase activity, while JH1 is responsible for tyrosine phosphorylation. JH1 paired in tandem with a weakly active JH2, which normally inhibits JH1 kinase activity in the absence of ligand binding. Following the binding of cytokines to their receptor, a change in receptor conformation takes place so that JAK2

phosphorylates in trans Y1007 and Y1008 in the activation loop of the JH1 domain, thus inducing JAK2 activation. JAK2 phosphorylates cytokine receptors and signal transducers and activators of transcription (STAT) bind to phosphorylated tyrosine residues on these cytokine receptors through their SH2 domains whereupon JAKs can themselves be phosphorylated by JAKs (Vainchenker et al., 2013). Phosphorylated STATs can translocate into the nucleus to be part of transcriptional complexes. In addition to activating STATs, JAKs also activate mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K) –AKT pathway. The hyperactivation of these three pathways induce survival, proliferation, and myeloid differentiation.

In 2005, Dr. Skoda's, Dr. Vainchenker's and Dr. Gilliand's labs, using different approaches, discovered a recurrent acquired mutation in JAK2 gene in a significant proportion of patients with MPN (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). This dominant gain-of-function mutation is a guanine-to-thymine substitution at nucleotide 1,849 in exon 14 of the JAK2 gene that results in a valine-to- phenylalanine substitution at codon 617 of JAK2. The JAK2V617F mutation maps to the JH2 pseudokinase domain, it rigidifies the α helix C of the domain, promoting the transphosphorylation of JH1, impairing its physiologic inhibitory influence. Tyr 1007 and Tyr 1008 in the JH1 domain are phosphorylated, so that JAK2V617F is constitutively active in the absence of cytokine (Silvennoinen et al., 2015).

In the heterozygous state, JAK2V617F-bearing receptors are still responsive to growth factors. Only with JAK2V617F homozygosity, usually due to 9p uniparental disomy, do these receptors become entirely autonomous with respect to growth factor.

Given that the mutant JAK2V617F is constitutively activated, it promotes pro-survival and anti-apoptotic signals as well as cytokine-independent growth (Kundrapu et al., 2008). In fact, when JAK2V617F is expressed in hematopoietic cells, several signaling pathways that are important for proliferation and survival are activated (Levine et al., 2005; Vainchenker et al., 2013).

In addition, mice transplanted with marrow cells transduced with a retrovirus expressing JAK2V617F invariably developed erythrocytosis eventually associated with leukocytosis, splenomegaly and later changes suggestive of transformation to post- polycythemic myelofibrosis (Wernig et al., 2006; Lacout et al., 2006; Bumm et al., 2006; Zaleskas et al., 2006).

More recently, transgenic mice presenting an expression of mutated allele lower than wild-type one have been generated, and found to develop an ET-like phenotype (Tiedt et al., 2008; Shide et al., 2008); overall these data suggest that the JAK2V617F mutation is an integral component of the myeloproliferative process that underlies the different classic MPNs.

JAK2 also serves as an endoplasmic reticulum chaperone for the erythropoietin and thrombopoietin receptors, transporting them to the cell surface, and increases the

total number of thrombopoietin receptors by stabilizing the mature form of the receptor, enhancing receptor recycling, and preventing receptor degradation. However, in contrast to its effect on the erythropoietin receptor, JAK2V617F appears to increase the quantity of immature MPL molecules while increasing MPL degradation through ubiquitination and reducing its cell-surface expression. In addition to functioning as a tyrosine kinase and chaperone, mutated JAK2 is sumoylated, permitting it to shuttle to the nucleus, where it deregulates gene transcription directly through histone phosphorylation and indirectly by phosphorylating and inhibiting PRMT5, a histone arginine methyltransferase (Spivak, 2017).

The mutation can be found on one or both alleles (homozygosity) as a result of a mitotic recombination process resulting in uniparental disomy that occurs in most patients with PV or PMF and a minority only of ET. Different studies suggested that in ET and PV, a higher allele burden is associated to disease progression to MF, but in contrast, a low allele burden is related to an inferior overall and leukemia-free survival in MF (Steensma et al., 2006; Guglielmelli et al., 2009; Vannucchi et al., 2011).

Approximately 3% of patients with PV have insertions or deletions in JAK2 exon 12 at the interface of the JAK2 SH2 and JH2 domains, which enable constitutive kinase activation, possibly also by altering the interface between the JH2 and JH1 domains (Scott et al., 2011). The JAK2 exon 12 phenotype is usually more benign

than that of JAK2V617F, often causing erythrocytosis alone, though a complete polycythemia vera phenotype can develop, as can homozygosity or coexistence with JAK2V617F (Passamonti et al., 2011).

CALR MUTATIONS. The gene that encodes calreticulin, is the second most common myeloproliferative driver gene. In 2013, whole-exome sequencing analyses contemporary performed by Dr. Green's and Dr. Kralovics's groups led to the identification of CALR exon 9 mutations in patients with MPNs who had no mutations in either JAK2 or MPL. (Nangalia et al., 2013; Klampft et al., 2013).

The *CALR* gene is located in the short arm of chromosome 19 (19p13.2). It contains 9 exons and spans 4.2 kb. So far, more than 50 different types of *CALR* mutations have been identified, all of which occur in exon 9. With the exception of a few non-recurrent point mutations (Wu et al., 2014), almost all of these mutations are small insertions and delection. The commonest mutations accounting for 85% of mutated cases are a 52 base-pair (bp) deletion (CALRdel52/Type 1; c.1092_1143del; L367fs*46; 45-53% of all cases) or a 5 base pairs (bp) insertion (CALRins5/Type 2; c.1154_1155insTTGTC; K385fs*47; 32-41% of all cases).

Interestingly, the frequency of type 1 mutation is significantly higher in PMF than in ET, suggesting a specific role of these mutations in myelofibrotic transformation (Rumi et al., 2014; Cabagnols et al., 2015). Most mutated genes have a mutation in

heterozygosity. Homozygous *CALR* mutations are very rare and they are most 5-bp insertions (Nangalia et al., 2013; Klampfl et al., 2013).

Remarkably, all mutations are exclusively +2/-1 base-pair frameshifts that cause a +1 frameshift in the reading frame, and thus generate a novel amino acid sequence (characterized by the acquisition of a minimal 36 amino acid stretch in place of 27 amino acids that are lost from the normal sequence) common to all mutant CALR proteins on their C-terminal end. As a result, mutant CALR proteins contains a number of positively charged amino acids; such variability may be associated with qualitatively different phenotypes of *CALR*-mutated MPNs.

CALR is a developmentally highly conserved luminal ER (endoplasmic reticulum) chaperone protein that ensures the proper folding of newly synthesizes glycoproteins; it has also been implicated in several other roles both within and outside of the ER, including calcium homeostasis, immunogenic cell death, proliferation, phagocytosis, apoptosis as well as the assembly and cell surface expression of major histocompatibility complex (MHC) class I molecules (Michalak et al., 2009; Chao et al., 2012). CALR is a 46 kDa protein that consists of multiple domains. The first 17 amino acids comprise a signal peptide sequence that is cleaved upon entering the ER (Denning et al., 1997). This sequence is followed by the amino-terminal domain (N-domain; residues 1-180), which contains amino acids required for carbohydrate binding, zinc binding, and

chaperone activity (Kapoor et al., 2003; Guo et al., 2003). Next comes the proline-rich P-domain (residues 181-290), which binds to calcium and ERp57 that cooperates with CALR for protein folding (Martin et al., 2006). The last C-domain (residues 291-400) shows a capacity for calcium storage and includes an ER retention signal sequence, the KDEL (Nakamura et al., 2001; Araki et al., 2017).

The mutant calreticulin protein C-terminus differs from the wild-type protein in two ways. First, a Golgi-to-ER retention signaling motif (KDEL) responsible for retrieving and retaining chaperone proteins back to the ER is lost in the mutant protein. Second, CALR mutations have been shown to be acquired at the level of the hematopoietic stem cell (HSC) and clonal characterization of MPN samples has shown that mutated CALR is present in the earliest clone, which is consistent with it being an initiating event in these malignancies.

Mutant CALR requires both its mutant C terminus and MPL for oncogenic transformation. Interestingly, it was found that CALR binds to the N-glycosylated residue of the extracellular domain of MPL in the ER, and the lectin-binding activity of CALR mutants as well as the new tail are required for MPL activation. This binding is reinforced by the new C-terminus and its positive charges, which lead to activation of the TPO receptor (Chachoua et al., 2016; Elf et al., 2016; Araki et al., 2016). Some studies have demonstrated that *JAK2/CALR* mutations and *MPL/CALR* mutations are not mutually exclusive (Ahmed et al, 2016; Tashkandi et

al., 2017; Bernal et al., 2017). Comparing clinical outcomes of CARL mutation vs. JAK2 or MPL positive cases, the former seem to have more benign prognosis in terms of survival and lower risk of thrombosis due to lower hemoglobin levels and leukocytes count than JAK2 positives (Rumi et al., 2014; Rotunno et al., 2014).

MPL MUTATIONS. In 2006, Gary Gilliland's research team identified in some patients with PMF JAK2 wild-type a new somatic point mutation in the MPL gene, coding for the thrombopoietin receptor (Pikman et al., 2006). MPL mutations are the least common myeloproliferative neoplasm driver mutations, occurring in <10% of PMF and ET. MPL is a unique type I hematopoietic cytokine receptor because it is the only one expressed in hematopoietic stem cells; it also has a reduplicated extracellular cytokine-binding domain. Somatic MPL mutations occur most often in exon 10 and result in a switch from tryptophan to leucine or lysine or, less frequently, to arginine or alanine at amino acid 515 (MPL W515L/K or W515R/A) in the MPL juxtamembrane domain (Pardanani et al., 2006).

They have been detected in 5% to 11% of patients with PMF and in up to 9% of JAK2V617F-negative cases of ET (Beer et al. 2008; Vannucchi et al., 2007). A less common mutation, S505N, in the MPL transmembrane domain, in which serine is switched to asparagine, can be inherited or acquired and causes ET (Williams et al., 2007) MPL mutations force a change in receptor conformation, activating JAK2 in the absence of thrombopoietin binding. Like JAK2 and CALR mutations, however,

MPL mutations require a hematopoietic growth factor, in this case thrombopoietin, for complete kinase activation in the heterozygous state. Myeloproliferative neoplasm driver mutations also occur in the MPL extracellular distal cytokine domain. For example, MPL S204P/F are acquired mutations causing ET or PMF, whereas the germline MPL variants, K39N (MPL Baltimore) and P106L, cause a benign form of thrombocytosis in African-American and Arab populations, respectively, which is most marked in the homozygous state. Interestingly in some patients, multiple MPL mutations or the coexistence with the JAK2V617F allele were described (Guglielmelli et al., 2007; Vannucchi et al., 2008; Lasho et al., 2006).

In patients with ET, MPL mutations are associated with greater myelofibrotic transformation, but there is no difference in overall or leukemia-free survival between patients with MPL mutations and those with JAK2V617F, and there appears to be no survival difference between patients with PMF who have MPL mutations and those who have JAK2V617F mutations (Tefferi et al., 2010; Guglielmelli et al., 2007).

JAK2V617F impairs MPL maturation, increasing the proportion of immature receptors in the plasma membrane; reduces MPL recycling; and increases its degradation. Impaired MPL cell-surface expression, which is also a feature of CALR and MPL mutations, results in elevated plasma thrombopoietin levels, as a result of reduced clearance of thrombopoietin from the plasma by megakaryocytes

and platelets, and may also be involved in the emigration of involved hematopoietic stem cells from their marrow niches.

Additional mutations in MPNs

High-throughput next generation sequencing (NGS) analyses have identified a high number of additional somatic mutations with a prognostic and therapeutic value, particularly in PMF. More than 50% of MPN patients harbor additional mutations (Grinfeld et al., 2018). These are not restricted to MPN but are also common in other myeloid malignancies as acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). These concomitant mutations are more frequent with increasing age, not only in patients, but also in healthy individuals in the context of age-related clonal hematopoiesis (ARCH) (Shlush et al., 2018). Affected genes are involved in DNA methylation (TET2, DNMT3A, IDH1 and IDH2), histone modification (ASXL1, EZH2), mRNA splicing (SFRB1, SRSF2, U2AF1 and ZRSR2), signaling pathways (LNK/SH2B3, CBL, NRAS, KRAS, PTPN11), transcription factors (RUNX1, NFE2 TP53, PPM1D).

TET2 enzyme is involved in demethylation through oxidation of 5-methyl-cytosine (5-mc) into 5-hydroxymetylcytosine (5-hmc), an important process in stem cell

gene regulation. TET2 loss of function mutations occur in 10-15% of MPN patients and 20-25% of blast-phase (BP) and are the most common co-occurring mutations with JAK2V617F (Tefferi et al., 2016; Lasho et al., 2018). Although TET mutations have no a defined impact on prognosis and thrombosis, recently it has been reported that the order of acquisition of JAK2 and TET2 mutations may affect MPN phenotype with "JAK2-first" is more commonly detected in PV and "TET2-first" in ET (Ortmann et al., 2015). DNMT3A mutations are frameshift/nonsense resulting in reduced methyltransferase activity and occur in 5-15% of MPN, with lower frequency in PV and ET compared to PMF and BP (Tefferi et al., 2016; Tefferi et al., 2016; Lasho et al., 2018). Like TET2, patients are more likely to present with PV when JAK2V617F is acquired before DNMT3A mutation, compared with patients who first acquired DNMT3A mutation which most commonly have an ET phenotype (Nangalia et al., 2015). Mutations in isocitrate dehydrogenase genes (IDH1 and IDH2) are heterozygous and occur mostly as point missense mutations at residues R132 in IDH1 and R140 or R172 in IDH2. These mutant proteins acquired the ability to convert alpha-ketoglutarate (a-KG) to 2 - hydroxyglutarate (2-HG), favoring leukemogenesis through epigenetic dysregulation of some genes. IDH1 and IDH2 mutations have been associated with worse prognosis in PMF with a high risk to transformation in BP, in fact they are reported in up to 6% of MPN and up to 30% of BP (Tefferi et al., 2010; Guglielmelli et al., 2014). ASXL1 and EZH2 encode for factors involved in histone

modifications. ASXL1 mutations are heterozygous nonsense and frameshift mutations in exon 12 and are more common in PMF and BP (18-37%) compared with PV and ET (5-10%) (Vannucchi et al., 2013; Guglielmelli et al., 2014).

