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Analysis of polymorphic variants and new mutations in patients with Chronic Myeloproliferative Neoplasms

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Dedicated to my family

SUMMARY

BACKGROUND

Chronic Myeloproliferative Neoplasms

Chronic Myeloproliferative Neoplasms (MPN) are clonal hematologic disorders arising from an alteration of the hematopoietic stem cell and characterized by abnormal proliferation of one or more hematopoietic lineages in the bone marrow as well as in extramedullary districts.

MPN were first described in 1951 by Dameshek who grouped under the name of Chronic Myeloproliferative Syndromes four individual entities that shared biological and clinical features. According to 2008 World Health Organization Classification, MPN include the classical forms, i.e. Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF), in addition to BCR-ABL1 positive Chronic Myelogenous Leukemia (CML) and other less frequent disorders (Tefferi, 2008).

Chronic Myelogenous Leukemia

Chronic Myelogenous Leukemia is now considered a separate entity since it has unique clinical features and a specific cytogenetic and molecular aberration. CML has an incidence of 1-2 cases/100.000 adults and accounts for 15% of newly diagnosed leukemias in adult population (Jemal, 2010).

This neoplasm is characterized by the presence of the Philadelphia Chromosome originating from the reciprocal translocation t(9;22) (q34; q11) which produces the chimeric fusion oncogene BCR/ABL1 encoding for an abnormal constitutively activated tyrosine-kinase. BCR/ABL1 promotes growth and replication through downstream pathways such as RAS, RAF, JUN kinase, MYC and STAT (Mandanas,1993; Okuda, 1994; Raitano, 1995; Sawyers, 1992; Shuai, 1996; Carlesso, 1996; Ilaria, 1996). Mutated cells display cytokine-independent growth and aberrant response to apoptotic signals. Several studies underlined the causal relationship between BCR/ABL rearrangement and CML development. For example mice expressing a BCR/ABL isoform harboring the K1176R mutation that inactivates the catalytic activity of the fusion protein do not

develop leukemia (Zhang, 1998). The breakpoints within the ABL1 gene can be upstream of exon 1b, downstream of exon 1a or between exons 1a and 1b (Melo, 1996). In most cases breakpoints within the BCR gene span a 5.b Kb region going from exon e12 to e16 which is called Major breakpoint cluster region (M-BCR). Alternative splicing produces either b2a2 or b3a2 transcripts both resulting in a 210-kDa protein (p210). In some cases BCR breakpoint resides in a 54 Kb area between exons e2' and e2 called minor breakpoint cluster region (m-BCR). This breakpoint generates the e1a2 transcript that originates the p190 protein (Faderl, 1999).

BCR/ABL contains several functional domains. The N-terminus of ABL is a "Cap" that exists in two different isoforms generated by alternative splicing of exon 1. The 1b isoform is myristoylated. ABL1 contains a tyrosine kinase domain preceded by an SH2 and SH3 domains (Hantschel, 2004). The region of the last exon contains proline-rich SH3 domains that serve as binding site for SH3 domains of adaptor proteins such as Crk, GRB2 and Nck (Feller, 1994; Smith, 1999), DNA-binding domain, nuclear localization signals and nuclear export signals. Also BCR has several important domains including a Ser/Thr kinase domain and a PH domain. Tyr177 of BCR is a docking size for GRB2, GRB10, 14-3-3 proteins and ABL1 through its SH2 domain (Ren, 2005). The myristoyl modification at the N-terminus of ABL1b has an important role in the autoinhibition property of ABL1 (Hantschel, 2003; Nagar, 2003). BCR/ABL1 activates several downstream signalling pathways such as JAK/STAT that on turn promotes transcription of BCL-X, RAS/MAPK leading to transcription of the anti-apoptotic BCL-2 and PI3K/AKT that causes increased transcription and stabilization of MYC (Quaintas-Cardama and Cortes, 2009).

CML is classified in three stages: Chronic Phase (CP), Accelerated Phase (AP) and Blast Phase (BP). Most diagnosis are made in CP with a consistent percentage being asymptomatic. Common signs and symptoms, mainly resulting from anemia and splenomegaly, include fatigue, weight loss, malaise, easy satiety, and left upper quadrant fullness or pain. More rarely bleeding, associated with thrombocytopenia, thrombosis, due to thrombocytosis and leukocytosis, gouty arthritis, retinal haemorrhages and upper gastrointestinal tract ulceration and bleeding can be present. Half of patients display splenomegaly and 10-20% hepatomegaly. Most patients evolve into AP before progressing to blast phase that is an overt acute leukemia with

worsening constitutional symptoms, bleeding, fever, and infections (Jabbour, 2012). The mechanisms responsible for disease progression are still poorly understood. Possible explanations may be the occurrence of additional chromosome abnormalities, other than Ph chromosome, up-regulation of genes involved in differentiation block, inactivation of tumor-suppressor genes and genomic instability (Quinats-Cardama, 2009).

Diagnosis of typical CML requires the demonstration of Philadelphia Chromosome or BCR/ABL fusion gene through either conventional cytogenetics, FISH or molecular analysis by amplifying the BCR/ABL splice junction (Jabbour, 2208; Kantarjian, 2008; Schoch, 2002). Quantitative RT-PCR is also used as a sensitive assay to assess minimal residual disease. 10-15% of patients have additional chromosomal changes such as trisomy 8, isochromosome 17, additional loss of material from 22q or double Ph, or others. In the past CML therapy consisted of nonspecific agents such as busulfan, hydroxyurea and interferon-alpha (Silver, 1999). Allogeneic stem cell transplantation was an option notwithstanding the high morbidity and mortality. Once tyrosine-kinase inhibitors were developed, the natural course of CML changed dramatically, as 10-years overall survival improved from 20% to 80-90% (Jemal, 2010; Deininger, 2009). These drugs indeed represent a specific therapy that targets BCR/ABL protein. The first one approved was Imatinib mesylate that acts through competitive inhibition at the ATP-binding site of BCR/ABL protein resulting in inhibition of phosphorylation of downstream proteins involved in signal transduction. Subsequently new TKI, such as dasatinib and nilotinib, were developed and can be used as first line therapy or to treat those patients who failed imatinib therapy mainly due to point mutations in the kinasic domain of BCR/ABL that impair the inhibition potential of the drug. TKIs are able to induce complete cytogenetic remission (CCyR) in most patients and in a large part also major molecular remission (MMR) which is defined as BCR/ABL transcript lower than 0.1% expressed in International Scale.

Philadelphia Chromosome-negative Myeloproliferative Neoplams

Philadelphia Chromosome-negative MPN are the most common hematological neoplasms and usually affect elderly persons with a predilection for male gender. A recent study (Rollison, 2008) based on the North American Association of Central Cancer Registries (NAACCR) comprising 82% of United States population reported in the period 2001-2003 a median MPN annual incidence of 2.1/100000 inhabitants and estimated for 2004 6328 new diagnosis in the total US population. Due to the usually indolent course is possible that many cases are not diagnosed. Age, male sex and white race have been shown to represent risk factors. In subjects more than 80 years old MPN frequency rises up to 13.3/100000 per year. The current incidence of these diseases seems to be steadily increasing probably due both to increasing age and better diagnostic procedures. A study investigating survival in 9364 MPN diagnosed in Sweden from 1973 to 2008 assessed that 10-years relative survival ratio compared to expected survival of the general population is 0.64 for PV, 0.68 for ET and 0.21 for PMF patients. Survival increased significantly in last decades but after 2000 the improvement was smaller and confined to PV and ET patients (Hultcrantz, 2012).

Common features of the MPNs are the involvement of a multipotent hematopoietic progenitor, the overwhelming of the transformed clone on the residual normal population, the abnormal proliferation of one or more blood lineages in absence of growth factors, extramedullary hematopoiesis especially common in Primary Myelofibrosis and the possibility of spontaneous evolution to Acute Myeloid Leukemia (Spivak 2003).

The main clinical characteristic of MPN, other than signs and symptoms related to the expansion of each hematopoietic cell lineage, is the high frequency of thrombotic and hemorrhagic events that represent the major cause of death. The clinical phenotype of the different forms of MPN can overlap, especially in the early stage of the disease.

Due to the lack of a specific cytogenetic and molecular alteration and the frequent phenotypic overlapping between the different entities and with

myelodysplastic/myeloproliferative disorders (i.e. atypical CML or Chronic Myelomonocityc Leukemia) or reactive forms, the differential diagnosis of MPN is sometimes complicated. Less than 30% of MPN cases harbor chromosomal alteration and none of these recurs often enough to be considered specific for these neoplasms. Chromosomes 8, 9 and 20 are frequently affected and acquired uniparental disomy of chromosome 9p is reported as the most frequent cytogenetic alteration in PV (Kralovics, 2002; Bacher, 2005). Chromosomic aberrations at diagnosis do not have a prognostic significance while their occurrence during the course of the disease is regarded as a sign of progression (Rowley, 1982; Testa, 1984). Some data point out the possible role of fusion genes with kinase activity involved in aberrant signalling transduction pathways such as PDGFRalpha, PDGFR, FGFR1 and JAK2 in rare cases of atypical CML, CMML and Hypereosinophilic Syndrome and KIT gene in Systemic Mastocytosis. Observation of the different types of MPN revealed hypersensitivity of myeloid cells to cytokines and growth factors, hyper expression of the anti-apoptotic factor Bcl-XL and activation of signal transducers STAT3 and STAT5 (Cross 2002; Delhommeau 2006; Cross 2008).

Many biological and clinical aspects of these diseases still need to be clarified. Experimental and clinical evidences indicate that MPN develop after neoplastic transformation of an immature hematopoietic progenitor with biased differentiation potential towards both myeloid lineages and B-lymphocyte lineage (involvement of T-lymphocytes is more rare) (Raskind 1985).

Through the years some markers of MPN were identified. MPN diagnosis was mostly elucidated after the identification in 2005 of the somatic point mutation V617F in the tyrosine-kinase Janus Kinase 2 gene (JAK2) that frequently occurs in these patients (Baxter 2005, Levine 2005, Jones 2005, James 2005, Kralovics 2005). The spontaneous formation of Endogenous Erythroid Colonies (EEC) was observed when culturing blood mononucleated cells in absence of EPO in 75-100% of PV patients and 50% of ET and PMF patients while it is absent in reactive forms (Klippel, 2004). The PRV-1 (Polycythemia Rubra Vera 1) gene, which is a member of the plasminogen activator receptors family, is overexpressed in PV granulocytes when compared to CML or reactive cases (Klippel, 2003) and in a subgroup of ET patients compared to secondary thrombocytosis (Teofili, 2002). The study of the inactivation of HUMARA

(Androgen Receptor) gene or other X- chromosome associated genes in affected females revealed clonal hematopoiesis in PV and PMF and in 50% of ET patients (Gale, 1997; Champion, 1997; Harrison, 1999). Monoclonality correlated with thrombotic events in ET females patients (Chiusolo, 2001; Vannucchi, 2004). Altered expression of Thrombopoietin (TPO) receptor c-MPL was demonstrated by immunohistochemistry and immunoblotting assays in platelets and megakaryocytes of MPN patients but not in CML or reactive erythrocytosis patients. Its correlation with the incidence of thrombotic events is still unclear (Horikawa, 1997; Moliterno, 1998; Moliterno, 1999; Tefferi, 2000; Teofili, 2002). TPO is the most important cytokine responsible for platelet production. Its binding to MPL causes activation and consequent dimerization of the receptor followed by JAK2 activation and downstream phosphorylation of STAT proteins which on turn migrate to the nucleus where they act as transcriptional factors. In PMF patients that do not display JAK2V617F mutation a somatic point mutation 1544G>T (W515L) of MPL gene was identified. This mutation determines activation of the downstream JAK2/STAT signalling cascade in the absence of TPO (Pikman, 2006). Afterwards other mutations, W515K, W515A and W515R, involving the same codon and with the same constitutively activation effect were identified (Pardanani, 2006; Beer, 2008; Chaligné, 2008; Vannucchi, 2008). MPL mutations can be found in 15% of ET and PMF patients (Pietra, 2011). Some studies revealed that MPL mutations can also be found in a small proportion of JAK2V617F positive patients and that the extent of the two clones is usually inverse. Both mutations seem to be an early event in the disease course and it has been hypotized that phenotypic variations are mostly due to the variation of the allele burden rather than to the mutation itself (Lasho, 2006). New mutations affecting exon 12 of JAK2 gene were identified in the small group of PV patients that do not harbor JAK2V617F mutation. These mutations are insertion, deletions and point mutation located in a region that links the SH2 domain to the regulatory JH2 domain and can somehow determine structural modification of JH2 domain. These mutations, similarly to V617F, determine proliferation in the absence of growth factors (Scott 2007, Pietra 2007)

Polycythemia Vera

Polycythemia Vera (PV) is characterized by abnormal expansion of erythroid lineage, possibly associated with leukocytosis and thrombocytosis.

Diagnostic criteria for PV were initially established by the Polycythemia Vera Study Group (PVSG) and were based only on clinical parameters; in 2001 bone marrow panmyelosis was included in the minor criteria of WHO classification. Absence of BCR/ABL fusion gene is needed to exclude CML as well as causes of reactive erythrocytosis must be excluded. Karyotypic alterations do not impact on prognosis but are considered a main diagnostic criterion in that they point to the existence of clonal hematopoiesis and allow to exclude reactive erythrocytosis. Cytogenetic abnormalities occur in 30% of patients and are predominantly represented by chromosomes 8 and 9 trisomy and 20q deletion; alteration of chromosome 5, 6, 12, 13 and 1q duplication were reported. After the identification of JAK2 mutations, that occurs virtually in >95% of PV patients, some of the previous criteria became useless. Currently, according to WHO 2008 criteria for PV (Tab.1), diagnosis requires both increase of red cell mass and presence of JAK2 mutation, as the two major criteria, together with one of the minor criterion or the first major criteria and two minor criteria.

Major Criteria
Hemoglobin > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume
Presence of JAK2 V617F or other functionally similar mutation such as JAK2 exon 12 mutation
Minor Criteria
Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation
Serum erythropoietin level below the reference range for normal
Endogenous erythroid colony formation in vitro

Tab. 1 – WHO 2008 diagnostic criteria for PV (Tefferi, 2009)

The incidence of PV is estimated at 0.79 cases/100.000 per year and it represents almost a half of all MPN with a median 3 year survival of 88% (Rollison 2008). Median age at diagnosis is 60 years and no more than 5% of patients is under the age of 40. (Berlin, 1975). A little prevalence in male gender was observed (Modan, 1995).

PV is characterized by global bone marrow hypercellularity, with a predominance of erythroid expansion which is largely independent from erythropoietin, the main erythrocytes growth factor. This is confirmed by the evidence of in vitro spontaneous formation of erythroid colonies in absence of EPO stimulation (Prchal, 1974). The identification of JAK2 mutation possibly explains the molecular basis of this phenomenon. Experimental data demonstrated the hypersensitivity of PV erythroid progenitors in response to growth factor such as EPO, GM-CSF, IL-3, IGF-1 and TPO, suggesting an underlying global defect of the intracellular signalling transduction system rather than a dysfunction of specific receptors. Mutations of EPO receptor have been demonstrated in Hereditary Erythrocytosis patients that in 20% of cases are transmitted as a dominant autosomic defect that causes increased erythropoiesis but retains normal leucocyte and platelet counts with reduced EPO levels. Unlike PV progenitors, mononuclear cells from Hereditary Erythrocytosis patients do not form EEC and are sensitive to low levels of EPO (McMullin, 2008). Cases of Hereditary Erythrocytosis on autosomic recessive basis have been described, arising from loss-of-function mutations in VHL gene that determine increased levels of HIF1alpha and consequently enhanced production of EPO (Kralovics and Prchal, 2000). Neither mutations of EPO receptor nor VHL mutations were found in PV. In PV erythroid progenitors display overexpression of the anti-apoptotic protein bcl-xl which is also expressed in mature cells unlike in normal subjects (Fernandez-Luna 1999) Some hematopoietic growth factors, such as IGF-1, partially suppress apoptosis. Hypersensitivity to growth factors could thus derive from an intrinsic resistance to physiologic apoptosis mechanisms.

Main clinical features of PV are direct consequences of the increased proliferation of the different hematopoietic lineages. 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. Costitutional symptoms such as weight loss, night sweats, and fever, are present al

diagnosis in <30% of cases. Hepatomegaly, splenomegaly, cyanosis and arterial hypertension are common. Main complications are thrombotic events that can occur also in uncommon sites such as principal abdominal vessels. PV is characterized by increased erythrocyte mass or hemoglobin values higher than 18.5 g/dL in men and 16.4 g/dL in women for at least 2 months. Increased plasma volume can lead to underestimation or failure to identify the increased erythrocyte mass (Spivak, 2002; Spivak, 2003, Spivak and Silver, 2008). Leukocytosis and thrombocytosis occur in 50% of patients. Erythrocyte morphology is usually normal and only in the advanced phase of the disease occasional erythroblasts or myeloid progenitor cells, such as myelocytes, can be found, suggesting transformation to post-PV myelofibrosis. Leucocyte alkaline phosphatase levels are increased in 70% of patients.

The most frequent cause of death in PV patients are cardiovascular events (41% of deaths). PV patients mortality increases with age being 1.6 fold higher than general population in patients under 50 years and 3.6 fold higher in patients older than 50 (Passamonti, 2004: Cervantes, 2008). PV can evolve to secondary myelofibrosis and more rarely in acute myeloid leukemia. Evolution is responsible for 13% of deaths (Marchioli, 2005). Frequency of evolution to secondary myelofibrosis is estimated at 5% after 15 years from PV diagnosis (Marchioli, 2005). ECLAP study estimated that progression to AML occurs in 1.3% of patients after an average of 8.4 years from diagnosis. Age, leukocytosis and duration of disease are risk factors associated to AML progression (Finazzi, 2005). Treatment with P32, clorambucil or polychemotherapy associated an increased risk of AML evolution (Murphy, 1995; Murphy 1997; Barbui 2004). Current treatment recommendations for PV suggest phlebotomy to maintain hematocrit value <45%. Most patients do not require additional therapy for long time. Indication for myelosuppressive treatment with hydroxyurea are an exceeding need for phlebotomy, increasing myeloproliferation or a condition of high thrombotic risk.

Essential Thrombocythemia

Essential Thrombocythemia (ET) is characterized by abnormal megakaryocyte proliferation that leads to increased platelets count. Diagnostic criteria for ET according to WHO 2008 classification are reported in Tab. 2 (Tefferi, 2008).

Major Criteria
Platelet count ≥ 450 x 10^9/L
Megakaryocyte proliferation with large and mature morphology. Absent or poor granulocyte or erythroid proliferation.
Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm
Demonstration of *JAK2*V617F or other clonal marker **or** no evidence of reactive thrombocytosis

Tab. 2 – WHO 2008 diagnostic criteria for ET (diagnosis requires all major criteria)

ET pathogenetic mechanism is still poorly known. Thrombocytosis is caused by augmented platelet production in the bone marrow where megakaryocytes display larger volume and increased nuclear lobulations and ploidy, rather than from prolonged platelet (Buss, 1994; Thiele, 2003). Clonality analysis in female patients affected from ET, who were heterozygous for G6PD isoenzymes, demonstrated the involvement of a pluripotent hematopoietic stem cell (Fialkow, 1981; Fialkow, 1990). Further studies, involving restriction-fragment length polymorphism analysis of X chromosome, evidenced that also granulocytes, platelets and occasionally B-lymphocytes are clonal (Chen, 2007). Nevertheless a subgroup of non-clonal lymphocytes has been demonstrated, indicating the presence of a residual normal population that is overwhelmed by the neoplastic clone during disease progression. These results suggest that neoplastic transformation can occur at different levels of the hematopoietic hierarchy.

TPO gene mutations have been described in cases of Hereditary Thrombocytosis. (Cazzola, 2000; Skoda, 2005: Liu, 2009; Skoda, 2009). Thrombopoietin is the main cytokine involved in megakaryocytes growth and differentiation and platelet production. Four different mutations have been reported, with autosomic dominant transmission and complete penetrance. The clinical phenotype of the disease is characterized by increased platelet count with normal hematocrit and high serum TPO

levels and it manifests since the very early childhood. All the mutations induce hyperproduction of TPO by reducing or abolishing the inhibitory action of regulatory regions. It has been hypothesized that such mutations might affect also some ET patients that display high serum TPO levels.

Studies on the TPO receptor MPL showed its reduced expression in platelets derived from ET patients thus probably explaining TPO high levels (Li, 2000). Reduced expression of MPL is associated with altered glycosylation that leads to defective activation of the downstream kinase cascade and subsequent altered phosphorylation of JAK2 and STAT5 (Moliterno, 1998). JAK/STAT pathway activation may occur after activating mutation of cytokines receptor, such as MPL. Most studies investigated the transmembrane domain which is critical for dimerization and receptor activation and the juxta-membrane domain which is needed for JAK2 binding. These studies lead to the identification of W515L and W515K point mutations that cause constitutive activation of the downstream signalling pathway in the absence of TPO (Pardanani, 2006; Pikman, 2006). MPL mutations were first estimated to occur in 1% of ET patients (Pardanani, 2006) but more recent studies, including from our group, demonstrated that the percentage of mutated cases is up to 5-8% (Beer, 2008; Vannucchi, 2008).

Mutations in p53 gene were identified in ET patients, probably associated with progression to acute leukemia (Neri, 1996).

The role of megakaryocytes (MK) in ET has been deeply studied. In vitro analysis on MK colonies growth demonstrated that in some patients it may be cytokine-independent (Grossi, 1987). Furthermore MK precursors are less responsive to the treatment with growth inhibitors such as TGF-β, that is one of the most powerful MK growth inhibitors (Zauli, 1993). TGF-β is produced by MKs themselves, thus its high levels could be a consequence of increased MK and platelets number. It has been hypothesized that in the bone marrow microenvironment TGF-β stimulates local production of TPO that in turn induces the expression of TGF-β receptor thus enhancing growth inhibition. Some studies suggest that spontaneous formation of MK colonies may be due to lack of platelet factor 4. It was demonstrated that this factor inhibits MK colonies growth in ET patients (Han, 1990). Both TGF-β and platelet factor

4 are stored in α granules that have known inhibition activity on MK colonies growth (Grossi, 1986).

It has been demonstrated that erythroid progenitors of ET patients can originate BFU-E colonies when cultured in absence of EPO as PV patients progenitors do (Eridani, 1984).

ET clinical phenotype is characterized by thromboembolic events affecting arteries and veins of large and medium calibre as well as peripheral microvessels (Vannucchi, 2007; Vannucchi, 2010). Microvascular involvement leads to acrocyanosis, paresthesias and erythromelalgia while involvement of the cerebral district causes neurological symptoms such as headache, tinnitus, dizziness, frequent TIA and only occasionally epileptic and seizure attacks (Cortellazzo, 1990). Haemorrhagic events mainly affect gastrointestinal tract but can also involve skin, conjunctivae, urinary tract and rhino-pharyngeal mucosa. Systemic symptoms such as weight loss, sweating, fever and itching affect 20-30% of patients. About a half of them displays splenomegaly and 20% has hepatomegaly.

Laboratory data suggesting ET diagnosis are platelet count higher than 600.000/μL for 6 months and usually moderate leukocytosis. Blood smear analysis may reveal altered platelet morphology and presence of megathrombocytes. Functional abnormalities have been described in ET platelets such as defective metabolism of arachidonic acid, decreased expression of prostaglandin D2 receptor and adrenalin receptor (Kaywin, 1978). Platelets derived from ET patients with previous history of thrombotic events display increased production of B2 thromboxane and improved affinity for fibrinogen (Landolfi, 1992). The increased levels of β-thromboglobulin and serum thromboxane suggest an enhanced platelets activation mechanism (Cortelazzo, 1981).

Bone marrow biopsy reveals hypercellularity associated with marked expansion of megakaryocytes that are pleomorphic and clustered. MKs are mature and do not show dysplasia as in pre-fibrotic myelofibrosis that must be considered in the differential diagnosis with ET. Bone marrow iron store are often reduced. In 25% of cases reticulin amount is increased. Cytogenetic abnormalities occur rarely, mainly affecting chromosomes 1 and 20.

Disease course is chronic and survival is similar to the healthy population adjusted for age and sex. Progression to acute leukemia is rare and usually associated with the onset of cytogenetic abnormalities. Progression to secondary myelofibrosis is also possible. Current ET treatment is based on antiplatelet drugs for patients with vascular involvement (Beer, 2009). Hydroxyurea is the most used myelosuppressive treatment since it quickly and steadily reduces platelet count thus preventing thrombotic events (Cortellazzo, 1995). Other chemotherapeutic agents such as busulfan, melphalan, chlorambucil and thiotepa have been used in ET although with lower efficacy and higher risk of blastic evolution (Berk, 1981). Anagrelide treatment inhibits MK maturation and platelet release (Fruchtman, 2005) but it has been demonstrated that hydroxyurea more efficiently reduces platelet count and risk of thrombotic events or progression to myelofibrosis compared to anagrelide (Harrison, 2005).

Myelofibrosis

Myelofibrosis has an incidence of 0.5-1.5 new cases over 100.000/year. Median age at diagnosis is 60 and both sex are equally affected (Cervantes, 2008). Higher prevalence is noticed in Ashkenazim Hebrew (McNally,1997; Mesa,1999; Chaiter,1992). Myelofibrosis can be primary (PMF) or derive from the progression of pre-existent PV or ET. Clinical features of the disease are splenomegaly, leukoerythroblastosis, erythrocyte anisopoichilocytosis, variable degree of bone marrow fibrosis and extramedullary erythropoiesis mainly occurring in spleen and liver (Barosi, 2003; Tefferi, 2003; Tefferi, 2006). 20% of patients are asymptomatic at diagnosis. Laboratory data can reveal anemia, thrombocytosis or thrombocytopenia and variable leukocytosis or leukopenia. Most cases are diagnosed in more advanced phase when hematological parameters are clearly altered with marked anemia, leukocytosis and usually thrombocytopenia. Medullar fibrosis worsens and hepatoslplenomegaly due to extramedullary hematopoiesis increases. Blasts may be found in peripheral blood implying evolution to acute myeloid leukemia. Diagnostic criteria for PMF according to WHO 2008 classification are reported in Tab. 3 (Tefferi, 2008)

Major Criteria
Megakaryocyte proliferation and atypia accompanied by either reticulin and/or collagen fibrosis, **or** In absence of reticulin fibrosis megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e. pre-fibrotic PMF)
Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
Demonstration of JAK2V617F or other clonal marker or no evidence of reactive bone marrow fibrosis
Minor Criteria
Leukoerythroblastosis
Increased serum LDH
Anemia
Palpable splenomegaly

Tab. 3 – WHO 2008 diagnostic criteria for PMF (diagnosis requires 3 major criteria and 2 minor criteria)

Myelofibrosis is characterized by abnormally increased number of mature cells but also by differentiation abnormalities mainly involving megakaryocyte lineage. MK clone is thought to release fibrogenic factors that promote fibers deposition in the bone marrow. Thus myelofibrosis may originate as a consequence of the augmented MK population whose defective maturation may lead to local release of growth factor stored in the α granules (particularly TGF-beta) (Chagraoui, 2002; Vannucchi, 2005).

Myelofibrosis originates when a multipotent hematopoietic stem cell is damaged. Monoclonality of erythrocytes, granulocytes, MK, monocytes and B-lymphocytes was demonstrated studying G6PDH expression, inactivation pattern of X-chromosome, polymorphisms and RAS family mutations (Jacobson, 1978; Buschle, 1988; Reilly, 1994). T-lymphocytes have been shown to be monoclonal in a subgroup of patients suggesting that abnormal proliferation originates from a partially committed progenitor (Tsukamoto, 1994). On the contrary, bone marrow fibroblasts are policlonal

therefore not being part of the malignant clone (Wang,1992; Castro-Malaspina,1982; Greenberg,1987; Pieri, 2008). Bone marrow of myelofibrosis patients shows increased amount of stromal cells, extracellular matrix proteins, angiogenesis and osteosclerosis besides the alrered expression of many cytokines (Reilly,1997; Mesa,1999; Martyre,1997).