They are associated with a worse prognosis in PV and PMF patients (Tefferi et al., 2016; Tefferi et al., 2014; Tefferi et al., 2018), also in those who underwent allogenic stem cell transplantation (allo-HSCT) (Kröger et al., 2017). Moreover, it has been recently reported that ASXL1 mutations are frequently acquired during ruxolitinib treatment (Newberry et al., 2017). EZH2 mutations are less frequent than those in ASXL1 and they are found in up to 10% of MPN and up to 15% in BP patients. EZH2 seems to be a tumor suppressor in MPN, and a high number of loss of function mutations have been identified that synergizes with JAK2V617F in initiating MPN and promoting myelofibrosis (Shimizu et al., 2016). In PMF, EZH2 mutations correlate with a higher leukocyte count, blast count and larger spleen size at diagnosis. Moreover, EZH2 mutations are an independent poor prognostic factor that importantly reduce overall survival (Guglielmelli et al., 2011).

Acquired mutations in genes encoding for spliceosome proteins and other regulatory splicing factors are reported in all myeloid malignancies, particularly MPN and MDS, and affected mainly SRSF2, U2AF1, ZRSR2 and SF3B1 genes. These mutations are typically mutually exclusive, occur in heterozygous and with a missense alteration, giving a dominant negative activity that affects RNA splicing

(Yoshida et al., 2011). SRSF2 mutations, mainly involved hotspot at position 95, are infrequent in PV and ET, but occur in 8-22% of PMF and BP, associated with a poor prognosis and leukemic transformation (Vannucchi et al., 2013; Lasho et al., 2012; Zhang et al., 2012; Tefferi et al., 2018; Tefferi et al., 2018). U2AF1 mutations, mainly at hotspot regions S34 and Q157, occur in 5-15% of PMF and BP and are associated with a significantly shorter overall survival (Tefferi et al., 2018). SF3B1 mutations are found in exons 14 -16, particularly in codon 700, and are more common in PMF and BP (up to 10%). Although rare in PV and ET patients, U2AF1 and SFRB1 mutations confer an inferior myelofibrosis-free survival (Tefferi et al., 2016). ZRSR2 mutations are very rare in MPN, among them they are more common in PMF without a clear impact on prognosis. LNK/SH2B3 is an adaptor protein that inhibits signaling through cytokine and tyrosine kinase receptors, including JAK2 (Bersenev et al., 2008; Tong et al., 2005). Many mutations are missense substitutions and they are identified in up to 10% of MPN and BP patients (Tefferi et al., 2016; Tefferi et al., 2016; Lasho et al., 2018). Although somatic *LNK* mutations are detected in MPN as acquired mutations all through the gene, *LNK*E208Q variant is the only one identified as a germline variant in cases of idiopathic erythrocytosis, and acquired in MPN cases, either alone or associated with a driver mutation (Maslah et al., 2017). Moreover, *LNK*E208Q is described as a germline variant in cases of familial MPN (Loscocco et al., 2016; Rumi et al., 2016). CBL mutations lead to increased STAT5 phosphorylation,

cytokine hypersensitivity and cell proliferation (Sanada et al., 2009); these mutations are mostly homozygous missense substitutions, detected in up to 6% of PMF and BP cases (Tefferi et al., 2016; Lasho et al., 2018). Heterozygous missense mutations in *NRAS*/*KRAS*, particularly in codons 12, 13 and 61 led to a constitutive activation of growth signaling (Braun et al., 2008); they are rare in PMF (up to 5%) and reported in up to 15% of BP (Tefferi et al., 2016; Tefferi et al., 2016). *CBL*/*NRAS*/*KRAS* mutations in PMF patients have been associated with adverse clinical features and overall survival and poor response to JAK inhibitor therapy (Coltro et al., 2020). *PTPN11* mutations are found in up to 8% of BP and are associated with a reduced overall survival (Lasho et al., 2018). Among transcription factors, *RUNX1* and *NFE2* are the most common mutated genes in MPN. Inactivating *RUNX1* mutations, occur in up to 10% of BP with a significantly shortened survival versus *RUNX1* wild type BP (Grinfeld et al., 2018). NFE2 mutations in MPN are rare and heterogenous with a prevalence of frameshift and deletion (Jutzi et al., 2013). In a recent paper, these mutations were slightly enriched in PV (8% of cases) and they do not appear to have meaningful hematological and clinical correlates, nor a prognostic value (Guglielmelli et al., 2020). TP53, is the transcription factor with a tumor suppression physiological function, with a key role in response to cellular stress and DNA damage. *TP53* mutations are frequent in BP, wherein biallelic loss of *TP53* has been reported in up to 35% of cases, with a poor outcome (Venton et al., 2018). Recently, a very low burden of *TP53* mutations has

been observed in the chronic phase of MPN, even for many years from diagnosis, associated with older age (Kubesova et al., 2018). The significance of these very low burden mutations, in relation to the risk of evolution into BP, is still unknown. Mutations in PPM1D, a regulatory inhibitor of TP53, have been recently described in MPN in 1.9% of patients, within the driver clone and also independently (Grinfeld et al., 2018). PPM1D are more frequent in cases of therapy-related AML and MDS, particularly after cytotoxic agents (Hsu et al., 2018), with an intrinsic chemoresistance to conventional therapies (Kahn et al., 2018).

Triple-negative MPN patients

The term triple-negative (TN) MPN has been coined and applied to patients without evidence of the 3 major mutation types of *JAK2* V617F, *MPL* W515L/K and *CALR* exon 9 indels. This particular signature has been reported to be associated with a relatively adverse survival (Figure 1) (Langabeer SE, 2016). In particular in approximately 10-15% of essential thrombocythemia (ET) and less than 10% of primary myelofibrosis (PMF), driver mutations are still unknown (Tefferi et al., 2017). Approximately 10% of TN ET and PMF patients have mutations outside of MPL exon 10 and JAK2 exon 14. These non-canonical MPL mutations include T119I, S204F/P and E230G in the extracellular domain and Y591D/N in the

intracellular domain (Cabagnols et al., 2016). Non-canonical JAK2 mutations include V625F, F556V, R683G and E627A93. As demonstrated in functional studies, most of these rare mutations lead to a constitutive activation of the JAK-STAT signaling (Cabagnols et al., 2016; Milosevic et al., 2016). In addition to somatic mutations also germline variants can contribute to the path hyperactivation with the possibility that many of these patients may have a form of non-clonal erythrocytosis or thrombocytosis with a variable family penetrance. Whereas the TN PMF have a poor prognosis, TN ET patients seems to have a good prognosis. However, it is presently unknown whether these TN MPN are a homogeneous entity.

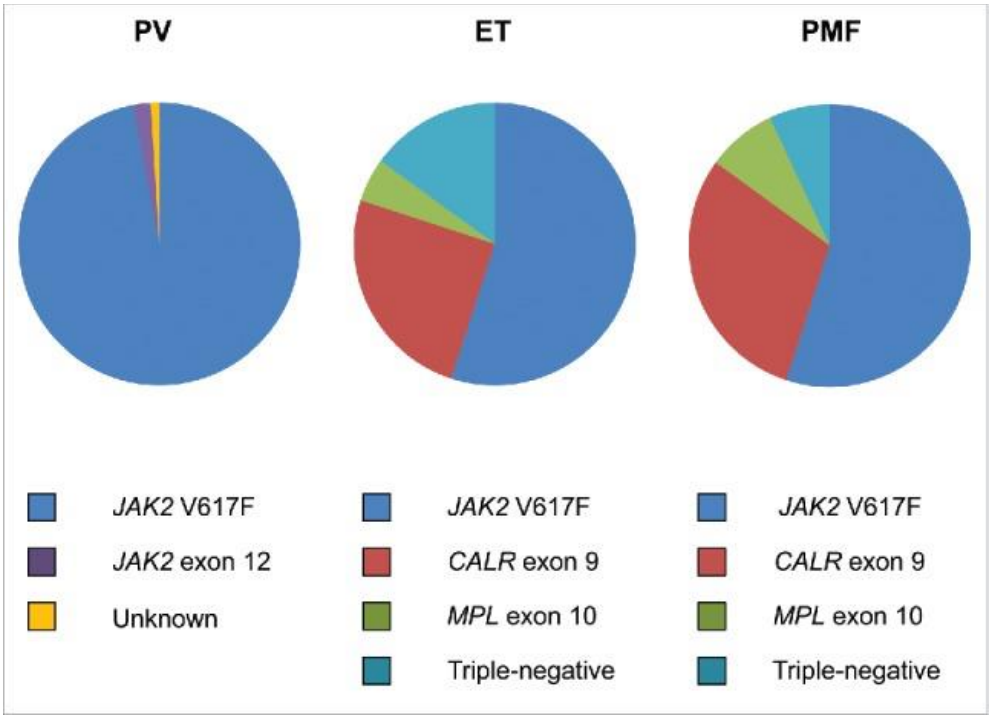

Figure 1: Distribution of driver mutations polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). [Langabeer SE. Chasing down the triple-negative myeloproliferative neoplasms: Implications for molecular diagnostics. JAKSTAT. 2016.]

CIRCULAR RNAs

Circular RNAs (circRNAs) were first found in pathogens. In 1976, Sanger and others (Sanger et al.,1976) described viroids that contain "single-stranded and covalently closed circular RNA molecules". A second study published in 1979 described RNAs that contained no free ends and their circularity was independent of associated proteins (Hsu and Coca-Prados, 1979). After these initial reports, sporadic studies identified and characterized circRNAs generated from endogenous RNAs. The first of these reports, in 1991, described the serendipitous discovery of transcripts produced from non-canonical splicing called "scrambled exons" that originated from the Deleted in Colon Cancer gene (DCC) (Nigro et al, 1991). A year later, two reports identified this type of non-polyadenylated RNA with scrambled exons as covalently closed circular RNAs. The first study demonstrated the specific circularization of DCC and EST-1 transcripts (Cocquerelle et al, 1993).

Although the authors refer to the molecules as mis-spliced, they raised the possibility that they might be functional. Cocquerelle and others authors also showed that the circularized RNAs were localized in the cytoplasm and were stable after a 48-h treatment of cells with actinomycin D. In the second study, the authors determined that the Sry RNA scrambled products are indeed circular. They showed that this circular RNA is mainly cytoplasmic, tissue specific and is present in three different mice species (Capel et al, 1993).
Later many studies classified other presumptive circular RNAs as scrambled-exon, exon-shuffling products, or just "non-linear mRNAs" (Dixon et al, 2005; Al-Balool et al, 2011).

The advancement of RNA-seq technologies in 2010 and the development of specialized computational pipelines, led to an explosion in circRNA research. Several studies revealed that thousands of types of circRNAs are expressed in metazoans. Despite the low expression levels of most circRNAs, some are highly abundant. Moreover, in several cases the circRNA can be the main product generated from the host gene (Salzman et al., 2012; Hansen et al., 2013; Jeck et al., 2013; Memczak et al., 2013). Moreover, two studies in 2013 not only identified thousands of circRNAs in different mammalian systems, but also showed that two circRNAs, CDR1as (also known as ciRS-7) and circSry, can bind to and likely modulate activities of specific microRNAs (miRNAs) (Hansen et al., 2013; Memczak et al., 2013). These and other works demonstrate that circRNAs are

expressed in a tissue and developmental stage-specific manner in humans, mice, and flies (Westholm et al., 2014; Rybak-Wolf et al., 2015; Gruner et al., 2016).

In 2014, Ashwal-Fluss and others showed that circRNAs are produced co-transcriptionally and in competition with regular splicing. Hence, biogenesis of circRNA results in reduced synthesis of mRNAs from the same locus. In this situation, production of the circRNA acts as an RNA trap for mRNA production. However circRNA has abundant biological functions and is involved in various physiological and pathological processes of tumor cells, including proliferation, apoptosis, invasion, and migration (Geng et al., 2018).

The making of a circRNAs

CircRNAs are generated by a specific type of splicing called backsplicing, where in the 5' terminus of a pre-mRNA upstream exon is non-colinearly spliced with the 3' terminus of a downstream exon. Two basic models of circRNA biogenesis have been proposed as follows: the first intron-pairing-driven circularization, also known as direct back-splicing, is the main form of ecircRNA production, in which the flanking intronic complementary sequences of the pre-mRNA form a lariat by direct base-pairing, forming an ecircRNA when introns are removed, and the second lariat-driven circularization, also known as exon-skipping, in which the pre-mRNA

is partially folded during transcription, allowing the 3′ splice donor of the downstream exon to connect to the 5′ splice acceptor of the upstream exon, resulting in exon-skipping and the formation of a RNA lariat containing both exons and introns (Geng et al., 2018). CircRNAs are predominantly found in the cytoplasm, and the lack of a 5' cap and 3' tail make the circular molecules more resistant compared than linear RNAs (Enuka et al., 2016). These structures can resist exonucleolytic degradation by RNase R (Jeck et al., 2014). CircRNAs are considered to be divided into four categories on the basis of their composition(Fig2): ecircRNAs; circular intronic RNAs (ciRNAs); exon–intron circRNAs (EIciRNAs); and tRNA intronic circular RNAs (tricRNAs), which are formed by tRNA introns. Over 80% of the identified circRNAs are ecircRNAs, and most are distributed in the cytoplasm (Memczak et al., 2013; Jeck et al., 2013). However, ciRNAs as well as EIciRNAs are predominantly localized in the nucleus (Zhang et al., 2013; Li et al., 2015), indicating that they may regulate gene transcription. CircRNAs are produced by the spliceosome in most eukaryotes, even if the exact mechanism seems to differ between yeast, plants, and metazoans. CircRNA can be generated either with the help of reverse complementary repeats or RNA-binding proteins and exported from the nucleus. Once in the cytosol, the circRNAs analyzed are not associated with ribosomes (Capel et al., 1993; Jeck et al., 2013; Guo et al., 2014) suggesting that circRNAs are not generally translated. In the cytoplasm, the circRNA might be bound by multiple factors. These can be

RNA-binding proteins, Argonaute proteins loaded with miRNAs as sponge or scaffold or for direct degradation, ribosomes or endonucleases that would cause degradation of the circRNA. From the non-degradative binding, the circRNA-factor complex might diffuse in the cytoplasm or been actively transported in into particular regions of the cell where it can release its bound cargo or starts to be translated. The enclosure of circRNAs or circRNA factor complexes in vesicle that would be released into the extracellular space would remove circRNAs from the cytoplasm. However, protected by the vesicle, the circRNAs or circRNA complexes could reach other cells or tissues and therefore act as messenger molecules or fulfill other unknown functions (Patop et al., 2019).

CircRNAs generally contain complete exons and are mostly generated from coding exons, particularly those located in the 5′ untranslated regions (UTRs) of protein-coding genes (Guo et al, 2014; Westholm et al, 2014; Rybak-Wolf et al, 2015). This results in back-splice junctions formed by coding sequence to coding sequence regions (CDS-CDS) and 5′ UTR-CDS regions and tends to include the second exon of the gene (Salzman et al, 2012; Westholm et al, 2014). This might be related to their biogenesis, which requires longer and less efficiently spliced introns than average; both criteria are usually met with the first introns (Ashwal-Fluss et al, 2014). While circRNA biogenesis might require inefficient canonical splicing, the introns within circRNAs seem to be mostly spliced out (Westholm et al, 2014; Guo et al, 2014; Gao et al, 2016; Rahimi et al, 2019; Ji et al, 2019). These findings

suggest that the inefficient splicing of the introns flanking circularizable exons is specific and not related to a poor processing of those transcripts in general.

CircRNAs are transcribed by RNA polymerase II and generated by the spliceosome (Cocquerelle et al, 1993; Zaphiropoulos, 1998; Ashwal-Fluss et al, 2014; Starke et al, 2015; Sun et al, 2016). Importantly, many of the exons forming circRNAs are not alternatively spliced (Aufiero et al, 2018). Hence, some highly abundant circRNAs regulate in cis the production of mRNAs from the host gene. Further evidence for the widespread importance of this cis-regulation is the fact that in addition to splicing circRNA production is also linked to inefficient cleavage and polyadenylation (Liang et al, 2017). Our understanding of circRNA biogenesis is hampered by the fact that circRNA production differs depending on the system used. In cell culture, circRNA biosynthesis seems to be post-transcriptional (Liang & Wilusz, 2014; Zhang et al., 2014). This might constitute an idiosyncrasy of a system (cell culture) in which circRNAs are known to be produced at very low levels compared to neural tissue (Ashwal-Fluss et al., 2014). If circRNA production is in competition with canonical splicing, changes in splicing efficiency should modulate the production of circRNAs. This could be achieved by modulation of trans-acting splicing factors or by changes in the kinetics of RNA polymerase II transcription, which are known to modulate alternative splicing (Kadener et al., 2001 and 2002; de la Mata et al., 2003). In this context, changes in circRNA production can be seen as a consequence of alterations in the efficiency of global

RNA processing events that are influenced by variations in transcription rates. CircRNAs have been confirmed to modulate parental gene expression, regulate alternative splicing and transcription as well as translation, act as miRNA sponges, act as RBP sponges or protein scaffolds, some may be translated into peptides/proteins and produce some pseudogenes (Geng et al., 2020).

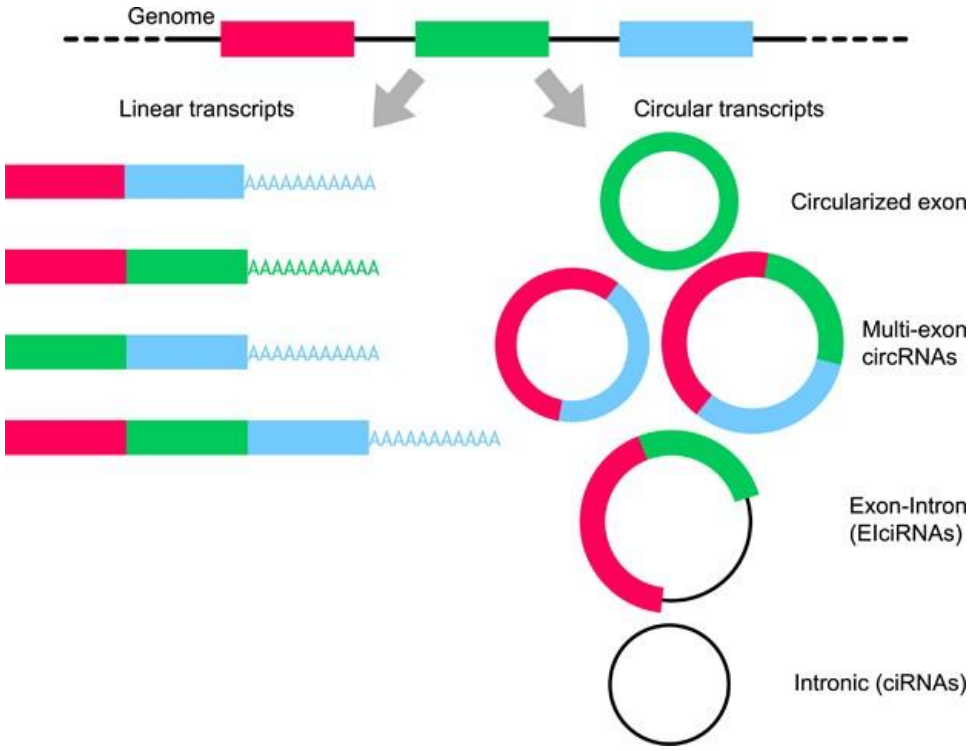

Figure 2: Shows Linear and circRNAs. Circular RNAs are produced by backsplicing, and combinations of exons and introns give rise to different products, including single circularized exons, circRNAs formed by two or more exons, by exon and retained intron sequences (EI-ciRNAs) and by intronic sequences only. (Bonizzato et al., 2016)

Growing evidence has shown that circRNAs can regulate parental gene expression through diverse mechanisms, such as transcription and splicing regulation, miRNA sponges, mRNA traps, translational modulation, and post-translational modification. The study of circRNAs and how circRNAs regulate the expression of parental genes facilitates the understanding their biological functions and new perspectives on their clinical application (Shao T et al., 2020).

Some nuclear circRNAs can interact with RNA Pol II or transcriptional factors. For example, ci-ankrd52 accumulates to its sites of transcription, interacts with an elongation RNA Pol II complex, and acts as a positive transcriptional regulator of its parental gene (Zhang Y et al., 2013). Certain nuclear circRNAs may activate parental gene transcription by inducing DNA hypomethylation in the promoter or by regulating intronic enhancer. For instance, a novel *FLI1* exonic circRNA, FECR1, utilizes a positive feedback mechanism to activate *FLI1* transcription by inducing DNA hypomethylation in the CpG islands of its promoter (Chen N et al., 2018).

It was found that a naturally expressed circRNA binds to miRNAs to suppress their function. Memczak et al. and Hansen et al. show that a mammalian circRNA, termed CDR1 antisense, contains >60 conserved miRNA-7 seed matches, suggesting that it can bind densely to this target miRNA (Du Toit A et al., 2013). Recent studies indicated that some cytoplasmic circRNAs can be effectively translated into detectable peptides, which underscore the importance of circRNAs in cellular physiology function. Internal Ribosome Entry site (IRES)- and N6-methyladenosines-mediated cap-independent translation initiation have been suggested to be potential mechanism for circRNA translation (Lei M et al., 2020).

Circular RNAs play a role in cancer

Strong evidence has revealed that circRNAs play critical roles in the development and progression of diseases, especially in cancers (Su et al., 2019). It is increasingly understood that circRNAs play a significant role in cancer pathogenesis and progression by regulating target genes and multiple signaling pathways. A variety of different pathways may find circRNAs involved in carcinogenicity mechanisms. As a result, proliferative signaling, epithelial-mesenchymal transition, angiogenesis and apoptosis or drug resistance can be affected and the progression of cancer can be impacted on directly (Geng et al., 2020). For example sponging of microRNA-7 (miR-7), circHIPK3 inhibits miR-7 activity, which cause an increase in the expression of FAK, IGF1R, EGFR and YY1 oncogenes. Because it is crucial for cell migration, FAK could trigger tumor invasion and metastasis through upregulated expression of VEGF, MMP2 and MMP9. Both IGF1R and EGFR are capable of activating PI3K/AKT and MEK/ERK signaling pathways, which stimulate cancer progression and cause drug resistance (Zeng et al., 2018).

Many malignant tumors may eventually develop resistance. Clinically, drug resistance is a major obstacle to cancer treatment and urgently needs a solution. Recently, many studies have revealed that non coding RNAs are very important in different biological processes in tumors and have been implicated in resistance to various antineoplastics (Xie et al., 2020; Liu et al., 2019). Furthermore, many studies have demonstrated that circRNAs are associated with cancer formation,

development, invasion and metastasis (Zhang et al., 2019; Dong et al., 2019) but the mechanism of circRNAs in anticarcinogen resistance has not been elucidated.

Lung cancer is an ordinary malignant tumor and the cause of cancer death worldwide. Chemotherapeutic resistance leads to poor therapy outcomes and recurrence of malignant lung cancer. Xu and others (Xu et al., 2018) discovered upregulation of hsa_circ_0071799 in taxol-resistant non-small-cell lung cancer cells (NSCLC), and also that hsa_circ_0071799 could play the role as a sponge of miR-141. Moreover, hsa_circ_0091931 was subject to downregulation and was associated with miR-34c-5p. The functions of circRNAs in Taxol chemoresistance in NSCLC were analyzed, and the results suggested that circRNAs may be involved in the regulation of Taxol resistance.

Breast cancer (BC) is the second leading cause of death among women. Tamoxifen (TAM) is an important treatment option for BC. In one study, researchers established a TAM-resistant cell line and obtained the circRNA expression profile. Hsa_circ_0025202 inhibited cell proliferation, colony formation and migration, and promoted apoptosis and TAM sensitivity. Mechanistic investigations revealed that hsa_circ_0025202 can sponge miR-182-5p and that the expression and activity of the target gene FOXO3a were further modified (Sang et al., 2019).

However more and more circRNAs have been reported to be dysregulated in many human malignancies, such as lung and breast cancer, gastric cancer, colorectal

cancer, and liver cancer and more. According to numerous studies, there is a significant difference in the expression of circRNAs among a variety of tumour tissues. Some specifically expressed circRNAs may potentially serve a new biomarkers for tumor diagnosis and prognosis (Geng et al., 2018).

Several circRNAs are considered responsible for the malignant biological behavior of cancer cells (Zhao ZJ et al., 2017).They can drive cancer development in many cancer types (Drula et al., 2020; Wu YP et al., 2020) as well in haematologic malignancies.

Circular RNAs in hematological malignancies

The presence of circRNAs in human peripheral blood, including in serum (Xu et al., 2018), plasma (Li et al., 2018) and peripheral blood mononuclear cells (Huang et al., 2018), and saliva (Bahn et al., 2015) and bone marrow (hu et al., 2018) indicates their potential as biomarkers. However, the roles and mechanisms of circRNAs in hematological malignancies have not been fully clarified. Hematological malignancies are diseases of stem and progenitor cells which originate from genetic and epigenetic changes resulting in the dysregulation of self-renewal, proliferation and differentiation of cells (Szymczyk et al., 2018). CircRNAs have been shown to be tightly regulated in the course of differentiation. Hundreds of circRNAs are, for example, differentially expressed during epithelial-

mesenchymal transition (Conn et al., 2015), and circRNAs are globally up-regulated during neuronal differentiation (Rybak-Wolf et al., 2015). Hematopoietic stem cell (HSC) differentiation into a broad spectrum of specialized blood cells is a tightly regulated process that depends on a multitude of transcription factors and other molecules, including noncoding RNAs. As circRNAs can drive a subset of cellular functions, their expression in different blood cell populations is being evaluated to determine their involvement in hematopoiesis (Dostalova et al., 2020). However, most roles of circRNAs in the regulation of HSC differentiation remain to be revealed.