Bone marrow fibrosis is caused by augmented production of extracellular matrix proteins that organize in fibers. This stack of fibers leads to loss of hematopoietic function of the bone marrow. Extramedullary hematopoiesis expands predominantly in the spleen and liver but it doesn't functionally replace medullar hematopoiesis. Splenectomy is indeed used as a therapeutic option to improve blood cells count by removing a collection district. Fibrosis is a secondary event caused by growth factor released by the malignant clone. This hypothesis is supported by the evidence of fibrosis regression after bone marrow transplantation or prolonged treatment with chemotherapy or interferon (McCarthy, 1985; Manoharan, 1984). Cytokines mostly involved in fibrosis development are platelet derived growth factor (PDGF), fibroblasts growth factor (b-FGF), platelet factor 4, transforming growth factor beta (TGF-β), β-thromboglobulin, calmodulin, interleukin-1 and vessel endothelium growth factor (VEGF). These cytokines are produced by MKs and monocytes. The role of megakaryocytes in the development of bone marrow fibrosis is supported by several observation: 1- in bone marrow biopsies of MF patients MK hyperplasia, dysplasia and necrosis are reported; 2- bone marrow fibrosis occurs in megakaryocytic acute leukemia; 3- fibrosis is also reported in cases of gray platelet syndrome which is an hereditary disorder affecting platelet α granules (Martyre, 1994; Reilly, 1994); 4- animal models of myelofibrosis always show increase MK proliferation. MKs and platelets display enhanced production and anomalous release of α granules containing PDGF, platelet factor 4, FGF, TGF-beta and calmodulin. Cytoplasmic fragmentation of MKs determines release of these cytokines in the medullar microenvironment without affecting α granules concentration in circulating platelets (Villeval, 1997; Taskin, 1998; Vannucchi, 2005). Hystomorphology and electronic microscopy studies underlined emperipolesis as a possible mechanism that leads to the stack of fibers in the bone marrow of genetically modified animal models of human myelofibrosis. These animals displayed in bone marrow and spleen increased occurrence of neutrophils

emperipolesis within megakaryocytes. Neutrophils merge their membranes with the demarcation membrane system of the megakaryocytes and undergo cytoplasmic lysis with consequent release of the granules content (Centurione, 2004). This phenomenon could partially explain bone marrow alteration since PDGF has a potent mitogen action on fibroblasts and is chemotactic for fibroblasts, neutrophils and monocytes while it poorly stimulates collagen production (Kimura, 1989; Terui, 1990). TGF-β induces production of many extracellular matrix proteins that are found greatly increased in MF patients bone marrow, such as type I, III, V and VI collagen, ialuronic acid, condroitin and dermatan-sulfate, proteoglycans and non-collagenic glycoproteins as fibronectin, vitronectin, tenascin and the components of the basal membrane type IV collagen and laminin (Reilly,1992). Reticulin fibrosis is caused by hyperproduction of the normal pattern of bone marrow fibers formed by type I and III collagen and fibronectin. Collagenic fibrosis occurring in myelofibrosis is on the contrary responsible for disruption of normal bone marrow structure. The altered balance between production and destruction of extracellular matrix proteins promotes fibrosis and reflects itself in the altered serum concentration of peptides derived from the catabolism of these proteins (Reilly, 1995; Hasselbach, 1986). In the early stage of fibrosis type III collagen synthesis predominates while in the advanced phase fibronectin, tenascin and citronectin load increases up to a condition of osteomyelosclerosis. TGF-β reduces the synthesis of metalloproteases that destroy extracellular matrix and increases the production of protease inhibitors such as the inhibitor of plasminogen activator I (Reilly, 1997). TGF-β also induces bone and vessels growth thus possibly explaining osteosclerosis and neoangiogenesis occurring in myelofibrosis (Rosen, 1988; Roberts, 1990). TGF-β acts on early hematopoietic progenitors by inhibiting their proliferation. Expression of type II TGF-beta receptor is indeed reduced in CD34+ cells derived from myelofibrosis patients (LeBousse-Kerdilès, 1996). Calmodulin is a calcium-binding protein that acts as mitogen for fibroblasts. In myelofibrosis patients urinary excretion of calmodulin is higher compared to that of patients affected by other myelopoliferative disease that do not display fibrosis (Eastham, 1994; Dalley, 1996). More recently a role in MF pathogenesis has been hypothesized for the clonal population of monocytes-macrophages because they produce TGF-β, PDGF and IL-1. Monocytes are increased in the bone marrow of MF

patients as well as serum levels of macrophage colony stimulating factor (M-CSF) that is involved in proliferation and differentiation of macrophages (Gilbert, 1989). Macrophages activation occurs partly through the interaction between adhesion molecules such as CD44 and extracellular matrix, that leads to up-regulation of intracellular TGF-β levels (Rameshwar, 1996). Studies on animal models demonstrated the role of macrophages in the onset of secondary myelofibrosis. In mice affected from various forms of hereditary immunodeficiency with TPO hyperexpression myelofibrosis occurred in mice with selective lymphocyte deficiency. On the contrary mice having also monocyte-macrophage immunodeficiency defects did not display any fibrotic alteration of bone marrow (Frey, 1998).

Myeloid Metaplasia is a characteristic of Myelofibrosis. It was first hypotized that it developed after reactivation of stem cells that remained quiescent since the fetal period in spleen or liver. This hypothesis was abandoned after the observation that unlike fetal spleen hematopoiesis that of MF patients is abundant and do not comprise only late-stage erythroid progenitors. Metaplasia can also occur in districts that are not involved in fetal hematopoiesis such as central nervous system, lung, gastrointestinal tract and genitourinary tract (Wilkins, 1994). According to the “theory of filtration”, extramedullar hematopoiesis could be a consequence of fibrosis since it warps sinusoid vessels allowing immature progenitor cells to reach blood circulation and move from the bone marrow to distant districts. Spleen and liver seem to be much more prone to filtrate progenitor cells from blood and permit the achievement of terminal differentiation. Hematopoiesis occurring into sinusoid vessels is frequently noticed in bone marrow biopsies of myelofibrosis patients (Wolf, 1985; Thiele, 1992).

Cytogenetical analysis revealed that the most common defects, representing 65% of all cytogenetic abnormalities in Myelofibrosis, are deletions of chromosome 13q which correlate with early leukemic transformation (Mesa, 2009), deletion of chr 20q and partial trisomy of 1q (Reilly, 2002). Chromosome 8 trisomy and chr 12p deletion seem to represent unfavourable prognostic factors (Tefferi, 2001). Many tumor-suppressor genes might be involved, such as RB-1 (retinoblastoma 1) located on chr 13q14 whose loss of heterozygosity has been demonstrated in 25% of PMF patients (Juneau, 1998). Occurrence of chromosomic alterations has been shown to own ominous prognosis in many studies (Demory, 1988; Dupriez, 1996), and to reduce

therapy response rate (Besa, 1982). Cytogenetic abnormalities can occur during disease progression and possibly contribute to leukemic transformation. Such role has been hypotized for p53 and RAS family genes lesions (Gaidano, 1994; Wang, 1998).

Circulating hematopoietic progenitor cells can be observed in MPNs, mainly in myelofibrosis. These cells can be identified by the expression of CD34 surface marker that is not present on differentiated cells. CD34+ cells can leave bone marrow at different maturation stages as suggested by their variable expression (23 to 99%) of CD38 transmembrane molecule which is poor or absent in hematopoietic cells with high self-renewal and differentiation potential (Barosi, 2001). Circulating CD34 positive cells count is meaningful for MF diagnosis since it is 360 fold higher compared to that of healthy population and 18 to 30 fold higher in respect with PV and ET count. Threshold value of $15x10^6$/L discriminates untreated myelofibrosis from other MPN with positive predictive value of 98,4% and negative predictive value of 85%. CD34+ cells count increase during disease progression and correlates with circulating myeloid blasts count and with splenomegaly (Barosi, 2001). CD34+ count decreases after cytoreductive therapy suggesting that it correlates with the extent of tumor mass. CD34+ value is associated with leukemic transformation as patients with more than $300x10^6$/L have 50% probability to develop leukemia in the next 11 months (Barosi, 2001). Myelofibrosis patients also display higher levels of pluripotent progenitors, such as megakaryocytes colony forming unit (CFU-MK), granulocytic/monocytic-CFU (CFU-GM), erythroid burst forming unit (BFU-E) and granulocytic/erythroid/monocytic/megakaryocytic-CFU (CFU-GEMM) when compared to healthy donors (Partanen, 1982; Wang,1983; Douer,1983; Hibbin,1984). The amount of circulating progenitors in MF patients is higher compared to cases of medullar fibrosis ascribable to other causes such as bone marrow metastasis of solid tumors. This suggests for MF an intrinsic defect of hemopoietic cells rather than the mere destruction of the microvascular architecture caused by fibrosis (Wang, 1983).

Molecular mechanisms responsible for clonal proliferation in myelofibrosis are still poorly understood. The proliferative advantage is demonstrated by cytokine-independent in vitro growth of mutated cells and hypersensitivity to cytokines (Taskin, 1998). For **stem cell factor receptor** (c-KIT) over-expression and a point mutation were both reported. The mutation occurs in the domain that stabilizes the binding to SCF

and could explain the increased tyrosine-kinase activity downstream of c-KIT. It has been hypothesized that myelofibrosis is caused by alteration of the signalling pathway involving the **fibroblasts growth factor** (b-FGF). This cytokine is produced by several cell types including hematopoietic and stromal cells and has mitogenic activity on bone marrow stromal cells. FGF is also a strong angiogenic factor and promotes stem cell growth acting through a tyrosine-kinase associated receptor. In CD34+ cells of myelofibrosis patients both b-FGF and its receptor are overexpressed in contrast with the reduced expression of TGF-beta (LeBousse-Kerdilès, 1996). Recent studies suggested the involvement of **JAK2/STAT** signalling pathway since the discovery of the point mutation JAK2 V617F that causes constitutive activation of the downstream pathway. Several experiments demonstrated that V617F leads to in vitro spontaneous growth of erythroid colonies (BFU-E) in PV patients. This mutation can indeed represent an important molecular marker for myelofibrosis although its higher frequency in PV suggests that other mechanisms may play an important role in the pathogenesis of myelofibrosis (Baxter, 2005; Ugo, 2005; Kralovics, 2005; Levine, 2005). Further studies identified mutations in the TPO receptor MPL. These mutations are found in 5% of myelofibrosis patients that do not display JAK2 V617F mutation and lead to constitutive activation of TPO signalling (Pardanani, 2006; Pikman, 2006).

JAK2*V617F* mutation

JAK2 is a member of the Janus Kinase family of tyrosine-kinase proteins that display a crucial role in intracellular signal transduction. Janus Kinases have 7 homologous domains. C-terminal JAK homology domain 1 (JH1) carries the kinase activity while adjacent pseudokinase domain JH2 is enzymatically inactive and acts as negative regulator of JH1 domain (Saharinen 2002, Feener 2004). The protein's name, referring to the double-faced roman god Janus, is due to the presence of these two symmetric domains. SH2 domain has two regions, JH3 and JH4, that are specific for phosphorylation while the 4.1 domain, formed by JH4-JH7 regions, displays the protein-protein interaction activity (Fig.1) (Vainchencker, 2008).

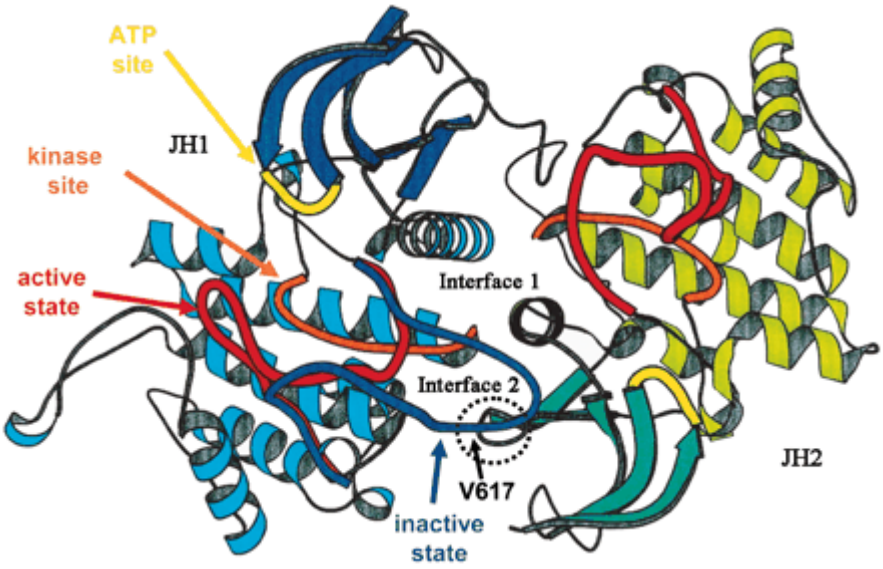

Fig 1 - JAK2 structure with JH1 and JH2 domains and V617F mutation pointed out.

JAK proteins are partially associated to membrane receptor in inactive form and act as intermediate with intracellular signal transducers. When the specific ligand binds the receptor, JAK proteins associated with the intracellular domain of the receptor become activated and phosphorylate each other and downstream molecules, particularly those belonging to STAT and MAP kinase families and to the PI3K-AKT pathway (Martens, 2007). Once activated STAT proteins dimerize and move inside the nucleus where they bind DNA in specific consensus sequences of target gene

promoters. JAK2 activation seems to occur mainly in response to binding of hematopoietic growth factor such as EPO, IL-3, IL-5, G-CSF, GM-CSF and TPO (Fig.2). JAK2 has been functionally associated to homodimeric growth factor receptors type I that do not display intrinsic kinase activity.

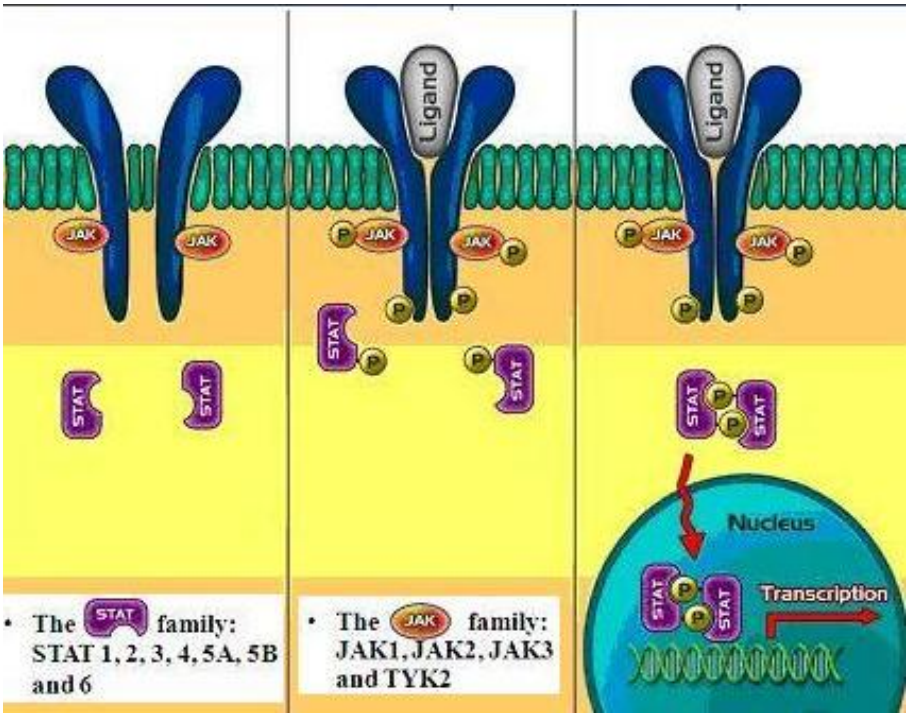

Fig 2 - JAK proteins role in signal transduction

JAK2 gene is located on chromosome 9p24. In 2005 the JAK2V617F mutation was identified. V617F is a somatic point mutation 1849G>T in exon 14 that causes valine to phenylalanine aminoacid change at position 617. V617F is an acquired somatic mutation present in the present in myeloid lineage and more rarely in lymphoid one. This mutation lies in JH2 pseudokinasic domain and it is believed to determine loss of auto-inhibitory function on the kinasic JH1 domain. JAK2 is consequently permanently phosphorylated and thus it activated even in the absence of receptor ligand. Cell lines usually dependent from cytokines once transduced with this mutation show abnormal proliferation and become independent from cytokines and hypersensitive to them mimicking in vitro growth of hematopoietic progenitors isolated from peripheral blood of MPN patients (James, 2005; Kralovics, 2005). This phenomenon explains EEC formation (Garcon

2005). Moreover, JAK2 mutated cells transplantation in mice provokes a PV-like disease with leukocytosis and some traits of myelofibrosis evolution (Wernig, 2006; Lacout, 2006; Bumm, 2006; Zaleskas, 2006). Modulation of JAK2 mutated gene expression allows the establishment of a TE-like phenotype in mice having low V617F allele burden (Tiedt, 2008). These models suggest that JAK2V617F mutation is enough to cause a MPN-like phenotype in mice and that mutation allele burden influences disease phenotype (Vannucchi 2008).

JAK2V617F mutation frequency is estimated to occur in around 95% of PV patients and 60% of ET and PMF patients (Szpurka 2006, Steensma 2006, Renneville 2006, Nishii 2007, Vizmanos 2006, Tefferi, 2006; Tefferi, 2007; Vannucchi, 2007). 25-30% of PV and PMF patients and 2-4% of ET patients display homozygous V617F mutation due to mitotic recombination (Levine 2005, James 2005, Kralovics 2005, Baxter 2005). Homozygosity is acquired during mitotic recombination through duplication of the mutated 9p chromosome region. Usually the highest mutation allele burden is displayed by PV, followed by PMF and ET that has the lowest V617F burden (Passamonti 2006, Antonioli 2008). Several studies evidenced the correlation between V617F burden and some clinical features of the disease although the allele burden does not discriminate the three different phenotypes. In familial MPN cases, patients with PV or ET displaying homozygous mutation have a higher risk of complications such as higher frequency of myelofibrosis and AML development (Bellanne-Chantelot 2006). Homozygous patients show higher leukocyte count, hematocrit level and more often present splenomegaly when compared to heterozygous or wild type patients. PV V617F homozygous patients have decreased platelet count and higher incidence of pruritus at diagnosis. Noteworthy homozigosity leads to 4 fold higher risk of thrombotic complications in ET patients compared to JAK2 wt patients (Vannucchi 2007). A significant association between homozygosity and disease duration was reported (Kralovics 2005); this observation was confirmed in subsequent studies suggesting that V617F homozygous state may be an evolutive event that occurs over time (Levine 2005). Kralovics proposed a two-step model for JAK2V617F mutation role in MPN: 1) somatic mutation is acquired in hematopoietic stem cell or progenitor leading to expansion of an heterozygous clone that overcomes the

residual normal population 2) the heterozygous mutated cell undergoes mitotic recombination and originates homozygous clone that due to the enhanced proliferative advantage quickly overcomes even the heterozygous clone (Fig.3).

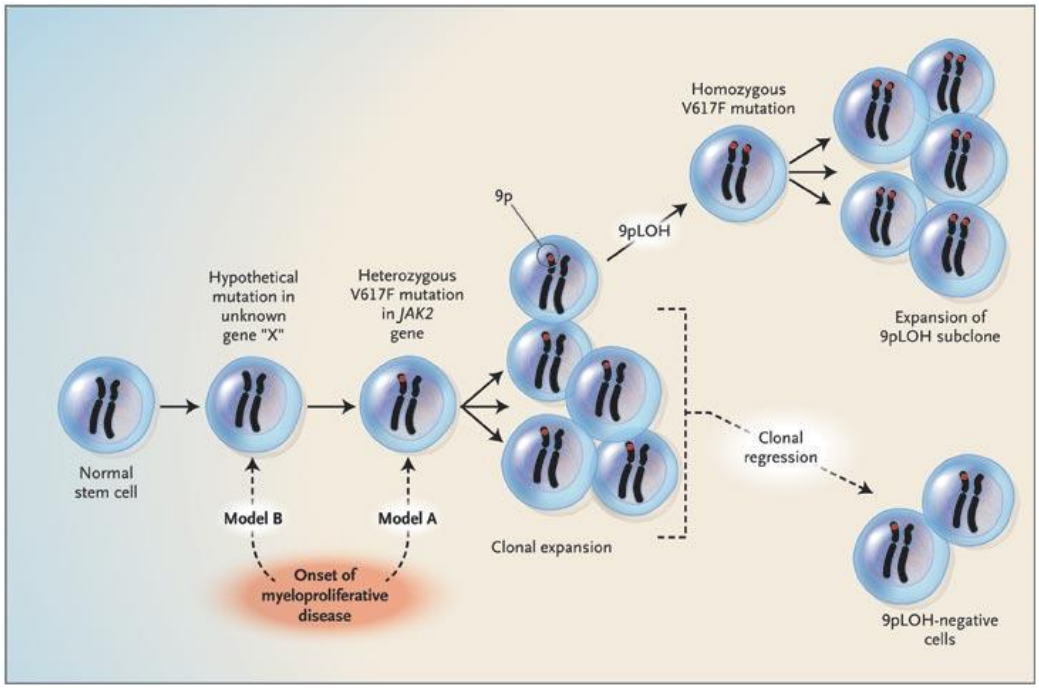

Fig 3: Possible role of JAK2V617F mutation and mitotic recombination in MPN pathogenesis.

In PV JAK2V617F allele burden correlates directly with hematocrit, leukocyte count, LDH, ALP and PRV-1 expression while there was an inverse correlation with MCV and platelet count. Moreover PV patients with high levels of V617F allele (>75%) display a worse disease phenotype more often characterized by splenomegaly, itching, thrombotic events, and need for cytoreductive therapy instead of phlebotomy alone for disease management (Vannucchi, 2007). ET JAK2V617F mutated display high hematocrit value, leukocytosis and reduced platelet count but without a clear correlation between these parameters and the mutation burden (Antonioli, 2008).

Finally, single nucleotide polymorphism (SNPs) of JAK2 locus have been associated with PV diagnosis (Pardanani, 2008) supporting the hypothesis that genetic hereditary factors may determine the phenotypic heterogeneity of MPNs. There are evidences that JAK2V617F is not the founding mutation and conversely a

“pre-JAK2” mutation exists (Skoda 2007, Nussenzveig 2007). For instance it was demonstrated that patients with JAK2V617F-positive MPN that evolve to AML can often have JAK2 wt blasts (Theocharides, 2007; Campbell, 2006). On the contrary JAK2 mutations are likely to be necessary to determine PV phenotype as they can be found in virtually all patients (Pardanani, 2007) and are sufficient to induce PV phenotype in mice.

Therefore, JAK2V617F is crucial in classical MPN, although its position in the hierarchy of disease pathogenesis is still unclear.

Concomitant Occurrence of JAK2 V617F and BCR/ABL rearrangement in CML

Since the discovery of JAK2 V617F isolated cases of JAK2 mutations were described in CML patients who were convincingly shown to harbor concomitantly the JAK2 V617F point mutation and the BCR/ABL rearrangement have been reported. In 2005, in a patients with typical CML a translocation t(9;22)(p24;q11.2) was evidenced cytogenetically that involved the BCR gene but not ABL1. A candidate gene approach led to the identification of JAK2 as the fusion partner of BCR (Griesinger, 2005). In a study evaluating the presence of JAK2 V617F in 374 cases of various hematologic neoplasms, V617F was found in 3 (19%) of 16 cases of Ph-negative CML but in none of 99 patients with BCR/ABL positive CML (Jelinek, 2005). The first case of co-occurrence of JAK2 V617F and BCR/ABL fusion gene was reported in 2007 in a patient with CML evolving to Myelofibrosis under Imatinib treatment. The patient was a 55 years old man that presented with splenomegalyleucocytosis, low hemoglobin value and increased LDH. Blood smear identified a typical CML morphology with 14% of blasts cells. Cytogenetical analysis revealed the presence of a rare BCR/ABL variant (e19a2). Imatinib therapy induced normalization of blood parameters and spleen size and cytogenetic and molecular response within 11 months. Bone marrow biopsies at 11, 14 and 23 months after therapy onset revealed increased megakaryocytes count and focal deposition of argyrophilic fibers. Although BCR/ABL transcript was undetectable,

hystomorphology suggested persistence or relapse of malignant clone. Patient's samples were then screened for the presence of JAK2 V617F mutation that was present at 5% of allele burden in the first biopsy and increased at 15% and 23% at 14 and 23 months respectively. Suppression of BCR/ABL clone by Imatinib therapy and contemporary increase of JAK2 V617F clone suggested the presence of two independent clones (Hussein, 2007). Subsequently a case of myelofibrosis harboring both JAK2 mutation and BCR/ABL rearrangement was reported. In this case BCR/ABL fusion gene was suggested as occurring as a secondary event in the myeloid lineage thus not affecting the stem cell compartment (Bornhäuser, 2007). Further studies identified several other cases of patients with both V617F and BCR/ABL mutations (Inami, 2007; Kramer, 2007; Bocchia, 2007, Kim, 2008).

New mutations in MPN

Despite the identification of JAK2 and MPL mutations represented milestones for the comprehension of MPN, it is accepted that these are not sufficient to completely uncover the pathogenesis of this diseases. As a matter of fact 30-40% of ET and PMF patients do not harbor any known genetic alteration. Recently new mutations in TET oncogene family member 2 (TET2), Additional Sex Combs-Like 1 (ASXL1), Casitas B-lineage lymphoma proto-oncogene (CBL), Isocitrate dehydrogenase (IDH1/IDH2), IKAROS family zinc finger 1 (IKZF1) and Enhancer of zeste homolog 2 (EZH2) genes have been identified (Tefferi, 2010). These mutations, although occurring at low frequency, demonstrated that MPN have a complex and multifactorial molecular pathogenesis that needs to be further clarified. Regarding the functional impact, such mutations seem to deregulate JAK2/STAT signalling pathway, to alter epigenetic modulation of transcription and to determine accumulation of oncoproteins. It remains to elucidate if these mutations contribute to the establishment and/or to the evolution of the disease since they can occur in different stages from the onset, even before JAK2 or MPL mutation acquisition, to the chronic phase and up to AML evolution. This latter case is interesting because molecular mechanisms that drive the disease from chronic phase to the terminal and untreatable acute leukemic stage are largely unknown. Recent studies on TET2, EZH2, ASXL1, CBL, IDH and IKZF1 mutations estimate their incidence

to be 0-17% of MPN patients. Nevertheless none of this mutations is specific for MPN since they can occur in other hematologic disease such as Chronic Myelogenous Leukemia (TET2, ASXL1, CBL), myelomonocitic leukemia (CBL), mastocytosis (TET2), myelodisplastic syndromes (TET2, ASXL1) and MPN-secondary AML (IDH, ASXL1, IKZF1).