Most important works in literature that discuss the possible mechanisms of circRNAs in the field of hematology are related to acute myeloid leukemia (AML), a biologically heterogenous disease. Although mechanistic studies clarifying the molecular underpinnings of AML have facilitated the development of several novel targeted therapeutics, most AML patients still relapse. Thus, overcoming acquired resistance to current therapies remains an unsolved clinical problem and for these reasons there is an ongoing demand to identify newer diagnostic, therapeutic and prognostic biomarkers for AML. Recent interest in exploring the role of circRNA in elucidating AML biology and therapy resistance has been promising (Singh V et al., 2021).

In 2017 Hirsch et al., investigated circRNA expression of one of the most frequently mutated genes in AML and they found that there are many different

circRNA variants for NPM1 that comprise non-canonical exon and intron sequences. In particular, quantification of hsa_circ_0075001, circNPM1 variant, and integration of global gene expression data revealed a distinct hsa_circ_0075001-associated gene expression signature, pointing to a biological relevance of this circRNA. High expression level was associated with a significantly lower expression of genes involved in the Toll-like receptor (TLR) signaling pathway. TLRs have recently been implicated in the differentiation of normal hematopoietic cells and moreover TLR1 expression has been linked to leukemic stem cell survival in AML.

A recent study in patients with AML compared to healthy patients reported the role of circATAD-1 in promoting AML cell proliferation through the downregulation of miRNA-34b. circATAD1 has been demonstrated be specifically expressed in the nucleus and the expression was found to be elevated and inversely correlated with that of miRNA-34b, in patients with AML. The circATAD1 overexpression in AML cells decreased miRNA-34b expression and increased miRNA-34b gene methylation. Moreover, AML cell proliferation was increased by circ-ATAD1 overexpression, but decreased by miRNA-34b overexpression. Together, these results have shown that circATAD1 promotes AML cell proliferation by downregulating miRNA-34b by methylation (Wu Y et al., 2021).

Furthermore, in a study where the focus was circular transcriptome in AML patients compared to healthy samples, was observed that hundreds of circRNAs are differentially expressed. In particular knockdown of circBCL11B showed a negative effect on leukemic cell proliferation and resulted in increased cell death of leukemic cells, suggesting circBCL11B as a novel functionally relevant candidate in AML pathogenesis (Lux S et al., 2021).
Studies related to the behavior of circRNAs in the field of MPN in the literature, are limited.
A recent interesting study regards the altered profile of circRNAs in bone marrow-derived exosomes of ET patients. In fact the identification of biomarkers within extracellular vesicles (EV) for clinical studies could be crucial in the diagnosis and prognosis of MPN (Catani et al., 2021). EVs are particles of double lipid layer produced by all types of cells that function as players in either short- or long-distance intercellular communication since EV structure can prevent degradation of bioactive molecules. Moreover EVs have been detected in various biological fluids including blood, urine and saliva. Their composition includes proteins involved in the formation of multivesicular bodies' formation and membrane transport and fusion, tetraspanins, cytoskeletal components and proteins of cytosolic origin. EVs also carry nucleic acids (mRNA, microRNA, long non-coding RNA, and double strand DNA) and selected lipids.

EVs interest both normal and malignant hemopoiesis and those derived from blood cancer cells or blood cancer's microenvironment result functionally regulate key processes including coagulation, angiogenesis, immunity and chemoresistance.

The analysis of nucleic acid cargo of EVs from ET patients in MPN showed that, as mentioned above, the profile of circRNAs was altered in bone marrow-derived exosomes of ET patients. In particular, circDAP3, circASXL1 and circRUNX1 were significantly decreased in exosomes of ET patients. Moreover circRNA coding genes and miRNA- mRNA networks targeted at these three circRNAs have been involved in various key biological processes and signaling pathways such as cell proliferation and apoptosis. (Wang et al., 2021).

PLOD2 and CircPLOD2

Primary myelofibrosis (PMF) is a myeloproliferative neoplasms characterized by progressive deposition of extracellular matrix components in the bone marrow. Besides the action of pro-fibrogenic factors with subsequent overproduction of extracellular matrix (ECM) components, the counteracting proteolytic environment, including collagenases/matrix metalloproteinases and their inhibitors, substantially contribute to the development of PMF (Xu et al., 2005; Bock et al., 2006). PMF cases were re-evaluated and subdivided into two groups depending on the degree of myelofibrosis (Thiele et al., 2005; Buhr et al., 2003). In brief,

hypercellular PMF cases showing no deposition of fibers were graded as mf 0, and a mild increase of reticulin fibers led to grade mf 1. Advanced PMF showing a manifest myelofibrosis with extended collagen deposits and intra- sinusoidal hematopoiesis were graded as mf 2. In addition, demonstrable osteosclerosis and bone apposition were classified as grade mf 3 (Bock et al., 2008). Collagen is the most abundant protein in our body, and present in both normal tissues and cancer. Collagen regulates tumor progression by modulating cancer cell migratioin, invasion, proliferation, survival and metastasis. (Xiong et al., 2014; Pollard, 2004; Cheon et al., 2014; Oudin et al., 2016; Sun et al., 2016).

All collagen is composed of a triple helix, and the most common motif of the triple helix sequence is Gly-X-Y (X and Y represent proline or hydroxyproline) (Albaugh et al., 2017). Collagen is synthesized in the rough endoplasmic reticulum (ER) as a precursor (Nimni, 1983). After peptide bond formation, proline and lysyl hydroxylation is catalyzed by prolyl 4-hydroxylase (P4H) and procollagen-lysine,2-oxoglutarate 5-dioxygenase (PLOD). The hydroxylation of lysyl residues is one of the critical steps of collagens biosynthesis. Three PLODs (PLOD1, PLOD2 and PLOD3) has been identified, catalyzing the lysyl hydroxylation to hydroxylysine (Hausmann, 1967; Rhoads and Udenfriend, 1968; Kivirikko Ki, 1998; Rautavuoma et al., 2004). PLODs catalyze hydroxylation of lysine (Lys) intracellularly before collagen is secreted, and then lysyl oxidase (LOX) binds to hydroxylysine (Hyl) residues in the extracellular collagen fibers and induces the

cross-link formation (Saito and Marumo, 2010). The mutation or overexpression of PLODs has been detected in many human diseases. In particular the overexpression of PLOD2 is detected in many types of cancer (Qi and Xu, 2018).

Proteins in the PLOD family are highly homologous; the overall identity in protein sequences among PLOD1, 2 and 3 is 47% (Valtavaara et al., 1998). PLOD protein has binding sites for cofactor Fe^{2+} and L- ascorbate. *PLOD2* gene is at chromosome 3q23-q24 (Szpirer et al., 1997) and also contains 19 exons. Two splice variants (LH2a and LH2b) have been identified in the *PLOD2* gene; LH2b differs from LH2a by incorporating the small exon 13A (Valtavaara, 1999).

PLOD expression is mainly regulated at the transcription level. A number of cytokines, signaling pathways, and microRNAs have been identified to be involved in transcriptional regulation of PLODs (Qi and Xu, 2018). TGF-β signaling is important regulator of PLOD2 expression (Remst et al., 2014). SP1 and SMAD3, as downstream targets of TGF-β signaling, recruit histone modifying enzymes to the PLOD2 promoter region and induced PLOD2 transcription (Gjaltema et al., 2015). In addition, transcription factor E2Fs (Hollern et al., 2014) and FOXA1 (Du et al., 2017) have been identified as regulators of PLOD2 during cancer progression.

The hsa_circ_0122319, called circPLOD2 for convenience, is a circular RNA that originates from the *PLOD2* gene through circularization of exon 3 and exon 2. Its length is 229 bp. The knowledge about the possible role of circPLOD2 in

hematological malignancies is completely unknown. There are only few works in the literature that study the involvement of circPLOD2 in Colorectal Cancer (Li A et al., 2021; Artemaki PI et al., 2020). In one of this studies the representative circRNAs play functional roles in the refined regulation of colon cancer progression improving the prognostic stratification for patients (Ju et al., 2019).

AIM OF THE PROJECT

The myeloproliferative neoplasms (MPNs) are a group of clonal stem cell diseases with heterogeneous clinical features and laboratory characteristics. The aim of this project is to explore new routes to possible novel prognostic treatments and alternative strategies for MPN patients with particular interest in triple negative who represent the most insidious category.

For this reason it is important to look for mechanisms that can explain the pathways related to these diseases and circular RNAs could be interesting for this purpose.

MATERIALS AND METHODS

Samples collection

Granulocytes and CD34+ cells were isolated from healthy donors' (n=12 and n=8, respectively) and from bone marrow or peripheral blood of MPN patients (n=29 and n= 47, respectively) (see Table 6 for patient characteristics).

CD34+ cells before (n=10) and after (n=10) the treatment with JAK2 inhibitor were isolated from MPN patients (see Table 7 for patient characteristics).

Collection and processing of human samples was approved by the Azienda Ospedaliera-Universitaria Careggi Institutional Review Board (#14560) after obtaining informed written consent.

Table 6: Patient characteristics

Patients ID	Driver Mutation	Disease	Sample Type	Source
9172	JAK2	PET	Granulocytes	PB
c930	CALR	PMF	Granulocytes	PB
A0629	JAK2	PV	Granulocytes	PB
A0287	JAK2	PV	Granulocytes	PB
A0274	JAK2	PV	Granulocytes	PB
A0585	JAK2	PV	Granulocytes	PB
9978	JAK2	MF	Granulocytes	PB
A0540	JAK2	MF	Granulocytes	PB
C5	JAK2	MF	Granulocytes	PB
3055	JAK2	TE	Granulocytes	PB
3054	JAK2	TE	Granulocytes	PB
C52	TN	PMF	Granulocytes	PB
1247	TN	Pre-PMF	Granulocytes	PB
9859	TN	Pre-PMF	Granulocytes	PB
7431	TN	PMF	Granulocytes	PB
A0255	TN	Pre-PMF	Granulocytes	PB
11058	TN	Pre-PMF	Granulocytes	PB
2421	CALR	Pre-PMF	Granulocytes	PB
843	CALR	PMF	Granulocytes	PB
5735	CALR	PMF	Granulocytes	PB
7047	TN	Pre-PMF	Granulocytes	PB
5017	TN	Pre-PMF	Granulocytes	PB
7508	TN	Pre-PMF	Granulocytes	PB
1838	TN	Pre-PMF	Granulocytes	PB
5952	TN	PMF	Granulocytes	PB
7625	TN	Pre-PMF	Granulocytes	PB
1382	TN	PMF	Granulocytes	PB
9160	TN	Pre-PMF	Granulocytes	PB
7405	TN	Pre-PMF	Granulocytes	PB
C1888	CALR	Pre-PMF	CD34+	PB MNC
C1461	CALR	Pre-PMF	CD34+	PB MNC
A0969	CALR	Pre-PMF	CD34+	PB MNC
C1280	CALR	Pre-PMF	CD34+	PB MNC
C1784	CALR	PMF	CD34+	PB MNC
7520	CAL	PMF	CD34+	PB MNC
7191	CALR	PMF	CD34+	PB MNC
C1080	JAK2	PMF	CD34+	PB MNC

A0875	JAK2	Pre-PMF	CD34+	PB MNC
5826	JAK2	PMF	CD34+	PB MNC
5754	JAK2	Pre-PMF	CD34+	PB MNC
7515	JAK2	PMF	CD34+	PB MNC
1653	JAK2	PMF	CD34+	PB MNC
1063	JAK2	PMF	CD34+	PB MNC
5752	JAK2	PMF	CD34+	PB MNC
7350	JAK2	Pre-PMF	CD34+	PB MNC
A0628	JAK2	PMF	CD34+	PB MNC
A0285	MPL	Pre-PMF	CD34+	PB MNC
7298	MPL	Pre-PMF	CD34+	PB MNC
A0673	MPL	Pre-PMF	CD34+	PB MNC
A0236	MPL	Pre-PMF	CD34+	PB MNC
C1434	TN	PMF	CD34+	PB MNC
5726	JAK2, MPL	Pre-PMF	CD34+	PB MNC
9932	JAK2	PMF	CD34+	PB MNC
C817	CALR	PET	CD34+	PB MNC
A0983	CALR	PET	CD34+	PB MNC
A0777	JAK2	PET	CD34+	BM
4806	MPL	PET	CD34+	PB MNC
A0490	MPL	PET	CD34+	PB MNC
A0587	MPL	PET	CD34+	PB MNC
7408	MPL	PET	CD34+	PB MNC
A0894	TN	PET	CD34+	PB MNC
A0884	CALR	ET	CD34+	PB MNC
C25	CALR	ET	CD34+	PB MNC
7773	CALR	ET	CD34+	PB MNC
5722	JAK2	ET	CD34+	PB MNC
3054	JAK2	ET	CD34+	BM
C1463	CALR	PV	CD34+	PB MNC
3209	JAK2	PV	CD34+	PB MNC
7539	JAK2	PV	CD34+	PB MNC
11074	JAK2	PPV	CD34+	PB MNC
C1540	JAK2	PPV	CD34+	PB MNC
A0721	JAK2	PPV	CD34+	PB MNC
C1595	JAK2	PPV	CD34+	PB MNC
C134	JAK2	PPV	CD34+	PB MNC
A0661	JAK2, MPL	PPV	CD34+	PB MNC
C68	JAK2, MPL	PPV	CD34+	PB MNC
DON1	-	-	GN	PB
DON2	-	-	GN	PB

DON3	-	-	GN	PB
DON4	-	-	GN	PB
DON5	-	-	GN	PB
DON6	-	-	GN	PB
DON7	-	-	GN	PB
DON8	-	-	GN	PB
DON9	-	-	GN	PB
DON10	-	-	GN	PB
DON11	-	-	GN	PB
DON12	-	-	GN	PB
MID1	-	-	CD34+	BM
MID2	-	-	CD34+	BM
MID3	-	-	CD34+	BM
MID4	-	-	CD34+	BM
MID5	-	-	CD34+	BM
MID6	-	-	CD34+	BM
MID7	-	-	CD34+	BM
MID8	-	-	CD34+	BM

Table 7: Patient characteristics before/after the treatment with JAK2 inhibitor

ID pre/post	Start date JAK2 inhibitor	post RUXO pick date	Driver mutation	Allele burden %	Time elapsed
#7505/#9589	From 15/3/2014 to date	14/12/2015	JAK2	91	1 year and 9 months
#4734/7692	From 24/11/2011 to date	12/12/2013	JAK2	11	2 years
#2570/C134	From 22/12/2008	10/9/2018	JAK2	99	10 years
A0644/C1595	From 31/05/2018	7/10/2019	JAK2	90	1 year and 5 months
11074/A0929	From 30/3/2017	4/6/2019	JAK2	75	2 years and 3 months
A0207/A0628	From 1/7/2017	13/4/2018	JAK2	44	9 months
#4133/A0123	From 25/2/2015	8/6/2017	JAK2	52	2 years and 4 months
#4153/C2375	From 2/o4/2015	25/05/2020	JAK2	40	5 years and 1 month
#1203/#4729	From 14/01/2010	1/7/2010	JAK2	80	6 months
#9217/C570	From 15/6/2015	15/1/2019	CALR	-	4 years

CircRNAs identification

RNA-seq data from 3 healthy donors (HD) and 8 Primary Myelofibrosis (MPN) CD34+ samples were analyzed by CirComPara tool (Gaffo et al. 2017) using 9 aligners and circRNA detection programs combinations. Only circRNAs detected by at least two programs and with at least two reads (for each program) were considered expressed.

All circRNAs with a raw count of at least 5 reads in at least one sample and detected in at least 2/3 HD or 5/8 MPN patients were analyzed. CircRNA annotation and all analyses were based on the Ensembl GRCh38 human genome and annotation v93.

CircRNAs differential expression and prioritization

CircRNA expression was normalized with the regularized logarithm. A group of differentially expressed circRNAs with log2FoldChange>2 in PMF versus HD, high expression (read count>200), or coming from cancer genes, has been prioritized in this study.

The search of the circular-RNAs differentially expressed has been carried out using the search engines: circBase (http://www.circbase.org) eCircFunBase (http://bis.zju.edu.cn/CircFunBase/).

Sample preparation

For the evaluation of molecular markers and for cellular studies, 30 ml of peripheral blood or 5-8 ml of bone marrow were collected to obtain mononuclear cells, granulocytes and cell subpopulations.
For the evaluation of molecular targets and for cellular studies peripheral blood (PB) or bone marrow (BM) samples were obtained from ET, PV or PMF patients, diagnosed according to the World Health Organization (WHO) criteria, under a protocol approved by Institutional Review Board of Azienda Ospedaliera-Universitaria Careggi and after obtaining a written informed consent. Briefly, in order to collect mononuclear cells (MNCs) 20 mL of PB was layered on Lymphoprep density gradient separation (Stemcell Technologies) and centrifuged at 1800 rpm for 20 min at room temperature. It was pick up with a Pipette Pasteur the ring of mononuclear cells, which were washed in sterile PBS at 4°C, and

centrifuged at 1200 rpm for 10 minutes at 8 °C for two times. The cells were counted using a Burker chamber with a dilution factor of 1:300.

CD34+ cells isolation

The purification of CD34+ cells from peripheral blood or cord blood was performed using immunomagnetic separation according to the Miltenyi procedure: the layer of mononuclear cells was washed with Buffer 1 (Ca-Mg free PBS, 2 mM EDTA), centrifuged at 1000 rpm for 10 minutes and then resuspended in Buffer 2 (Ca-Mg free PBS, 0.5% BSA, 2 mM EDTA) to a final volume of 300 µl containing up to 10^8 mononuclear cells. To this suspension were then added 100 µL of FcR blocking reagent (Human IgG) and 100 µL of MACS CD34 microbeads (microbeads super-paramagnetic conjugated to murine anti-human CD34 monoclonal antibodies (isotype: mouse IgG1) up to 10^8 of total cells; the whole was incubated for 30 minutes at 4°C and agitated every 10 minutes. After incubation, Buffer 2 was added up to a volume of 10ml and centrifuged at 1000 rpm for 10 minutes at 8°C. Very carefully was removed the supernatant and the pellet was resuspended in a final volume of 500 µl Buffer 2 up to 10^8 total cells. For separating CD34 positive cells was taken a column MACS MS+ (2x 10^8 total cells, 1x 10^7 positive cells). After washing with 500 µl of Buffer 2, the column MACS MS+ was inserted in the magnetic support, the cell suspension was applied to the column which was then allowed to drain by gravity the CD34 negative fraction; then three washes of the column with 500 µl of Buffer 2 were performed. Then it was added further 500 µl

of Buffer 2 in the column, which was quickly detached from the magnetic media to recover the CD34 positive fraction by pressure with a piston-column. Sometimes it was necessary to perform additional steps of purification of CD34 positive fraction recovered in a new column.
Control CD34+ cells were obtained from discarded cord blood units.

CD34+ cells were plated at the concentration of 5x 10^5cells/mL in IMDM medium (Iscove's Modified Dulbecco's Medium, Lonza, Belgium) with the addition of 20% Human Serum, Penicillin/Streptomycin 1% L-glutamine 1%; plus a cytokines cocktail allowing CD34+ proliferation: human SCF 50ng/mL; FLT3L 50ng/mL; human TPO 20ng/mL; human IL-3 10ng/mL; human IL-6 10ng/mL.

Granulocytes separation

For the recovery of the granulocytes (GN), after the centrifugation on the density gradient and the recovery of the mononuclear fraction, the supernatant was aspirated, and the pellet was resuspended in 40 ml of 0.2% NaCl hypotonic solution for erythrocyte lysis. After centrifugation at 1500 rpm for 10 minutes at 8 ° C, the supernatant was removed to obtain the granulocyte pellet, which was subjected to a second lysis in 40 ml of 0.2% NaCl hypotonic solution and new centrifugation at 1500 rpm for 10 minutes at 8 ° C. The resuspended pellet in 2 ml of PBS was then transferred to two sterile eppendorf eppendors and centrifuged at 3000 rpm for 5 minutes.

Hematopoietic cell lines

SET2. This cell line was derived from peripheral blood of a 71 year old woman affected by essential thrombocythemia evolved into megakaryoblastic leukemia. The cells are found mostly individually suspended and some of them grow losing adhesion. It can be note the rare presence of giant cells (Uozumi et al., 2000; Gozgit et al., 2008). The SET2 cell line are heterozygous for the mutation JAK2V617F.

K562. This cell line was derived from the pleural effusion of a Caucasian woman of 53 years old affected by chronic myelogenous leukemia; the cells have a lymphoblastic morphology. They have a diploid karyotype with terminal deletion of the long arm of chromosome 22 (del (22) (q12)), the Philadelphia (Ph) chromosome. In addition to the Ph chromosome they also exhibit a second reciprocal translocation between the long arm of chromosome 15 with chromosome 17. The majority of the mononuclear cells are undifferentiated and do not produce immunoglobulin, show no alkaline phosphatase activity and myeloperoxidase and they are not able to phagocytize inert particles (Baker et al., 2002). The cell line K562 is WT for the mutation JAK2V617F.

HEL. This cell line was derived from the peripheral blood of a 30 year old man with acute erythroblastic leukemia (AML-M6) arose after treatment for Hodgkin's lymphoma. The cells show a rounded morphology, appear great, and occasionally giant and multinucleated; they show capacity for induced and spontaneous

synthesis of globin. The HEL cell line presents in its genome multiple copies of the mutation JAK2V617F (Quentmeier et al., 2006).

UT-7. This cell line was established from the bone marrow of a 64-year-old man with acute myeloid leukemia (AML M7) at diagnosis in 1988; cells are constitutively cytokine-dependent and responsive to various cytokines.

NB-4. established from the bone marrow of a 23-year-old woman with acute promyelocytic leukemia (APL = AML FAB M3) in second relapse in 1989; patented cell line; cells carry the t(15;17) PML-RARA fusion gene.

The K562, HEL, UT-7 and NB-4 cell lines used were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany, www.dsmz.de). SET2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, US, www.atcc.org).
The cells lines were maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS; Lonza, Verviers, Belgium) (20% for the SET2 cells), 1% L-glutamine and 1% Penicillin-Streptomycin.

Non-hematopoietic cell lines

The non-hematopoietic PC-3 (human prostatic cancer) and A375 (human malignant melanoma) cell lines, a gift from Prof. F. Paoletti, were also used to evaluate the selected circRNAs.

The JAK2 inhibitor (Ruxolitinib)

Ruxolitinib (INCB018424) is an inhibitor of tyrosine kinases JAK1 and JAK2; the JAK-STAT pathway is a key component of cytokine signalling and growth factors that regulate cell proliferation, hematopoiesis, and immune response. Deregulation of this pathway, upregulation of JAK1 or JAK2 or gain of function mutations (such as JAK2V617F, MPLW515L, and JAK2 mutations on exon 12) are involved in MPN pathogenesis. Ruxolitinib binds and inhibits JAK1, JAK2 (both wild-type and JAK2V617F) and also JAK3, though with lower affinity. Ruxolitinib treatment leads to inhibition of the cell signalling mediated by growth factors and the neoplastic cell proliferation (Verstovsek et al 2010, Quintás-Cardama et al., 2010).

The JAK2 inhibitor treatment, was performed on CD34+ cells as control after separation from cord blood, at concentration of 0.5 μM and 1μM.

Cell culture techniques

Erythroid and megakaryotocyte differentiation.

Liquid culture

To obtain the growth of erythroid or megakaryotocyte synchronous cells during their in vitro differentiation cultures have been used in two phases: a first proliferative phase of expansion of the progenitor pool and a second differentiating phase. Positive CD34 cells isolated from cord blood were platted at a concentration of 2x105/ml in SYN medium. H (serum-free medium for expansion of hematopoietic cells, Abcys S.A.) with the addition of Penicellin/Streptomycin at 1%, L- Glutammin at 1%.

MEDIUM SYN.H: Clinical grade human albumin, synthetic iron carrier, rh-insulin, nucleosides, L-Glutammin, α-monothioglycerol, synthetic lipids, synthetic Iscove base medium.

In cultures for erythroid differentiation was added a cytokines cocktail for the proliferative phase, consisting of: Human IL-3 2ng/mL;Human IL-6 2ng/mL;

SCF 10ng/mL

After 6 days of culture, the cells were washed in PBS, counted and plated for a further 6 days at a concentration of $1,5x10^5$/ml for the differentiating phase. The STEM-α medium was used for the latter phase (serum-free medium without

citokines for human hematopoietic cells, STEM-α) with added Penicillin/Streptomicin (ICn Biomedicals Inc, USA) all'1%, L-Glutammin (Cambrex,Belgium) all'1%.

MEDIUM STEM-α: Clinical grade human albumin, synthetic iron carrier, rh-insulin, nucleosides, L-glutamine, α-monothioglycerol, synthetic lipids,synthetic Iscove base medium.

In order to obtain erythroid differentiation, a cytokines cocktail was added for the differentiating phase, consisting of: Human IL-3 2ng/mL;Human IL-6 2ng/mL;

Human EPO 3U/mL

Semisolid colture

Have been added 2,5 x 10^5 MNCs / mL at rates of 2,5 mL of semisolid medium

(methylcellulose), in medium culture IMDM (Cambrex, Belgium),Penicillin/Streptomicin e L-Glutammin 2mM; the cells were subsequently platted in Petri dishes.

Two semi-solid soils were used:

a methylcellulose preparation containing an optimal mixture of cytokines (METHOCULT GF H4434, Stemcell Technologies Inc, USA) to induce the growth of myeloid colonies (with rh SCF, rh GM-CSF and rh IL-3) and erythroids (with rh IL-3 and rh EPO); a preparation of methylcellulose "base" (METHOCULT H4531,

Stemcell Technologies Inc, USA), without any cytokine, for the growth of spontaneous eitroid colonies (EEC).

METHOCULT GF H4434 to which we have been added:
2mM L-Glutammin

50ng/mL rh Stem Cell Factor

10ng/mL rh GM-CSF
10ng/mL rh IL-3
3 unit /mL rh Erythropoietin (EPO)

METHOCULT H4531 to which we have been added:
2mM L-Glutammin

10 % Agar Leukocyte Conditioned Medium

After 14 days of incubator culture at 37℃ and 5% CO2, the haematopoietic colonies were recognized as belonging to the myeloid or erythroid line based on conventional morphological criteria and the presence or absence of hemoglobin pigment with inverted microscope examination and counted.
For the evaluation of megakaryotic colonies Megacult-C serum-free soil (Stem Cell Technologies Inc, USA) has been used, to which CD34-positive cells purified by immunomagnetic separation at concentration of 1 x 10^3/ml, have been added was used for the evaluation of megakaryotic colonies, in a final volume of 3,3 ml

consisting of 0,4 ml of cell suspension, 1,7 ml of Megacult-C Medium not supplemented with cytokines and 1,2 ml of Collagen Solution. They were then added to the medium 50 ng/ml of thrombopoietin, 10ng/ml of human IL-6 and 10ng/ml of human IL-3.