EZH2 (Enhancer of zeste homolog 2) is a member of the Polycomb group proteins (PcG) that were first idenified in Drosophila as a master regulator of the expression of homeotic homeobox-containig genes (Hox) togheter with the Trithorax group. Polycomb and Thritorax proteins act in multi-proteic complexes on the chromatin with opposite effects: they activate transcription of homeotic genes (Thritorax proteins) or they inactivate it (Polycomb proteins) (Cardoso, 1998; Cardoso, 2000). EZH2 is a member of the Polycomb Repressive Complex 2 accounting for its metiltransferase activity against Lys27 of H3 histone which determines transcription silencing (Pasini, 2004; Pasini, 2007, Simon, 2008). Differential levels of H3K27 methylation have an important role in many biological processes included hematopoiesis (Clui, 2009). EZH2 point mutations have been identified in MPN patients. These mutations lead to the inactivation of the catalytic domain of the protein and subsequent alteration of histone methylation that perturbs the regulation of gene silencing (Ernst, 2010). In a study considering 90 MPN patients, EZH2 mutations were harbored by 13% of PMF and 3% of PV patients (Ernst, 2010). Lately the presence of EZH2 mutations has been associated with decreased overall survival in patients with PMF (Guglielmelli, 2011)

ASXL1 (Additional sex combs-like 1) gene is also involved in epigenetic regulation of transcription since it is a member of the polycomb group protein (Fisher, 2010). ASXL1 represses retinoic acid receptor-mediated transcription trough modulation of histone 3 demethylation (Lee, 2010). Mutations in ASXL1 disrupting the C-terminal domain have been identified in MPN patients, mainly in Myelofibrosis and post-MPN AML groups (Abdel-Wahab, 2011).

TET2 (Ten-Eleven Translocation family member 2) protein catalyzes in a ketoglutarate-dependent manner the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxylmethylcytosine (5hmC) (Ko, 2010). TET2 mutations span all over the gene and can be missense/nonsense substitutions, insertion or deletion resulting in frameshift

or premature stop codons. Most mutations are predicted to determine loss of protein function thus pointing to TET2 as a tumor-suppressor gene (Ko, 2010; Bacher, 2010). TET2 mutations have been found in 5% of ET patients and in around 15% of PV, PMF and blastic phase MPN patients (Tefferi, 2009).

IDH1 and IDH2 genes encode for two isocitrate dehydrogenase. IDH1 protein is mainly located in cytoplasm and peroxisomes while IDH2 acts in mitochondria; both are involved in the citric acid cycle where they catalyze carboxylation of isocitrate to α-Ketoglutarate (Gross, 2010). IDH1/2 normal activity seems to contribute to protection from oxidative damage. Somatic mutations of IDH1/2 were first identified in gloms, glioblastomas and AML (Parson, 2008; Yan, 2009; Mardis, 2009). Subsequent studies demonstrated that IDH1/2 mutations occur in normal karyotype de novo AML (Chou 2010; Gross, 2010; Marcucci, 2010; Ward, 2010). Their incidence in MPN is estimated 0.8 % in ET, 1.9 % in PV, 4.2 % in PMF, and 21 % of post-MPN AML cases (Pardanani, 2010; Tefferi, 2010). These mutations have been shown to promote leukemic transformation and are associated with shortened survival in PMF (Tefferi, 2012). Many studies demonstrated that IDH mutated proteins display decreased affinity for isocitrate and predominantly convert α-ketoglutarate to 2-hydroxyglutarate that accumulates in the cell (Gross, 2010: Ward, 2010). Through this mechanism IDH1/2 mutations may inhibit TET2 activity that requires α-ketoglutarate as a cofactor. TET2 inhibition leads to decreased hydroxylation of 5-methyl-cytosines and demethylation (Figueroa, 2010; Xu, 2011). Metabolism abnormality thus seems to promote malignant transformation.

CBL is a regulator of signal transduction since it can act as an adaptor protein or as an E3 ubiquitin ligase that promotes proteasomal degradation of cytokine receptors, such as EPOR and TPOR, or tyrosine kinases, such as JAK2 (Schmidt, 2005; Saur, 2010). CBL mutations have been found in AML, MDS and MPN patients (Sargin, 2007; Caligiuri, 2007; Abbas, 2008; Dunbar, 2008; Grand, 2009; Sanada, 2009). Particularly they can occur in 6% of PMF but rarely in PV or ET (Grand, 2009). Although CBL mutations have been found in 13% of post-MPN AML (Beer, 2010; Milosevic, 2012), their role in promoting progression to acute leukemia is still to be clarified.

IKZF1 gene encodes for transcriptional factors involved in lymphoid differentiation. It is thought to modulate the expression of lineage-specific genes allowing normal lymphoid development and thus acting as a tumor-suppressor (Mullighan, 2008). IKZF1 mutations occur rarely in MPN chronic phase but are present in 19% of blast-phase MPN patients (Jager, 2009).

Finally new mutations involving DNMT3A have been discovered in MPN patients. DNMT3A is a member of the DNA methyltransferase family, together with DNMT1 and DNMT3B, and catalyzes de novo methylation of cytosines in the CpG islands. Mutations of DNMT3A have been first reported in 22% of AML patients (Ley, 2010). DNMT3A mutations occur in around 10% of MPN patients, mostly in PMF and in patients progressing to advanced phase (Stegelmann, 2011; Abdel-Wahab 2011) and can co-occur with JAK2, ASXL1, TET2 and IDH1/2 mutations. It is still unclear whether DNMT3A mutations determine gain or loss of gene function and what is their contribution to disease pathogenesis (Milosevic, 2012).

Very recent studies identified in MPN patients mutations involving the RNA splicing machinery. These mutations, predominantly involving SF3B1 and SRSF2 genes, are very frequent in MDS and have been reported in 9.4% of MPN (Yoshida, 2011). SF3B1 mutations occur in 3-6% of MPN patients and their association with thrombotic events has been hypothyzed (Lasho, 2012; Papaemmanuil, 2011; Visconte, 2012). MPN patients with SF3B1 mutations often show ring sideroblasts as in MDS with ring sideroblasts (Yoshida, 2011). SRSF2 mutations have been shown in 17% of PMF patients and 19% of post-MPN AML and often cluster with IDH1/2 mutations (Lasho, 2012; Zhang, 2012). SRSF2 mutations associate with reduction of overall and leukemia-free survival (Lasho, 2012; Zhang, 2012).

Genetic Risk Factors

JAK2 46/1 haplotype

In order to further clarify possible factors contributing to the specific phenotype of PV, ET and PMF, that is well established can not be merely attributed to the presence of JAK2 V617F mutation, recent studies investigated the possible role of hereditary factors in the development and clinical presentation of MPN.

A set of SNP involving JAK2 locus were analysed in MPN patients with V617F allele burden greater than 50% implying the presence of an homozygous mutated clone. This clone originated by acquired uniparental disomy of chromosome 9p (9p-UPD), that often occurs in PV and MF but is rare in ET. SNP analysis revealed that 77% of V617F mutated alleles displayed the same haplotype, named 46/1 (Tab.4), suggesting that homozygosity did not occur randomly but conversely occurred preferentially when the mutation was carried on this specific allele.

Haplotype number	rs7864782	rs10124001	rs10758669	rs1327493	rs6476934	rs10815144	rs7046736	rs2149556	rs12342421	rs10974944	rs10119004	rs10974947	rs12343867*	rs12340895*	rs2031904	rs10491652	rs11793659	rs17425637	rs17425819	rs10815160
1	G	C	C	C	T	A	A	T	G	G	A	G	C	G	G	C	G	T	T	T
8	G	C	A	C	T	A	C	T	C	C	A	A	T	C	A	C	A	C	C	G
32	G	C	.	C	T	G	.	C	.	C	G	.	T	C	.	C	A	C	C	T
38	G	C	A	G	G	A	C	T	C	C	A	A	T	C	G	C	A	C	C	G
46	A	C	C	C	T	A	A	T	G	G	A	G	C	G	G	C	G	T	T	T
55	A	C	.	C	T	A	.	T	.	C	A	.	T	C	.	C	A	C	C	G
71	A	C	A	C	T	G	C	C	C	C	A	G	T	C	G	C	A	C	C	T
76	A	C	A	C	T	G	C	C	C	C	G	G	T	C	G	C	A	C	C	T
88	A	T	A	C	T	G	A	T	G	C	G	G	T	C	G	C	G	T	T	T

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Tab. 4 - 46/1 haplotype (grey shadowed) is the combination of the two more frequent haplotypes that differ just for one SNP

Assessment of 46/1 haplotype frequency in healthy donors compared with a group of V617F heterozygous MPN demonstrated that its prevalence among the latter (Jones, 2009). Two hypotheses have been proposed to explain the association between MPNs and 46/1 haplotype (Campbell, 2009):

1- hypermutability hypothesis, suggests that 46/1 haplotype increases mutability of JAK2 locus leading to preferential acquirement of V617F on 46/1 allele;

2- fertile ground hypothesis, suggests that although V617F occurs randomly, 46/1 haplotype might be in linkage disequilibrium with an unknown functional variant that by interacting with V617F increases the probability of developing a clinically manifest disease

In order to determine if JAK2 harbouring 46/1 haplotype may be functionally different the ability of this haplotype to influence myeloid colonies formation in healthy subjects was evaluated. It was thus demonstrated that individuals carrying at least one 46/1 allele displayed reduced CFU-GM growth while there was no effect on erythroid colonies (Jones, 2009).

Another study revealed that MPN patients acquire JAK2 mutations with a high frequency that could not be explained with a random mutagenesis phenomenon. Multiple occurrence of JAK2 V617F mutation on both alleles in the same patient was demonstrated in 3% of MPN through genotyping of the JAK2 SNP rs12343867. Multiple acquisitions of oncogenic mutation in the same gene have not been previously demonstrated in hematological malignancies although it was a known mechanisms for KIT and KRAS in solid tumors. This study also revealed that 85% of V617F mutations occurred on the allele that carried the C variant of rs12343867. The frequency of rs12343867 C variant was higher in V617F positive MPN compared to a JAK2 wt MPN group. This observation and further analysis on other SNPs lead to the identification of the GGCC haplotype that increases susceptibility to JAK2 V617F mutation. Acquired 9p UPD occurs in JAK2 V617F mutated clone through loss of residual JAK2 wt allele and duplication of mutated allele that leads to homozygosity for both V617F and the haplotype on which the mutation occurred. In a subset of 17 patients with 9p-UPD, 88% of cases acquired V617F mutation on GGCC haplotype that comprises rs3780367, rs10974944, rs12343867, rs1159782 SNPs and partially overlaps with 46/1 haplotype (Olcaydu, 2009). Subsequent study demonstrated that JAK2 V617F mutation occurs preferentially in cis with rs10974944 G allele, suggesting a direct connection between the specific

haplotype variant and the acquisition of mutations on the same strand (Kilpivaara, 2009). Lately 46/1 haplotype was found to be associated with JAK2 exon 12 mutation and with MPL mutations, this latter case possibly supporting “fertile ground hypothesis” (Jones, 2010).

The prognostic role of 46/1 haplotype in MPN is still controversial. Some studies identified 46/1 haplotype nullizygosity as being an unfavourable prognostic factor for survival in PMF (Tefferi, 2009). In ET 46/1 haplotype does not seem to influence clinical phenotype or prognosis (Pardanani, 2009). A study of my group on the contrary failed to identify any correlation between 46/1 haplotype configuration and rate of leukemic transformation, overall survival or risk category in PMF (Guglielmelli, 2010). In a recent study 46/1 haplotype homozygosity seems to determine in untreated PV a progressive increase in JAK2 V617F allele burden during disease progression (Alverez-Larràn, 2012).

In a Swedish registry-based epidemiological study almost 25.000 relatives of 11.039 MPN patients were analyzed. Relatives of MPN patients displayed an increased risk of developing any MPN (RR=5.6). The assessment of risk for specific MPN revealed an increased risk for first-degree relatives of developing PV (RR=5.7), ET (RR=7.4) and not-otherwise specified MPN (RR=7.5) compatible with a model of autosomal recessive inheritance. The risk estimates were significantly higher for siblings and the female sex of both the patients and relatives. An increased relative risk, although not statistically significant, was also evidenced for CML development in MPN relatives (RR=1.9) while there was no significantly higher risk of AML and MDS (Landgren, 2008).

Chromosome 12q24 haplotype

A recent study of Genome Wide Analysis in over 4500 subjects for the first stage and more than 9000 for the replication set identified on chromosome 12 in the q24 region a 10 SNPs common haplotype (Fig 4) that in normal population is associated with platelet count and higher risk of coronary artery disease (CAD). The association region spans 1.6 Mb and includes 15 genes. One of the 10 SNPs is an Arg262Trp nonsynonymous change in the gene SH2B3 (rs3184504), seven are

intronic within four genes (ATXN2, C12orf30, C12orf51 and PTPN11) and two are intergenic (Soranzo, 2009).

Table 2 Association with disease and signatures of natural selection at the ten core SNPs in the 12q24 region

		Platelet count association				CAD		Natural selection						
SNP	Gene annotation	Increaser allele	Beta (s.e.m.) (10^9/l)	*P*	Risk allele	OR (95% CI)	*P*	Ancestral/ derived allele	DAF[c] CEU	DAF[c] YRI	DAF[c] CHB	Standardized iHS	Fay and Wu's *H+*	F_{ST}[d]
rs3184504[a]	*SH2B3* (Arg262Trp)	T	7.22 (1.28)	1.6×10^{-8}	T	1.176 (1.120–1.238)	4.23×10^{-11}	C/T	0.41	0	0	–2.756	–35.656	0.39**
rs4766578[a]	*ATXN2* (intron)	T	7.33 (1.28)	1.0×10^{-8}	T	1.158 (1.100–1.218)	1.16×10^{-8}	A/T	0.42	–	–	–2.761	–37.062	–
rs10774625[a]	*ATXN2* (intron)	A	7.33 (1.28)	9.9×10^{-9}	A	1.158 (1.100–1.219)	1.16×10^{-8}	G/A	0.42	0	0	–2.761	–36.22	0.40**
rs653178[a]	*ATXN2* (intron)	C	7.25 (1.27)	1.2×10^{-8}	C	1.171 (1.117–1.227)	5.69×10^{-11}	T/C	0.41	0	0	–2.882	–34.185	0.39**
rs11065987	Intergenic	G	7.52 (1.31)	8.3×10^{-9}	G	1.152 (1.104–1.202)	7.05×10^{-11}	A/G	0.34	0	0	–3.038	–36.295	0.34*
rs17696736[b]	*C12orf30* (intron)	G	6.89 (1.24)	3.1×10^{-8}	G	1.144 (1.098–1.192)	1.41×10^{-10}	A/G	0.35	0	0	–3.212	–57.263	0.34*
rs17630235	*TRAFD1* (3′ of gene)	A	7.07 (1.25)	1.7×10^{-8}	A	1.145 (1.096–1.196)	1.33×10^{-9}	G/A	0.33	0	0	–3.206	–61.348	0.32*
rs11066188	*C12orf51* (intron)	A	7.22 (1.25)	8.5×10^{-9}	A	1.150 (1.103–1.199)	5.28×10^{-11}	G/A	0.32	0.008	0	–3.227	–60.794	0.30*
rs11066301	*PTPN11* (intron)	G	7.48 (1.25)	2.3×10^{-9}	G	1.144 (1.095–1.196)	2.52×10^{-9}	A/G	0.35	0.008	0	–2.646	–56.342	0.33*
rs11066320	*PTPN11* (intron)	A	7.48 (1.25)	2.3×10^{-9}	A	1.149 (1.101–1.198)	1.18×10^{-10}	G/A	0.35	0	0	–4.341	–59.37	0.34*

The PLT summary statistics are relative to the discovery sample. The derived and ancestral allele status was obtained from the UCSC annotations. Derived allele frequencies in the three HapMap Phase 2 samples were obtained from the HapMap repository. The natural selection statistics iHS and Fay and Wu's *H+* for HapMap Phase 2 were obtained from Haplotter.
[a]CAD data not available for WTCCC-CAD; T (derived) allele at rs3184504 increases risk for celiac disease. [b]All cohorts with genotyped calls; G (derived) allele increases risk for T1D.
[c]DAF = derived allele frequency. [d]Global F_{ST} for the comparison of CEU/YRI/CHB+JPT calculated from HapMap Phase II data. The symbols * and ** indicate SNPs exceeding the 95th (F_{ST} = 0.309) and 99th (F_{ST} = 0.37) percentiles of the empirical genome-wide distribution for this MAF bin (0.05–0. 1 worldwide).

Tesi van.

Soranzo, 2009

Fig. 4 - SNPs defining 12q24 haplotype

Some of these SNPs have been previously associated with other diseases. The G variant of rs17696736 in *C12orf30* is a known risk factor for type 1 diabetes (WTCCC, 2007; Todd, 2007) and the T allele of rs3184504 in SH2B3 gene has been associated with celiac disease (Hunt,2008). This latter variant was also reported to be associated with eosinophils count and risk of myocardial infarction (Gudbjartsson, 2009) and with blood pressure (Levy, 2009). In a genome-wide association analysis of 24167 individuals of European ancestry for erythrocytes traits, the minor allele of rs11065987 which is located between SH2B3 and ATXN2 has been associated with hematocrit value while the 12q24 haplotype has been globally associated with hematocrit and hemoglobin value (Ganesh, 2009). Some of the genes, other than SH2B3 that is extensively discussed later, included or near the haplotype region may have a possible role in hematopoiesis.

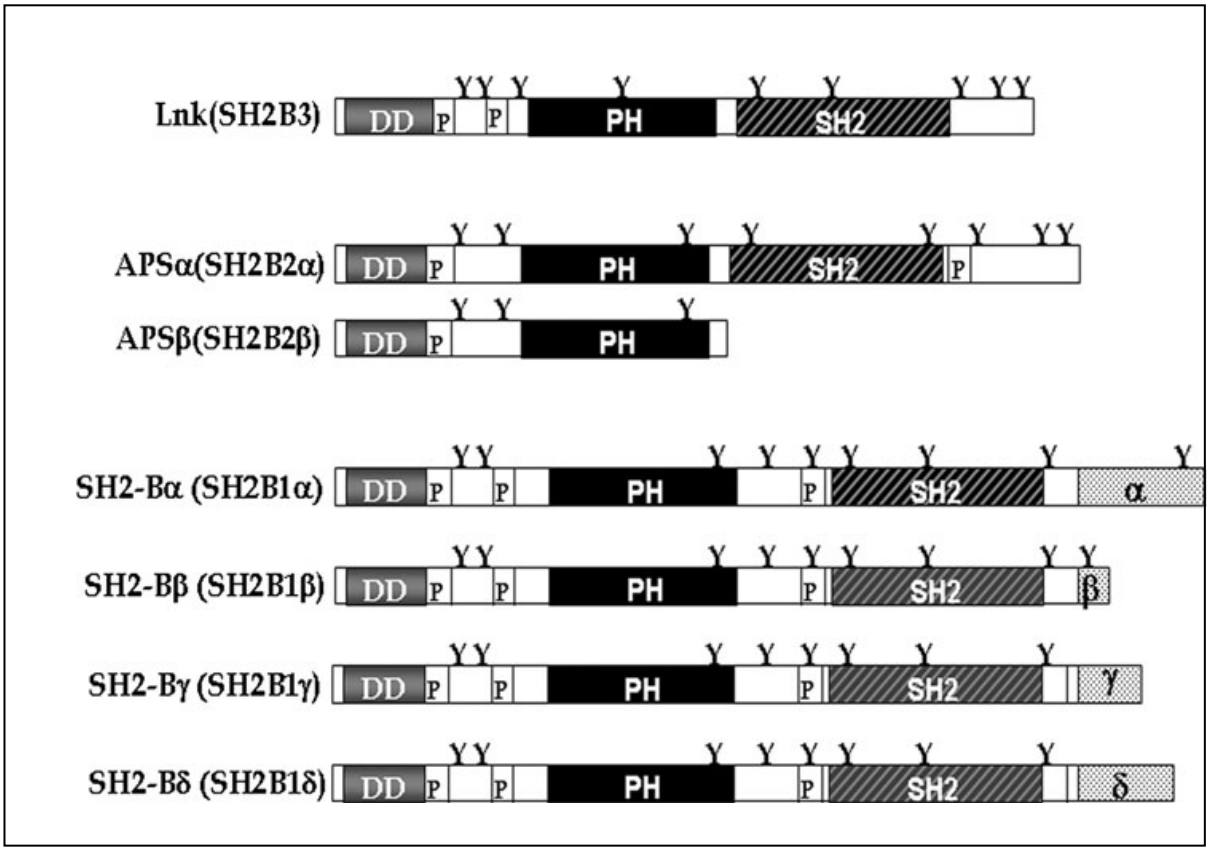
PTPN11 encodes for a tyrosine phosphatase called Shp2 that has two SH2 domains and a Tyr-phosphatase domain. In the inactive form the N-terminus SH2 domain exerts an autoinhibitory effect on the protein by preventing substrate access to catalytic domain. Mutations of this gene occur in 50% of children affected by Noonan syndrome (Tartaglia, 2001) which is a congenital disease characterized

by facial dismorphism, short stature, cardiac anomalies and in some cases by a myeloproliferative disorder resembling juvenile myelomonocitic leukemia (JMML) that however tends to spontaneously resolve. Subsequent studies identified PTPN11 mutations in 35% of de novo non-syndromic cases of JMML (Loh, 2003; Tartaglia, 2003). Comparison of mutations occurring in Noonan syndrome and in de novo JMML revealed that although the same codons are frequently affected the mutation type is mostly different leading to the hypothesis that transformation power of mutations occurring in Noonan syndrome is weaker thus allowing these alterations to be tolerated as germ-line events (Loh, 2010) PTPN11 mutations have been found in a small percentage of myelodisplastic syndromes and in rare cases of acute myelogenous leukemias (Tartaglia, 2003). Animal models of Shp2 knockout in hematopoietic tissues demonstrate that this protein is needed for HSC repopulating capacity. Lack of Shp2 leads to reduced quiescence and enhanced apoptosis rate of HSC. The germline gain of function mutation D61G enhances HSC activity and induces myeloproliferative disease in vivo as well as somatic mutations D61Y and E76K. This latter mutation can induce acute leukemia in recipient animals (Nabinger, 2012). Shp2 knockdown has been demonstrated to reduce CD34+ cells proliferation, survival and differentiation towards both myeloid and erythroid lineages. Depletion of Shp2 also downregulates ERK1/2, AKT and STAT5 phosphorylation (Li, 2011).

CUX2 (CUT-like homeobox 2) belongs, together with CUX1, to a family of transcriptional factors involved in the control of proliferation and differentiation. CUX2 contains three CUT domains and a homeodomain. CUX1 is expressed in most tissues while CUX2 has been demonstrated to be expressed mainly in nervous system. Nevertheless CUX2 might be of interest considering that the other member of its family, CUX1, has been shown to be deleted in a case of MPN harboring a 7qUPD and deletion of 7q were shown to associate with disease progression in MPN (Klampfl, 2011). In the same study the ch12q region containing CUX2 was identified as a commonly deleted region in MPN patients. In a recent study a missense mutation of CUX1 was identified in 1/15 cases of post-MPN AML in association with deletion of 7q (Thoennissen, 2011).

LNK

LNK, also known as SH2B3, gene maps in the 12q24 region and encodes for a 68 KDa protein that function as an adaptor protein (Li, 2000; Takaki, 2000). The LNK adaptor proteins family (Fig 5) comprises LNK (SH2B3), APS (SH2B2) and SH2-B (also known as PSM or SH2B1) (Rudd, 2001). These proteins lack enzymatic activity and contain a dimerization domain (DD), proline-rich motifs at the N-terminus, a Pleckstrin Homology domain (PH), a Src Homology 2 domain (SH2) and several possible tyrosine phosphorylation sites.



(Velazquez, 2012)

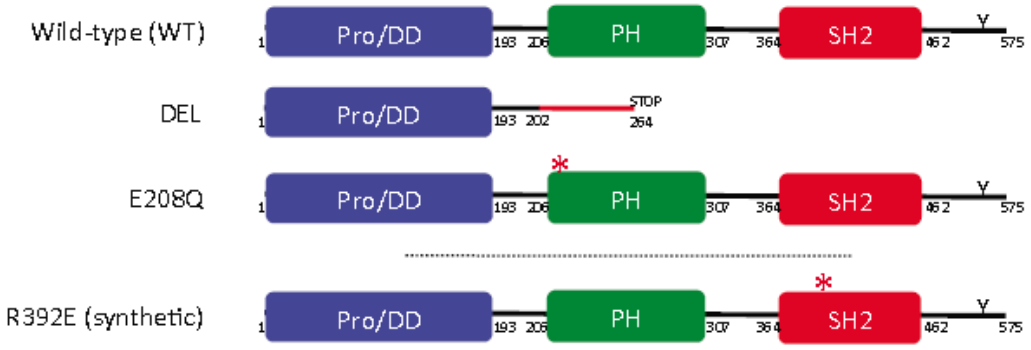
Fig 5 - Structure of LNK family members, comprising SH2B3 and the different isoforms of APS and SH2B

LNK is highly expressed in hematopoietic cells, particularly in hematopoietic stem and progenitor cells (Takaki, 2002) where it acts as a negative regulator of cytokine signalling. Furthermore its expression is up-regulated by some hematopoietic cytokines such as EPO, SCF, and TPO (Buza-Vidas, 2006; Gery, 2009; Baran-Marszak,

2011). LNK binds several targets through its SH2 domain. The first LNK binding partner identified was KIT, the SCF receptor. LNK SH2 domain-binding site is pTyr567 in the juxtamembrane region of KIT receptor (Simon, 2008; Gueller, 2008). The juxtamembrane region of KIT has been shown to be critical for recruiting positive and negative signalling regulators (Chan, 2003). Thus LNK binding might prevent association of KIT activators resulting in inhibition of SCF downstream pathway. This hypothesis is supported by the evidence that expression of a SH2 defective LNK abolishes its negative regulatory activity on cell proliferation induced by SCF (Simon, 2008). LNK family also binds JAK2 with different outcomes. APS or SH2B binding determines activation while LNK binding causes inhibition. LNK SH2-binding site is pTyr813 in the pseudokinasic regulatory JH2 domain of JAK2 (Kurzer, 2004; Kurzer, 2006; Bersenev, 2008). Furthermore LNK has been shown to associate to the JAK2 V617F mutant protein with higher affinity compared to wt JAK2 (Bersenev, 2008; Gery, 2009; Baran-Marszak, 2010). Recently some studies demonstrated that LNK is involved in regulation of integrin-mediated adhesion and migration of hematopoietic cells. In HSC LNK regulates interaction with vascular cell adhesion molecule 1 (VCAM1) thus possibly affecting their motility and appropriate homing in the BM hematopoietic niche (Takizawa, 2006). Experiments on LNK-deficient mice models showed that LNK /- HSC display enhanced self-renewal potential, hypersensitivity to TPO due to increased activation of Akt and STAT5 and inactivation of p38 MAPK (Seita, 2007). LNK deficient HSCs display increased repopulating ability in irradiated hosts (Takaki, 2002). LNK has been shown to regulate the TPO/MPL pathway thus affecting megakaryopoiesis and platelets production. LNK -/- megakaryocyte display abnormal proliferation due to lack of negative regulation of TPO signalling. LNK indeed inhibits MPL downstream signalling by binding via its SH2 domain the three main effectors: JAK2/STAT, MAPK, AKT (Tong, 2004; Takizawa, 2008). Furthermore LNK binds and regulates the MPL W515L mutated protein (Gery, 2007). LNK has a similar role in erythropoiesis. LNK -/- mice show enhanced recovery after erythropoietic stress compared to wt animals and primary LNK deficient erythroblasts display increased proliferation and survival due to lack of EPOR downstream signaling inhibition (Tong, 2005). LNK deficiency has been shown to accelerate JAK2 mutated-induced myeloproliferative disease in mice by enhancing cytokine-independent JAK/STAT signalling and to favour CML progression.