Elements and final concentration:

Collagen 1,1 mg/ml
L-glutammin 2mM

2-Mercaptoethanol 10^{-4} M

Iscov's MDM

After 12 days of incubator culture at 37℃ e 5% CO2, megakaryotic colonies (CFU-MK) have been recognized and counted based on their typical morphology.

Cytofluorimetric techniques

For the evaluation of erythroid differentiation the Antibodies used:

anti-Glycophorin A PE;

$1x10^5$ cells should be taken for each individual evaluation (although I had less available for this experiment), washed in 1 ml of PBS 4°C by centrifugation at 3000

rpm for 5 minutes and incubated with 2 µl of antibody on ice and protected from light for 20 minutes.

For the evaluation of erythroid differentiation the antibody anti-Glycophorin A (GPA) (Becton Dickinson, USA), marked with fluorophore PE. GPA is a late marker of erythroid differentiation.
The evaluations were carried out at the FACS Scan (Becton Dickinson) using the software Cell Quest Pro. Data were processed with Flow-Jo software (Tree Star, Ashland, OR, USA).

RNA Extraction

RNA purification was carried out by extracting the resuspended sample in thiocyanate/phenol Trizolguanidine, which scans the cells and inhibits RNases (Invitrogen-Life Technologies, Inc.), according to the manufacturer's protocol. Briefly, the granulocyte pellets resuspended in trizol are incubated at room temperature for 2-3 minutes, after which 0.2 ml of chloroform is added and centrifuged at 12000 for 15 min at 4°C. This results in three phases: one red, one white and one interface. The upper aqueous phase is recovered and 3 µl glycogen and 0.5 ml isopropanol are added to precipitate RNA. It incubates at room temperature for 10 min shaking by inversion then centrifuges at 12000 rpm for 10 min at 4°C. The RNA pellets were washed with 75% ethanol and finally centrifuged at 7500 rpm for 5 minutes at 4 ºC. They are dried for 5 minutes and resuspended in

20 µl sterile water (Eurobio Scientific). The resulting RNA concentration and purity is quantified by spectrophotometric assay assessed as 260/280 nm and 260/230 nm ratios by NanoDrop technology ND-1000 spectrophotometer (NanoDrop Techn., Wilmington, DE, USA).

RNase R Treatment

For each sample half of the isolated RNA was treated for 15 min a 37°C with 4 units/µg Ribonuclease R (RNaseR) (Lucigen RNR07250) in water supplemented with the digestion buffer provided by the manufacturer. Both RNaseR treated and untreated samples were purified with RNA Clean & Concentrator (Zymo Research). Reverse Transcription for cDNA synthesis was performed from 500 ng with Super Script III (Invitrogen), with random primers (Invitrogen) according to the manufacturer's protocol.

Primers, PCR and qRT-PCR

cDNA obtained using 500 ng of RNA, was synthesized using the High capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies; Carls-bad, CA). Primer sequences used to detect linear RNA were designed with the NCBI primer blast tool. Divergent primers for selective amplification of circRNAs were designed with Primer3 v. 0.4.0 and synthesized by Sigma-Aldrich, circRNA

ID confirmed using CircBase database. The primers used are included in Table 8 as follow.

Table 8: Primers used in this study

RNA ID	Primer Forward	Primer Reverse
PLOD2	GAGTCACCACGAAAAGGCTG	TGGCCCAAAGTGAAGTTGTT
18s rRNA	CGGCTACCACATCAAGGAA	GCTGGAATTACCGCGGCT
circPLOD2 hsa_circ_0122319	TGGAGAGGTGGTGATGGAAT	ATCCATCACTTTCTTTTGTTGCT
circRUNX1 NA	TTGTGACTGAGAAGGTGGGG	CCTGAGGTTAGAAGATGGTGC
circNPM1 hsa_circ_0075011	ACTTGCTGCTGATGAAGATGA	AACCTTGCTACCACCTCCAG
circNRIP hsa_circ_0115639	CCGGATGACATCAGAGCTACT	GGCTGTGTTTCTCCCAAATG
circHNRNPH1 NA	TGAAGTCAAATTGGCCCTGA	GGCGAGGCTTTTGTTGAACT
circSMARCC1 hsa_circ_0065251	GGAGACTGAAGAGAACAAAGAAC	GTGCTTTATCACCTTCATCCGT
circL3MBTL4 hsa_circ_0108802	AGAGGAAAAGAAGCCCAAGGA	TGGTCTGACACGTATTCTTGG
circL3MBTL4 hsa_circ_0000824	GCATGGTCTTGGGAGTGGTA	TGGTCTGACACGTATTCTTGG
circL3MBTL4 hsa_circ_0046760	TAGCCCTTATGTCCAGCCAG	TGGTCTGACACGTATTCTTGG
circR3HDM1 hsa_circ_0001070	TATGCCCACAACAGGAATGC	CCACTGACTAGGCTCTGACT
circLRBA hsa_circ_0071185	AGGCCTTGCTTTTATCGAACT	TTTCACAAGATCCGGTTTCAAAT

Untreated/treated with RNaseR RNA extracted from healthy donor samples was used for circRNA validation.

Circularity was confirmed by the absence of sensitivity to exonuclease RNaseR. PCR was performed with Master Mix 360 (AmpliTaq Gold 360, Applied Biosystems), primers 200 nM and product were run on 2% agarose gels. Sanger Sequencing (SeqStudio Genetic Analyzer, ThermoFisher) was performed on the PCR product to confirm the presence of the exact backsplicing junction (BSJ). Successively quantitative Real-Time PCR (qRT-PCR) was performed with technical triplicates with Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) in 10 µl per well, from 10 ng cDNA, 500 nM primers. The reaction was incubated in Step One Plus (Applied Biosystems, Thermo Fisher Scientific). Ct thresholds were determined by the software. The fold-change of expression was calculated using the $2^{-\Delta\Delta Ct}$ method and 18S ribosomal RNA (18S rRNA) was used as the internal control gene to normalize the relative expression. The data were plotted using GraphPad PRISM.

SiRNA Transfection

RNA silencing was performed by siRNA transfection according to Amaxa Nucleoflector® technology (Lonza). Briefly, 500 pmol of siRNA was used to transfect $2x10^6$ cells, previously resuspended in a volume of 0.1 mL. The suspension was then immediately transferred to a flask containing a preheated

culture medium. SiRNA custom, siRNA validated and non-targeting control siRNAs were purchased from Thermo Fisher Scientific. The transfection efficiency was assessed after 24 and 48 hours of culture by analysis of linear transcript and circRNAs levels with real-time PCR.

circPLOD2 overexpression

The cDNA encoding circPLOD2 was been amplified by PCR with modified primers containing the endonuclease recognized sequences (F: 5'-GATATCATAAATTATTAGTCATAACTGTTAG-3': R: 5'GTTAACCATTCAGTAAACATGACAACCA -3'). Subsequently, the purified circPLOD2 PCR products were ligated to pcDNA3.1(+) CircRNA Mini Vector (Addgene plasmid) according to the manufacturer's instructions. The One Shot® TOP10 Competent Cells (Invitrogen, Life Technologies) was transformed with circRNA Mini Vector-circPLOD2 expression construct or with the empty circRNA Mini Vector. The colonies were selected and analyze by plasmid isolation, PCR and Sanger sequencing. The circRNA-expressing plasmid was used to transfect SET-2 cell line and CD34+ cells. At 24 and 48 hours after transfection cells were collected for the relevant assays.

JAK2 wt/ mut overexpression

The pRP[Exp]-EGFP-CMV>hJAK2 [NM_004972.3] and pRP[Exp]-EGFP-CMV>hJAK2 V617F vectors expressing respectively human wild type JAK2 and mutated JAK2V617F mutated gene were customize by VectorBuilder. As a negative control, cells were also transfected in the absence of an ectopic construct (mock).

Assessment of apoptosis

Quantification of apoptotic cells was accomplished using Annexin-V-FLUOS Staining kit (Roche, Basel, Switzerland). 5 x10^5 cells were removed from the wells after 24 and 48 hours of incubation with the various compounds and washed with PBS by centrifugation at 1200 rpm for 5 minutes. The pellet was resuspended in 100 µl of Incubation Buffer with the addition of 2µl of Annexin-V-FLUOS labeling solution (Roche) and 2µl of propidium iodide and incubated at room temperature for 15 minutes. Then 300 µl of Incubation Buffer were added and we proceeded to the evaluation of at least 100.000 events in a FACS SCAN flow cytometer (Becton Dickinson). Data were processed with Flow-Jo software (Tree Star, Ashland, OR, USA).

Statistical Analysis

The comparison between continuous variables was carried out by t-test using GraphPad InStat software (GraphPad Software, Inc., San Diego, http:// www. graphpad.com) or Origin software (OriginLab, Massachusetts, USA). Data were expressed as mean ± standard deviation (SD). The level of significance from two-sided tests was $P<0.05$.

RESULTS

The circRNAome is dysregulated in MPN

Analysis of RNA-seq data conducted on CD34+ cells of cord blood (n=3) and CD34+ cells of PMF patients (n=8) were conducted in collaboration with Professor Stefania Bortoluzzi and Dr.ssa Anna dal Molin of the University of Padova. Analysis with Circompara (Gaffo E et al., 2022) detected 48,775 circRNAs. 3,455 were considered for further analysis. Unsupervised sample analysis based on circRNA expression profiles indicated a deep dysregulation of the circRNAome in CD34+ cells of MPN patients compared with controls. Next, we identified 1,166 circRNAs differentially expressed in MPN, 1,116 up- and 50 down-regulated in malignant cells, including 1,073 that were present only in MPN and 40 that were expressed in normal CD34+, but absent in MPN. Eleven among the circRNAs most dysregulated in MPN were prioritized for further study (Table 9).

Table 9: CircRNAs with log2FoldChange>2 in PMF versus HD, high expression (read count>200), or coming from cancer genes, prioritized in this study. LFC, Log2FoldChange (PMF vs HD); Padj, adjusted p-value; Aver.HD, average expression in HD samples; Aver.PMF, average expression in PMF samples

CircBase ID	CircRNA coordinates	Gene Name	LFC	Padj	Aver.HD	Aver. PMF
hsa_circ_0001070	2:135675332-135680324: ±	R3HDM1	12,48	2,81E-16	0	775,85
hsa_circ_0065251	3:47635190-47636136:-	SMARCC1	11,66	1,58E-12	0	452,27
hsa_circ_0075011	5:171392710-171392978:+	NPM1	10,57	2,73E-08	0	211,74
hsa_circ_0115639	21:14987742-15043574:-	NRIP	-10,36	2,87E-04	385,25	0
hsa_circ_0122319	3:146121112-146124229:-	PLOD2	12,35	2,17E-11	0	708,49
hsa_circ_0108802	18:6301903-6312056:-	L3MBTL4	12,29	4,05E-17	0	702,53
hsa_circ_0000824	18:6263947-6312056:-	L3MBTL4	11,44	1,32E-12	0	384,17
hsa_circ_0046760	18:6237964-6312056:-	L3MBTL4	10,84	5,13E-10	0	261,32
hsa_circ_0071185	4:150761783-150808398:-	LRBA	10,73	7,00E-09	0	240,83

NA	5:179621242-179621370:-	HNRNPH1	-9,92	0,00242	236,57	0
NA	21:34846291-34846510:-	RUNX1	-10,06	0,00664	256,88	0

We tested with PCR and Sanger sequencing on granulocyte (GN) RNA samples (thanks to greater availability of the sample) of a first set MPN patients. From the first qualitative analysis we have selected n=6/11 circRNAs (hsa_circ_0122319-*PLOD2*; hsa_circ_0075011-*NPM1*; hsa_circ_0115639-*NRIP*; hsa_circ_0108802-*L3MBTL4*; hsa_circ_0000824- *L3MBTL4*; hsa_circ_0046760-*L3MBTL4*) because backsplicing validation frequencies result more markedly different in MPN compared to controls. In particular hsa_circ_0122319, or circPLOD2, was detected in 40% of patients with myelofibrosis and absent in healthy donors. Thus we confirmed that circPLOD2 is somewhat specific of MPN. Moreover we have observed that GN does not represent the most adequate and rich fraction of circular RNA compared to CD34+ sample (Figure 2). So we continued the experiments on CD34+ fraction: CD34+ patients (n = 47) and CD34+ healthy donor control cells (n = 8).

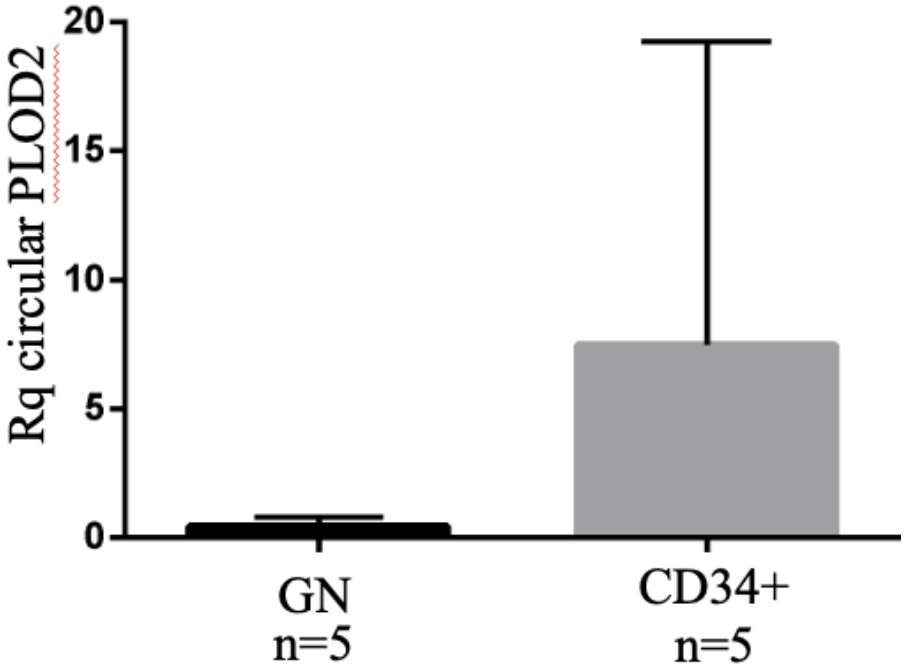

Figure 2: Figure shows the relative expression levels of circular PLOD2 in two different fractions of the same patients: Granulocytes (GN) and CD34+ cells.

Study of circPLOD2 in MPN patients

Further quantitative analysis in Real-Time PCR was conducted for circPLOD2 and linear PLOD2 mRNA. Differential expression analysis in qRT-PCR showed that

both circPLOD2 (Figure 3A) and the linear transcript (Figure 3B) are significantly overexpressed (p=0.0004; p= 0.001) in patients compared to healthy donors respectively.

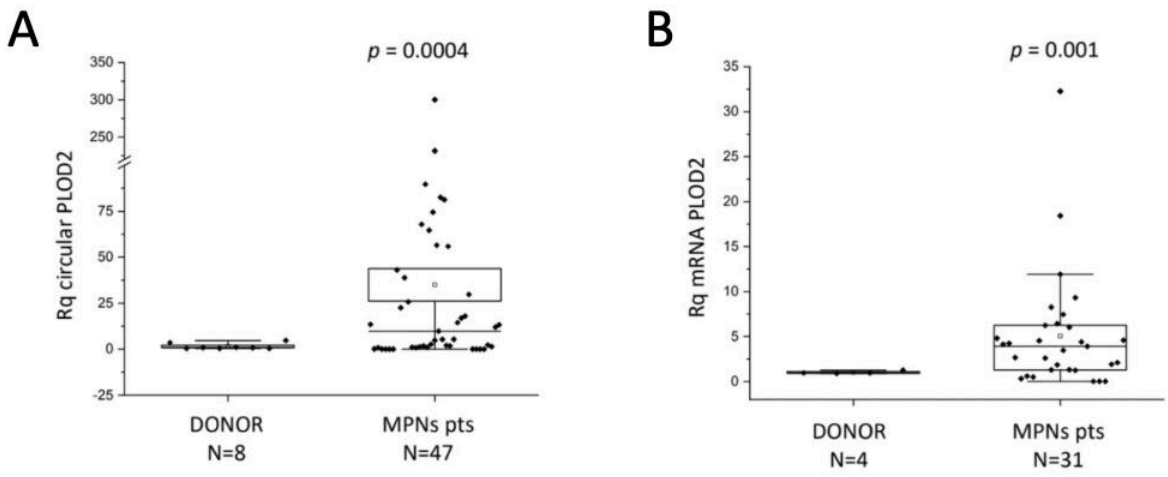

Figure3: The figure shows differential expression levels statistically significant of A) circPLOD2 in MPN pts vs Donor (p= 0.0004) and B) linear PLOD2 MPN pts vs Donor (p= 0.001).

Splitting patients by MPN group we have demonstrated that the expression level results higher in myelofibrosis patients and at some extent in PV cases; in particular, p=0.006 in PMF patients and p=0.01 in patients after myelofibrotic transformation compared to healthy donor as reference (Figure 4A). Moreover dividing patients by

driver mutation, we observed a statistically significant increase in patients with JAK2 and CALR mutations (p=0.01 and p=0.03, respectively) (Figure 4B).

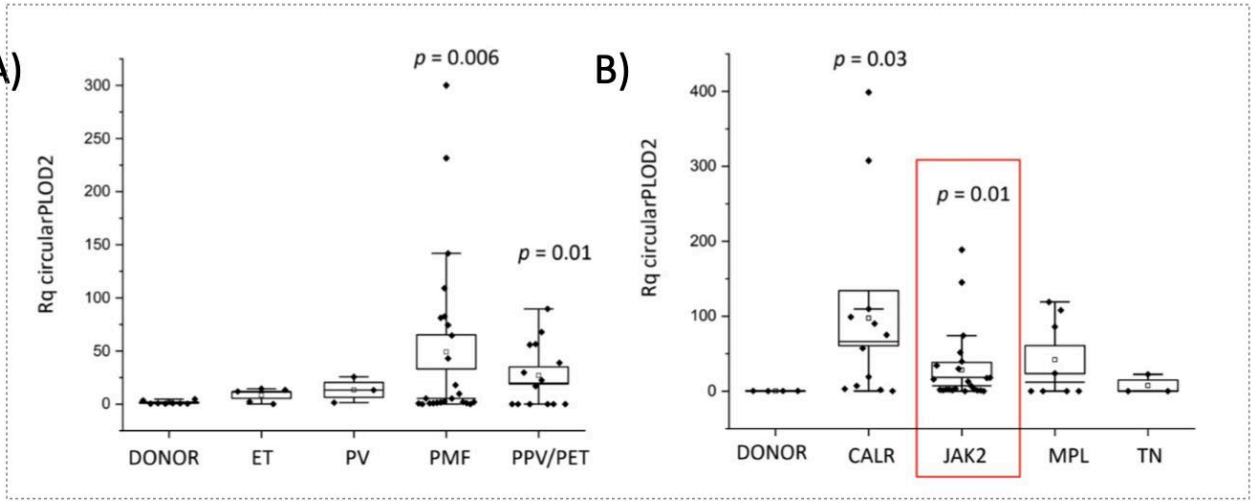

Figure 4: The figure shows relative expression levels of circPLOD2 in patients classified for diagnosis **A)** and for driver mutations too **B)**.

Study to understand the interaction between the JAK-STAT pathway and circPLOD2 in MPN

JAK-STAT pathway has a crucial role in regulation and homeostasis in hematopoiesis. Unregulated JAK/STAT signaling contributes to proliferation, survival, inflammation, invasion, new blood vessel formation, and metastasis, which are implicated in cancer initiation, progression, and advancement (Bose et al., 2020).

In order to understand if the increase of circPLOD2 expression in MPN is related to the JAK-STAT pathway activation, we quantified the relative expression in patients (n=10) pre and post treatment with the JAK2 inhibitor Ruxolitinib. In all cases (100%), we observed a decrease of circPLOD2 after the treatment (Figure 5A).

Differently, we have treated the control CD34+ cells with JAK2 inhibitor at increasing concentrations without results on circPLOD2 level. We thus concluded that the JAK2 inhibition has an effect on circPLOD2 expression in CD34+ cells of patients but not in those healthy donors (Figure 5B).

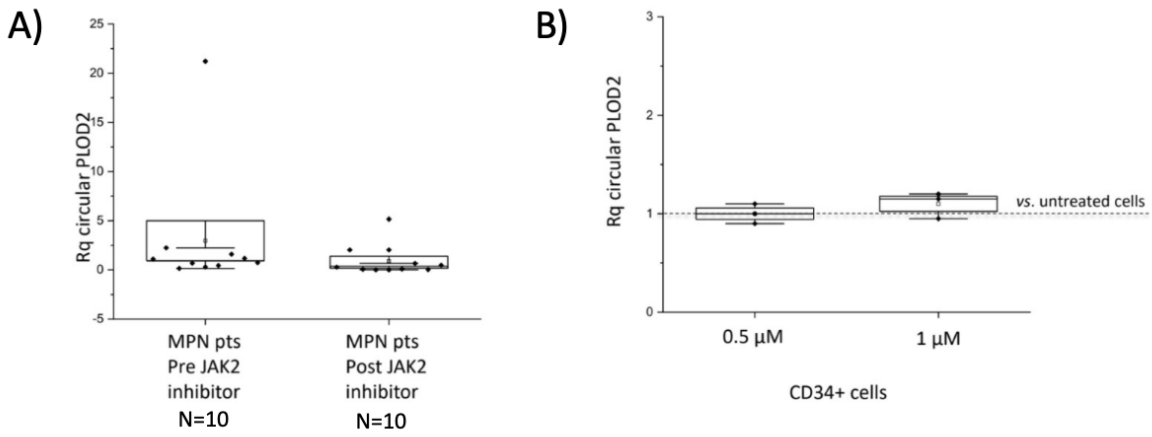

Figure 5: The figure shows the relative expression of circPLOD2 **A)** in n=10 pts pre and post JAK2 inhibitor treatment **B)** in CD34+ control cells after increased concentration of JAK2 inhibitor.

In addition, we transfected CD34+ control cells with either a plasmid containing a wild type JAK2 construct or with a plasmid containing a mutated JAK2V617F construct. After 48h of transfection (after confirmation of transfection efficiency of at both 24 and 48h) we observed a significant increase of circPLOD2 expression upon transfection with the mutated JAK2V617F construct (p=0.001) compared to the expression of circPLOD2 in transfected cells in the absence of ectopic construct (Figure 6). This corroborated the dependence of circPLOD2 expression from JAK/STAT hyperactivation.

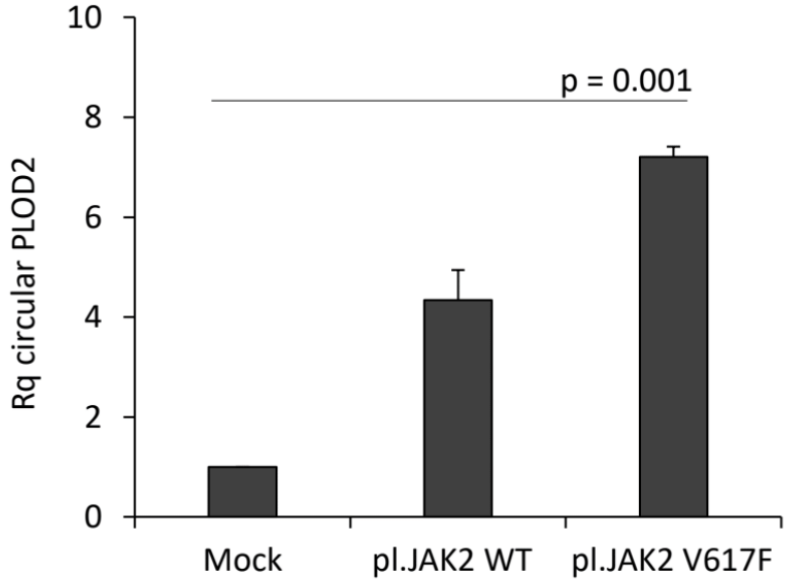

Figure 6: The figure shows the differential expression of circPLOD2 after transfection of CD34+ control cells with plasmid JAK2 wt and with plasmid JAK2V617F mutated.

Later we treated the SET-2 cell line (which carries the mutation of JAK2V617F in heterozygosity) with the JAK2 inhibitor. Again after 48h of treatment at concentrations of 0.5 µM and 1 µM and after having confirmed its effectiveness by means of the growth curve, we observed a marked significant decrease of both circular and linear PLOD2 ($p \leq 0.001$) after the treatment, with more intense variations at higher Ruxolitinib concentration(Figure 7). This additionally backs up the concept that circPLOD2 expression is dependent by JAK/STAT activation.

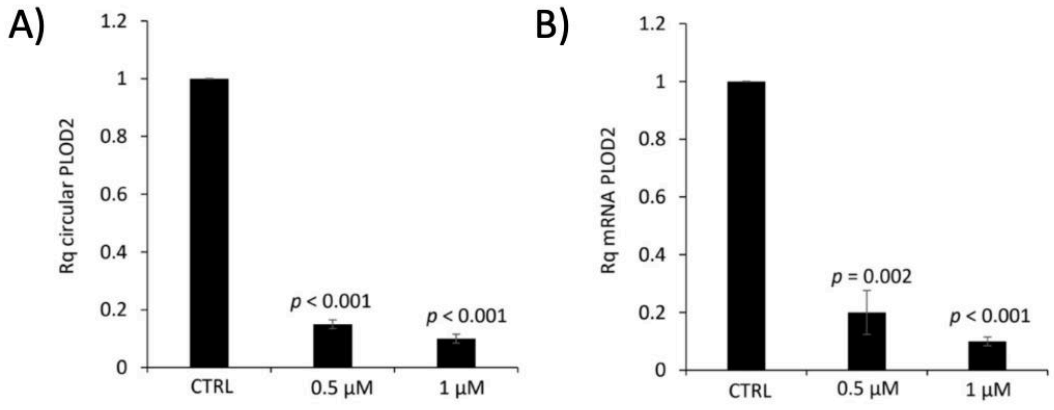

Figure 7: Relative expression of A) circPLOD2 B) linear PLOD2 upon SET-2 cell line treatment with the JAK2 inhibitor Ruxolitinib 0.5 and 1 µM.