JAK2 V617F maintains the ability to bind LNK suggesting that LNK could modulate JAK2 V617F MPN phenotype. Indeed a mutant form of JAK2 V617F unable to bind LNK leads to enhanced myeloid proliferation in comparison to JAK2 V617F without LNK-binding defects. Lethally irradiated mice transplanted with LNK -/- JAK2 V617F were shown to develop PV earlier and to progress more quickly to myelofibrosis compared to mice transplanted with WT LNK/ JAK2 V617F cells (Bersenev, 2010).

These data promoted investigation of LNK function in Myeloproliferative Neoplasms. Since 2010 several studies reported the occurrence of LNK mutations in patients with MPN. In the first study direct sequencing of LNK genomic region coding for PH and SH2 domain in 33 patients with JAK2 V617F-negative MPN evidenced two mutations in exon 2. A 5-bp deletion and a missense mutation [603_607delGCGCT; 613C>G] (referred to as DEL) was identified in a PMF patient leading to premature stop codon formation and subsequent loss of PH and SH2 domain. The second mutation was a 622G>C change determining the E208Q mutation in the PH domain and occurred in an ET patient (Fig 6). Both mutations were confirmed as somatic since they were absent in skin fibroblasts. LNK mutations frequency in this study is 6%, similar to that of MPL W515 mutations in ET and PMF.

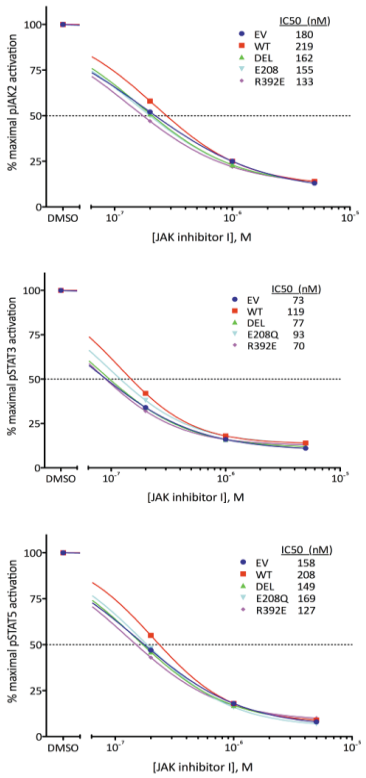


Oh, Blood, 2010

Fig 6 – LNK WT structure and E208Q, DEL and R392E synthetic mutant structure

TPO-induced growth of BaF3-MPL cells was inhibited by the expression of wt LNK while expression of a synthetic LNK mutation disrupting the SH2 domain (R392E) abolished LNK inihibition. Likewise DEL mutant displayed loss of TPO-dependent growth inhibition while E208Q had residual partial inhibitory activity.JAK2/STAT3-5

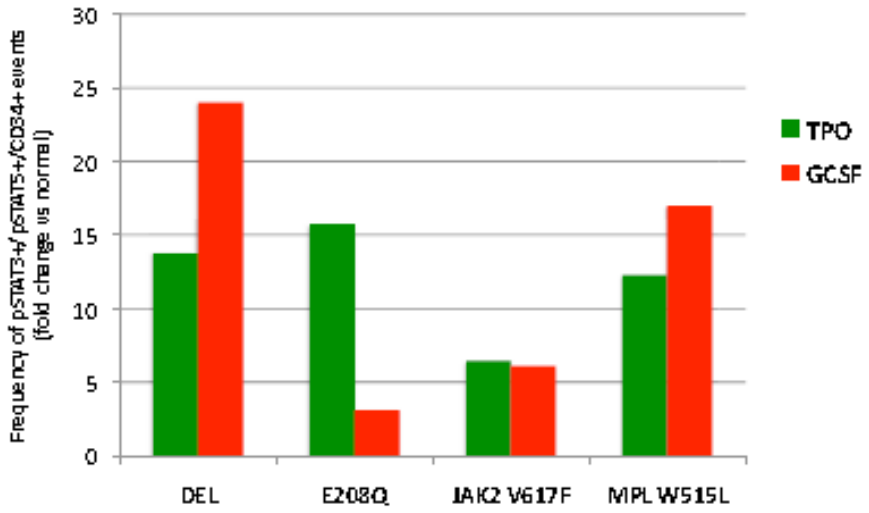
activation in BaF3-MPL cells transduced with LNK DEL mutant was as strong as that of cells transduced with empty vector or R392E mutant while on the contrary cells transduced with wt LNK displayed reduced JAK2/STAT3-5 activation. The E208Q mutation retained nearly-complete inhibitory ability. BaF3-MPL cultured in the presence of a pan-JAK2 inhibitor and transduced with LNK mutant are more sensitive to the treatment compared to cells transduced with wt LNK as demonstrated by the more pronounced decrease in JAK2/STAT3/STAT5 phosphorylation (Fig 7).



Oh, Blood, 2010

Fig 7 – Percentage of JAK2, STAT3 and STAT5 phosphorylation in BaF3 cells transduced with empty vector, WT or mutant LNK, in response to treatment with a pan-JAK2 inhibitor.

Furthermore in primary cells from LNK mutated patients a higher frequency of CD34+ cells responsive, to TPO or G-CSF stimulation in terms of fold change of STAT3/STAT5 phosphorylation compared to normal controls, was observed. The frequency of these cells was comparable or higher in respect to PMF patients with JAK2 V617F or MPL W515L mutation (Fig 8). Analysis of the abnormally increased CD34+ cells revealed the presence of DEL mutation suggesting that LNK mutation might be an early genetic event in MPN (Oh, 2010).



Oh, Blood, 2010

Fig 8 – Frequency of CD34 positive cells responsive to TPO and G-CSF in LNK mutant and in JAK2 V617F and MPL W515L mutant

A subsequent study in a larger cohort of MPN patients in chronic and blast phase evidenced new LNK mutations, mainly affecting the PH domain. LNK mutations can occur in both phases and can be present together with JAK2 V617F and IDH mutations (Pardanani, 2010). In 2010 the first report on LNK mutations in subjects with JAK2 mutations negative erythrocytosis was published (Lasho, 2010). In this study 8 patients with isolated erythrocytosis that were WT for JAK2/MPL/EpoR and displayed subnormal serum EPO levels were screened to assess whether a LNK mutation could explain the clinical phenotype. Two patients were found to harbor a mutation in the PH domain. One was the non-synonymous A215V alteration while the E208X mutation caused protein truncation with loss of both PH and SH2 domains A215V mutation was absent in patient's lymphocyte. Cytogenetical studies on this patient revealed monosomy X that has been reported in cases of myeloid malignancies (Gangat, 2009). Subsequent studies identified other LNK mutations mainly affecting exon 2 but also exons 4, 5, 7 and 8 (Oh, 2010; Lasho, 2010; Hurtado, 2011; Ha, 2011). Lately another study analyzed LNK in 23 patients with idiopathic erythrocytosis with subnormal serum EPO levels and no mutations in JAK2 or EPO receptor (McMullin, 2011). Mutational analysis failed to identify LNK mutations in these patients but 2 patients harbored the non-synonymous SNP E400K (rs72650673) whose frequency in the general population is unknown. Further studies identified a total of 5/96 patients with Idiopathic

Erythrocytosis harboring this variation compared to 0/200 healthy controls (p=0.0033). These data suggest that this particular LNK variant may be associated with Idiopathic Erythrocytosis.

14-3-3 proteins

14-3-3 proteins are a family of highly conserved proteins. In mammals there are seven isoforms, β, γ, ε, σ, ζ, τ and η, that recognize phosphoserine/phosphothreonine residues present in specific sequence motifs on target ligands This property allows 14-3-3 to bind several molecules, including kinases, phosphatases, cytoskeletal proteins, receptors and transcriptional factors, thus affecting many biological process (Zhao, 2011; Wilker, 2004; Fu, 2000; Muslin, 2000; Morrison, 2009; Mackintosh, 2004). 14-3-3 proteins can form homo- and heterodimers which may influence their ability to bind specific targets (Aitken, 2002). Two high-affinity 14-3-3 binding motifs have been described in 14-3-3 target proteins: RSXpSXP and RXXXpSXP (with pS meaning phosphoserine) (Muslin, 1996; Yaffe, 1997). 14-3-3 proteins may affect target protein function through several mechanisms such as modulation of enzymatic activity, protein stability, cellular localization or ability to bind other proteins (Yaffe, 2002; Tzivion, 2001).

The role of 14-3-3 proteins in cancer has been extensively investigated. 14-3-3 σ is suggested to have a tumor suppressor activity since it is silenced by abnormal methylation in solid cancers (Luo, 2010; Zurita, 2010) and is activated by p53 in response to irradiation or other DNA-damage agents (Hermeking, 1997). 14-3-3 sigma itself positively regulates p53 stability by inhibiting Mdm2-mediated ubiquitination of p53 and potentiates p53 transcriptional activity (Yang, 2003). One of the mechanisms possibly explaining 14-3-3 sigma tumor suppressor activity might be also its inhibition ability on AKT mediated cell-growth, transformation and tumorigenesis. AKT activity is increased in several types of solid tumor such as breast, ovarian and lung cancer (Vivanco, 2002). Decreased 14-3-3 sigma expression in primary breast cancer correlates with increased AKT activation (Yang, 2006). On the contrary the other isoforms are looked at as oncogenic proteins. Indeed they are overexpressed in breast cancer (Neal, 2009; Frasor, 2006; Sun, 2008; Cortesi, 2009). In breast cancer overexpression of of 14-3-3 ζ, is associated with poor prognosis (Neal, 2009). 14-3-3

zeta binds to the regulatory subunit of PI3K leading to Akt activation and enhanced tumor cell survival (Neal, 2011). Furthermore 14-3-3 zeta inactivates tumor suppressors like p53 and p21 (Danes, 2008; Wang, 2010). 14-3-3 zeta is also implicated in lung cancer where its marked overexpression associates with disease progression (Zang, 2010). Downregulation of 14-3-3 zeta strongly impairs anchorage-independent growth in lung cancer cells (Li, 2008). Other isoforms, such as 14-3-3 theta, beta and gamma, were found upregulated in lung cancer (Pereira, 2007; Qi, 2007; Qi, 2005). 14-3-3 zeta was reported as overexpressed in consequence of a gene amplification in 30-40% of head and neck squamous carcinoma cases (Lin, 2009). Patients with head and neck cancer showing overexpression of both 14-3-3 sigma and zeta displayed decreased median disease-free survival (Matta, 2008). 14-3-3 zeta mRNA has been shown to be a target of microRNA 451. Mir-451 is implicated in erythroid differentiation (Dore, 2008; Papapetrou, 2010) and its expression is regulated by GATA-1. GATA-1 is an important transcriptional factor involved in hematopoiesis whose activity is induced downstream of EPO receptor activation (Zhang, 2007). Furthermore its reduced expression in the GATA-1(low) mutant mice has been shown to contribute to myelofibrosis onset (Vannucchi, 2002) Mir-451 and mir-144 are encoded by a bicistronic transcript selectively expressed in erythroid cells. Combined knockdown of these miRNA in mice result in altered erythroid differentiation (Papapetrou, 2010). Mice models of mir-451 -/- display reduction in hematocrit, defective erythroid differentiation and ineffective erythropoiesis in response to oxidative stress. Mir-451 depletion leads to strong up-regulation of YWHAZ in erythroblasts and inhibition of 14-3-3 zeta restores proper differentiation (Patrick, 2010). 14-3-3 zeta accumulation in mice lacking mir-451 enhances relocalization of the transcriptional factor FoxO3 in the cytoplasm (Yu, 2010) thus hindering its activity of promoting transcription of pro-apoptotic genes (Alvarez, 2001; Dijkers, 2000). A role for deregulation of mir-451 and 14-3-3 zeta interaction in promoting cancer has been recently pointed out in breast cancer. Up-regulation of 14-3-3 zeta was induced by tamoxifen therapy leading to reduced relapse-free time due to endocrine therapy resistance. This study demonstrated that up-regulation of 14-3-3 zeta was caused by tamoxifen-induced down-regulation of mir-451. Indeed forced overexpression of mir-451 decreased 14-3-3 zeta levels and consequently suppressed cell proliferation and

colony formation, increased apoptosis and restored sensitivity to endocrine therapy in resistant cells (Bergamaschi, 2012).

A recent study demonstrated that LNK is a target of 14-3-3 proteins in mice (Jang, 2012). Two serine residues, S13 and S129, near the N-terminus of LNK, were shown to be critical for 14-3-3 binding to LNK since the double mutant where the two serine residues are converted to alanine (2SA) display complete abolishment of binding. 14-3-3 binding to LNK depends on phosphorylation of these critical serine residues that is moderately increased after TPO stimulation. LNK 2SA double mutant display an enhanced growth inhibition effect compared to wt LNK. Therefore, the ability of LNK to associate with 14-3-3 inversely correlates with its growth inhibitory activity. Since LNK slows cell growth by inhibiting JAK2 this study investigated the possible antagonizing effect of 14-3-3 on LNK inhibition of JAK2 demonstrating that the LNK 2SA mutant causes a stronger inhibition of JAK2 compared to WT LNK. 14-3-3 inhibit LNK activity on JAK2 by preventing LNK/JAK2 interaction partially through LNK sequestration in the cytoplasm. 14-3-3 proteins were shown to have a role in hematopoietic stem and progenitor cells (HSPC) function. Concurrent, but not individual, depletion of 14-3-3 proteins beta, zeta and epsilon through shRNA inhibited HSPC ability to reconstitute bone marrow after BMT experiments. LNK deficiency mitigated the disadvantageous effect of 14-3-3 depletion on BM repopulation. Furthermore 14-3-3 depletion was shown to reduce cytokine-induced STAT5 activation in HSPC. These data globally suggest that 14-3-3 proteins play an important role in the regulation of LNK/JAK2 pathway in HSPC.