Functional Experiments to explore the effects of circPLOD2 in MPN

Further functional investigation of circPLOD2 was carried out to assess a possible contribution of this circRNA to malignant cell behavior. We have silenced, using specific siRNAs, both the PLOD2 mRNA and the circPLOD2 in the JAK2 mutated SET2 cell line. After confirming the transfection efficiency, we have evaluated the effect of the silencing on cell proliferation and on induction of apoptosis. We did not observe significant changes in cell proliferation upon silencing, neither suppressing circPLOD2 not PLOD2 mRNA (Figure 8). Similarly, apoptosis rate remained stable, as shown by cytometric analysis for the evaluation of ANNEXIN V/ Propidium Iodide (Figure 9).

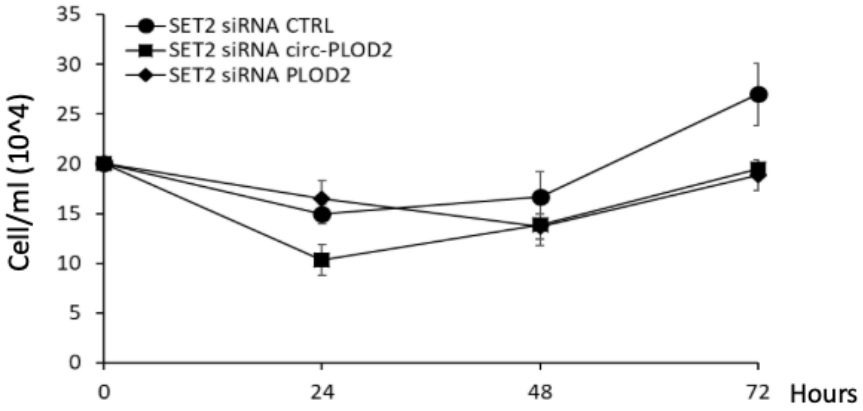

Figure 8: SET-2 cell proliferation

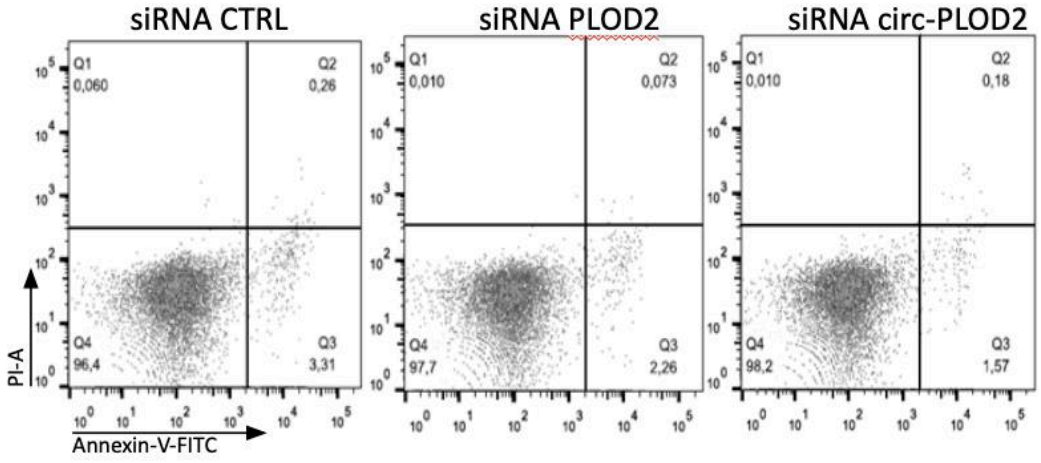

Figure 9: The apoptosis induction with the evaluation of ANNEXIN V/ Propidium Iodide

Additional experiments were conducted by overexpressing circPLOD2, by means a custo-developed plasmid. CD34+ cells from healthy donors were transfected with the circPLOD2 overexpressing plasmid, and with mock ectopic plasmid, as control. The effect of overexpression circPLOD2 on megakaryocyte and erythroid differentiation was evaluated after confirming the transfection efficiency through circPLOD2 expression in qRT-PCR.

To achieve the growth of erythroid and megakaryotic cells, two-stage cultures were used during their in-vitro differentiation. A proliferative phase of expansion of the progenitor pool and the second phase of differentiation.

After 10 days, we observed a significant increase of the megakaryotic colonies upon circPLOD2 overexpression (Figure10).

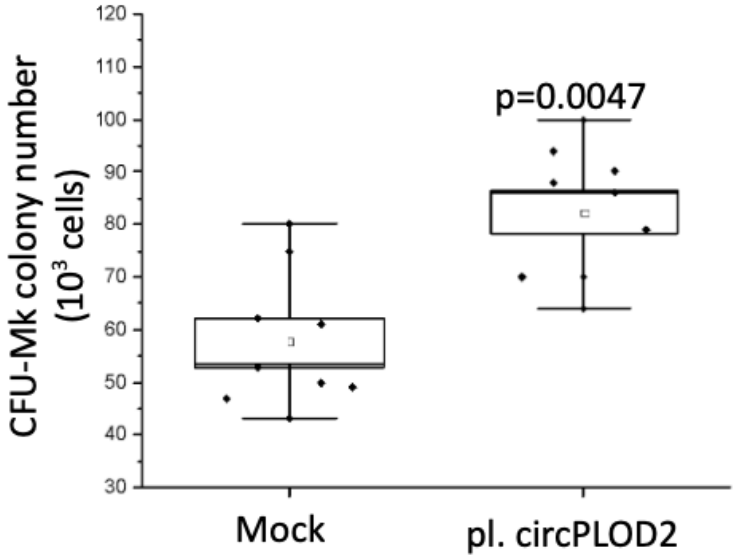

Figure 10: Number of megakaryotic colonies after transfection with the circPLOD2 expression plasmid compared to the control colonies.

After 12 days, we observed a tendency toward decrease of the erythroid colonies upon circPLOD2 overexpression, with a more marked decrease of the BFU-E than CFU-E colonies (Figure11A), indicating an impact of circPLOD2 expression particularly on early erythroid precursors.

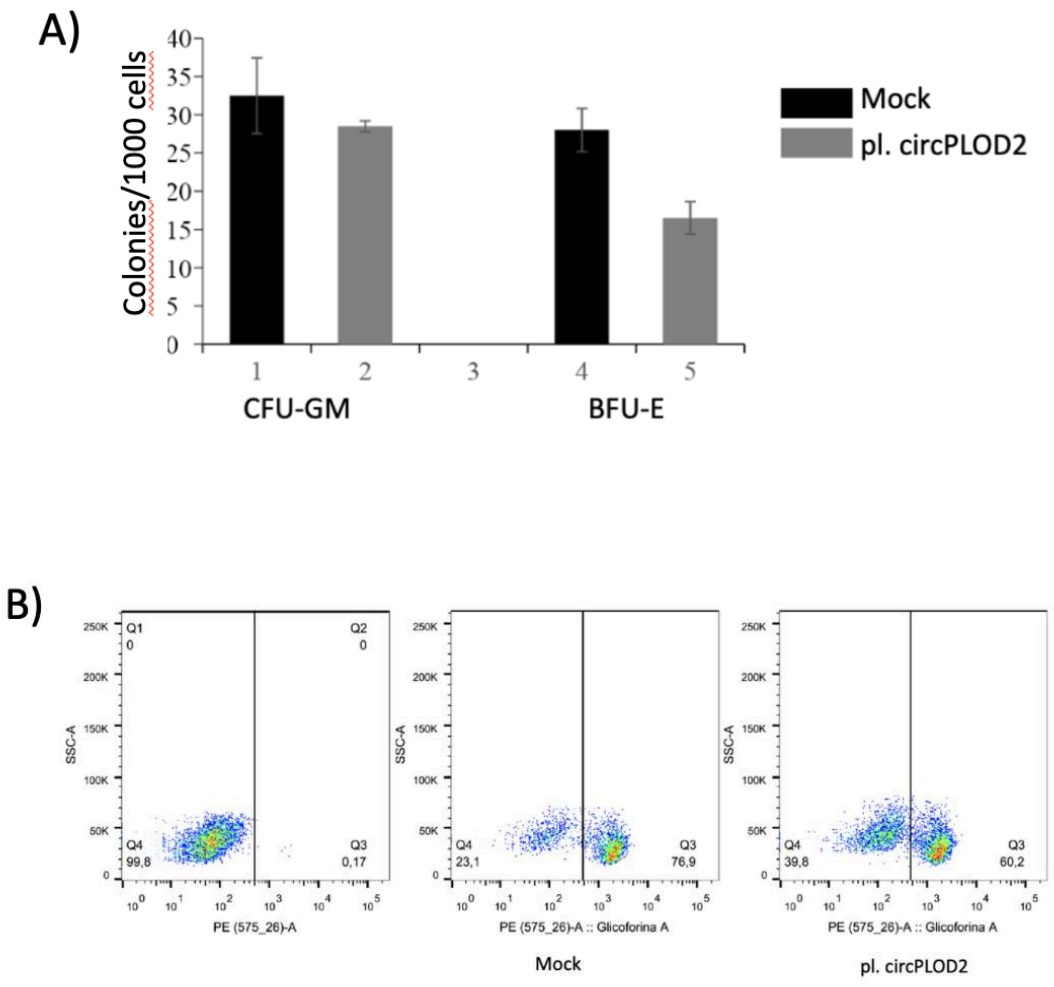

Figure 11: A) Number of erythroid colonies CFU-E and BFU-E **B)** flow cytometry analysis for the evaluation of erythroid differentiation with anti-GPA after transfection with the circPLOD2 overexpressing plasmid compared to mock ectopic plasmid.

DISCUSSION

Myeloproliferative Neoplasms (MPN) are one of the five categories of myeloid neoplasms according to the World Health Organisation (WHO) classification. The first milestone in the knowledge of the genetic basis of myeloproliferative neoplasms was reached in 2005 with the identification of a mutation of the *JAK2* gene, involved in the intracellular signalling pathway JAK-STAT. Not long after, further somatic mutations were identified against the MPL gene, which encodes for the thrombopoietin receptor, also involved in the same intracellular signalling pathway.

From the functional point of view, these are mutations that give a gain of function to the mutated protein, which is therefore constitutionally activated. Despite such scientific advances, however, a not insignificant proportion of patients with TE and MF remained without a known molecular marker.

The discovery in 2013 of the mutation of the gene *CALR* has partially fill this gap. Somatic mutations of *JAK2*, *MPL* and *CALR* behave as driver mutations, as they result in the acquisition of a selective advantage in a self-renewing cell, leading to the formation of a mutated clone. These scientific milestones led to the introduction of the term "triple negative" to identify that subtype of patients (about 20-30%) not

affected by the three mutations drivers, but with a clinical and histopathological picture compatible with a chronic myeloproliferative neoplasm. Efforts must focus on this not negligible group of patients for which known molecular markers are lacking.

To date, there are no therapies for MPN patients able to ensure the recovery of the disease, except for the transplantation of hematopoietic stem cells, which is, however, burdened by a high toxicity.

The aim of this project is to explore new routes to possible novel prognostic treatments and alternative strategies for MPN patients with particular interest in triple negative patients who represent the most insidious category for which the diagnosis is most inauspicious.

For this reason it is important to look for mechanisms that can explain the pathways related to these diseases and circular RNAs could be interesting for this purpose.

It's already been reported that more and more circRNAs result dysregulated in many human malignancies and have been shown that they could be tightly regulated in the course of differentiation.

In this regard, we have shown that circPLOD2 is significantly overexpressed in MPN patients compared to control samples.

After dividing the patients by MPN types and genetic lesions, we focused our interest on JAK2- mutated patients. In order to understand whether the increased

expression of circPLOD2 in patients was linked to the JAK-STAT pathway, which plays a crucial role in the regulation of hematopoiesis and immunity, we evaluated the expression levels of circPLOD2 in patients before and after the treatment with JAK2 inhibitor and observed a downregulation, although not significant, after the treatment. In contrast, in control CD34+ cells the expression remains unchanged. To strengthen the data obtained, we transfected CD34+ control cells with a plasmid JAK2V617F and we observed a significant increase in the expression levels of the circPLOD2 after the transfection, assuming a relationship with the constitutive activation of the JAK-STAT pathway. The same result was confirmed by the treatment of the hematologic cell line SET-2 with increasing concentrations of Ruxolitinib which showed a strongly significant decrease in circPLOD2 after the treatment.

No significant effects of upregulation of circPLOD2 were observed in term of proliferation and apoptosis.

Finally, and more interestingly, we observed that the overexpression of circPLOD2 in control CD34+ cells, increases the number of CFU-Mk colonies to indicate a stimulation towards megakaryotocyte differentiation. No significant differences were observed during the erythroid differentiation, as shown by BFU-E forming colonies and by Glycophorin A cytometric analysis.

In summary, we demonstrated that circPLOD2 expression is dependent on the JAK-STAT pathway activation. CircPLOD2 silencing did not impact proliferation

and apoptosis of malignant cells, suggesting that this circRNA cannot be associated with a generalized oncogenic role in MPN. Of interest, enforced circPLOD2 expression significantly boosted differentiation of megakaryocytes and, to some extent early erythrocytes, suggesting a role of this circRNA in the regulation of myelopoiesis in myeloproliferative neoplasms. RNAi assay will be used to inhibit the expression of circPLOD2, might contribute to downregulates JAK-STAT pathway in MPNs. Integration of bioinformatic predictions and experimental data to identify circRNA-miRNA or circRNA-protein associations will allow us to understand even better the role of circPLOD2 in pathogenesis of MPNs or more in general in hematopoiesis regulation.

Although further studies are needed to understand the role of circular RNAs in the context of myeloproliferative neoplasms, this project provides a good starting point. The data presented suggest that circular RNAs can be a useful tool for developing new diagnostic and therapeutic strategies in MPN.

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