AIM OF THE STUDY

1. Study of JAK2 gene in CML

- 1A. to evaluate JAK2 46/1 haplotype frequency in patients with CML to determine if it may represent a genetic risk factor as it is for Ph-negative MPN

- 1B. to study in a large cohort of CML patients the possible concomitant occurrence of JAK2 V617F and BCR/ABL mutations previously reported in sporadic cases in the literature

2. Study of chr12q24 region

- 2A. to study the frequency of chr12q24 SNPs associated with hematological parameters in MPN patients and their clinical impact in these disorders

- 2B. to analyze by deep sequencing the chr12q24 region in a patient with LOH

- 2C. to study the role of LNK mutations as possible new genetic marker that reveals clonal disease in patients with idiopathic erythrocytosis

- 2D. to study the expression of 14-3-3 proteins as a possible mechanism for hyperproliferation in absence of activating mutation of JAK2

METHODS

Study of JAK2 gene in CML

Study of JAK2 V617F mutation occurrence in CML patients

Patients enrollment and sample preparation

Analysis was performed after patients gave informed consent on 314 patients affected by Chronic Myelogenous Leukemia in treatment with tyrosine-kinase inhibitor (TKI) who were referred to Florence, Turin and Bari centres for routine BCR-ABL quantification assessment. Clinical data were revised in order to confirm diagnosis according to WHO criteria.Whole peripheral blood DNA was extracted from patients samples through column-based technique (QIAmp DNA Blood kit, Qiagen Germany). Quantification of DNA was performed using a precision spectroscopy (Nanodrop, NanoDrop Technologies USA) by measuring 260 nm absorbance and purity was assessed by evaluation of A260/A280 nm ratio.

Real time PCR

Real time PCR is based on the 5' esonucleasic activity of AmpliTaq Gold DNA polymerase that during amplification allows probe cleavage. The probe is an oligonucleotide that is marked in 5' position with a reporter fluorescent dye (FAM dye) and in 3' position with a quencer dye (TAMRA dye or MGB dye or NFQ dye). The probe specifically binds a target sequence comprised in the amplicon generated by specific primers designed for each reaction. When probe is intact reporter fluorescence is absorbed by quencher through the Förster-type energy transfer phenomenon (Förster, 1948; Lakowics, 1983). During reaction the probe binds the target sequence if it's present in the sample. Afterwards the 5'-3' esonucleasic activity of the enzyme cuts the probe only if it is perfectly hybridized to the target sequence and if the target is amplified thus warranting a high specificity. Probe cleavage allows the quencher to go away so that the reporter can fluoresce. Once the probe has been cut its fragments secede and the target is amplified (Fig 9).

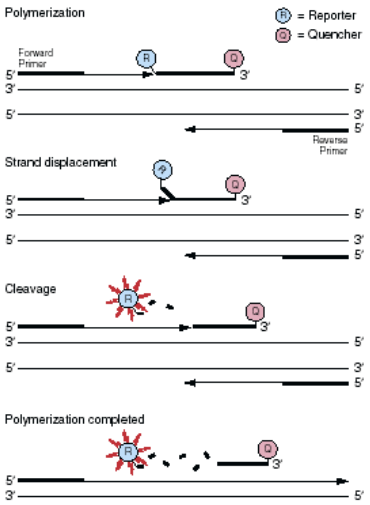


Fig 9 – Real time PCR mechanisms

In every cycle fluorescence increases as PCR products accumulate. Fluorescence variation is detected by ABI 7300 instrument. Reaction buffer contains a reference passive marker (ROX) that without being part of reaction functions as an internal standard for normalization of reporter signal during data analysis. Normalization is needed to correct fluorescence fluctuation due to variation in samples volume and concentration and is obtained from the ratio between reporter emission and ROX emission that gives the normalized reporter (Rn) for each reaction well. Every DNA sample reaches during amplification a threshold cycle that is defined as the first cycle showing a statistically significant change in Rn. Threshold is the average standard deviation of Rn for early cycles multiplied for a correction index. Threshold cycle verifies when the sequence detection system first reveals signal increase associated with exponential increase of PCR products (Fig 10).

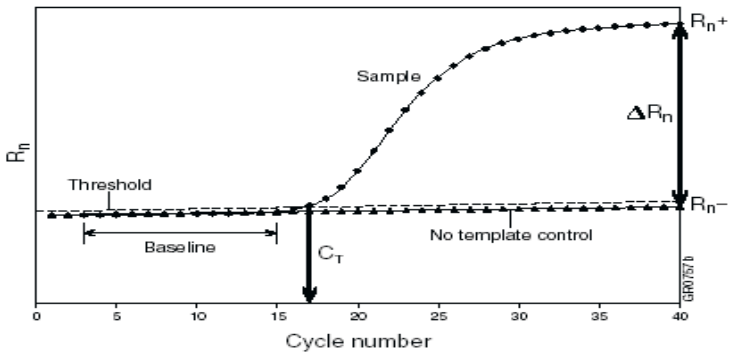


Fig 10 - Real time PCR reaction kinetic

Results are expressed as a difference of amplification between the levels of target gene and a reference gene. For each well ΔC_t is calculated as the difference between threshold cycle C_t of reference gene and the C_t of the target gene.

Quantitative evaluation of JAK2 V617F mutation

Presence of JAK2 V617F mutation was evaluated through quantitative real time PCR using specific primers and probes (Tab 5) (Lippert, 2006).

Primer	5'-3' sequence
Forward WT Primer	5'-GCGCGGTTTTAAATTATGGAGTATGTG-3'
Forward MUT Primer	5'-GCGCGGTTTTAAATTATGGAGTATGTT-3'
Reverse Primer	5'-GCGGTGATCCTGAAACTGAATTTTC-3'
Probe	5'-TGGAGACGAGAGTAAGTAAAACTACAGGCT-6FAM-MGBNFQ

Tab 5 - Primers and Probe sequence for JAK2 V617F quantitative assessment

Each sample was amplified in two different reactions, one using the WT specific forward primer and the other using the V617F specific primer. Both reactions use the same fluorescent probe. For each reaction 100 ng of DNA were amplified in a total volume of 20 µL containing 300 nM of each primer, 200nM of the probe and 1X TaqMan Universal PCR Master Mix (Applied Biosystem) with the following protocol: 95° C for 10 minutes, 50 cycles of 95° C for 15 seconds and 60° C for 1 minute.

Results were expressed for each sample as ΔC_t, calculated as the difference between C_t of mutated sequence and C_t of wt sequence. The mutated allele burden for each sample was assessed comparing ΔC_t value with a standard curve of serial dilutions of JAK2 V617F mutated plasmidic DNA and corresponding wt plasmidic DNA, using linear regression method on InStat3-GraphPad Software.

BCR/ABL quantitative assessment was performed as described (White, 2010).

Study of JAK2 46/1 haplotype in CML

Genotyping of rs12343867

The single nucleotide polymorphism rs12343867 is part of 46/1 haplotype and is located at the 5074189 base position in intron 14 of JAK2 gene. Ancestral allele of this SNP is T while minor allele that is in complete linkage disequilibrium with 46/1 haplotype is C.

rs12343867 was analyzed on whole blood DNA of 84 patients with Chronic Myelogenous Leukemia, referring to our centre and 235 volounteer healthy controls (come abbiamo scritto nel lavoro) after collecting informed consent.

Genotyping of rs12343867 was performed through Allelic Discrimination assay on ABI PRISM 7300 Sequence Detection System (Applied Biosystem) using SNP Genotyping Assay C_31941689_10 (Applied Biosystem) that contains specific primers for amplifying the target sequence and two specific probes, one for the ancestral allele (FAM-labeled) and one for the minor allele (VIC-labeled).

Allelic Discrimination is an application of real time PCR that allows genotyping of a polymorfic variant. The pre-designed assay contains two fluorogenic probes, each one specific for one of the SNP variant, that are marked with different reporter dyes in order to discriminate different PCR products accumulation. Reaction has three steps: (1) a pre-read reaction when the plate is analyzed to determine initial fluorescent background, (2) a standard amplification reaction and (3) a post-read reaction when the plate is analyzed to assess final fluorescence given by the amount of each PCR product present in each well. According to fluorescences revealed the instrument software elaborates a result plot where sample are distributed in three groups corresponding to the three possible genotypes: X allele homozygous, Y allele homozygous and heterozygous (Fig 11).

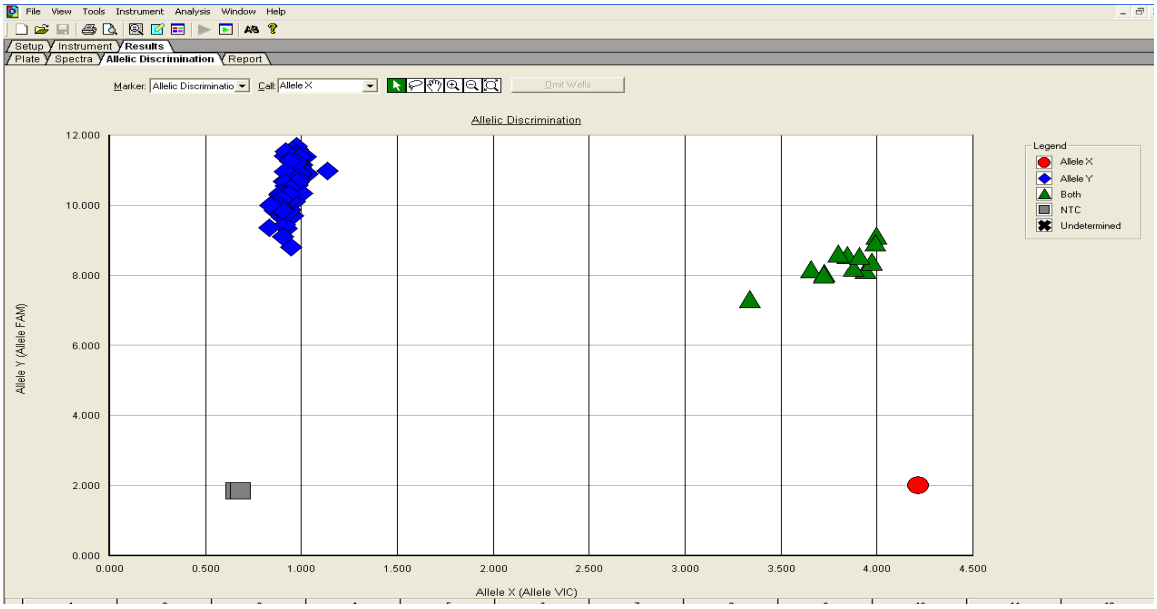


Fig 11 - Allelic discrimination plot of rs12343867 displaying the three genotypes T/T (blue), C/T (green) and C/C (red).

For each patient 20 ng of DNA were amplified in a reaction volume of 20 µL according to the following protocol: 95° C denaturation for 10 minutes followed by 40 cycles of amplification (92°C 15 sec, 60° 1 minute). Reaction mix contained rs12343867 SNP genotyping assay comprising primers and probes and 2X Taqman Universal PCR Mater Mix NO AmpErase UNG (Applied Biosystem).

Statistical analysis was performed using Mann-Whitney, Kruskal-Wallis and contingency table tests on SPSS software version 19.0 (IBM SPSS Statistics).

Study of chromosome 12q24

Genotyping of 12q24 SNPs

Patients enrollment and sample preparation

Analysis was performed after patients gave informed consent on 210 patients affected by Myeloproliferative Neoplasms (Tab 6) and 95 volounteer healthy controls on granulocyte or whole blood DNA. Clinical data were revised in order to confirm diagnosis according to WHO criteria.

Diagnosis	Number of Subjects
Healthy Donors	95
MPN (total)	**210**
MPN JAK2 V617F	**157**
MPN JAK2 wt	**53**
PV	64
PPV-MF	27
ET JAK2 V617F	40
ET JAK2 wt	26
PMF JAK2 V617F	26
PMF JAK2 wt	14
PET-MF JAK2 wt	13

Tab 6 – chr12q24 SNPs study cohort

Peripheral blood samples underwent a density gradient separation with Ficoll Hypaque (Lymphoprep, Eurobio France) to obtain granulocytes that were then lysed twice with ammonium chloride based solution (ammonium chloride 150 mM, potassium hydrogen carbonate 10 mM and EDTA $6x10^{-5}$ mol /liter) to eliminate erythrocyte contamination. Cells have been washed in saline buffer Dulbecco's PBS and stored at –20° C. Once pellet was thawed granulocyte DNA was extracted and quantified as described above. Whole blood DNA was extracted and quantified as

already described.

Genotyping of rs3184504, rs11065987, rs11066301

The analysis focused on three SNP comprised in the 12q24 haplotype that was recently identified and shown to be associated with platelet count and increased risk of coronary artery disease in normal population (Soranzo, 2009). The SNP rs3184504 is a C/T variant, with T being the minor allele, located at base position 111884608 in exon 3 of SH2B3 (LNK) gene. This SNP is a nonsynonimous variation since it determines the R262W aminoacid change. The SNP rs11065987 is an intergenic A/G variant, with G representing the minor allele. The SNP rs11066301 is located at position 112871372 in an intronic region of PTPN11 gene and is a A/G variation where G is the minor allele.

The three SNPs were genotyped as described above by Allelic Discrimination assay on ABI PRISM 7300 Sequence Detection System (Applied Biosystem) using pre-designed SNP Genotyping Assays C_2981072_10 (rs3184504) C_1243839_10 (rs11065987) and C_1243885_10 (rs11066301) (Applied Biosystem).

Statistical analysis was performed using Mann-Whitney, Kruskal-Wallis and contingency table tests on SPSS software version 19.0 (IBM SPSS Statistics).

Deep sequencing of chr12q24 region

In the occasion of a SNP array analysis made in collaboration with the group of Prof Martinelli at the University of Bologna in a group of MPN patients through Genome-Wide Human SNP Array 6.0 (Affymetrix), a small region of loss of heterozygosity affecting chromosome 12q24 was identified in a subset of 52/73 (71%) patients affected by MPN. The minimal shared region comprises SH2B3, ATXN2, BRAP, ACAD10, ALDH2, MAPKAPK5, TMEM116, ERP29 and C12ORF30 genes (Fig 12).

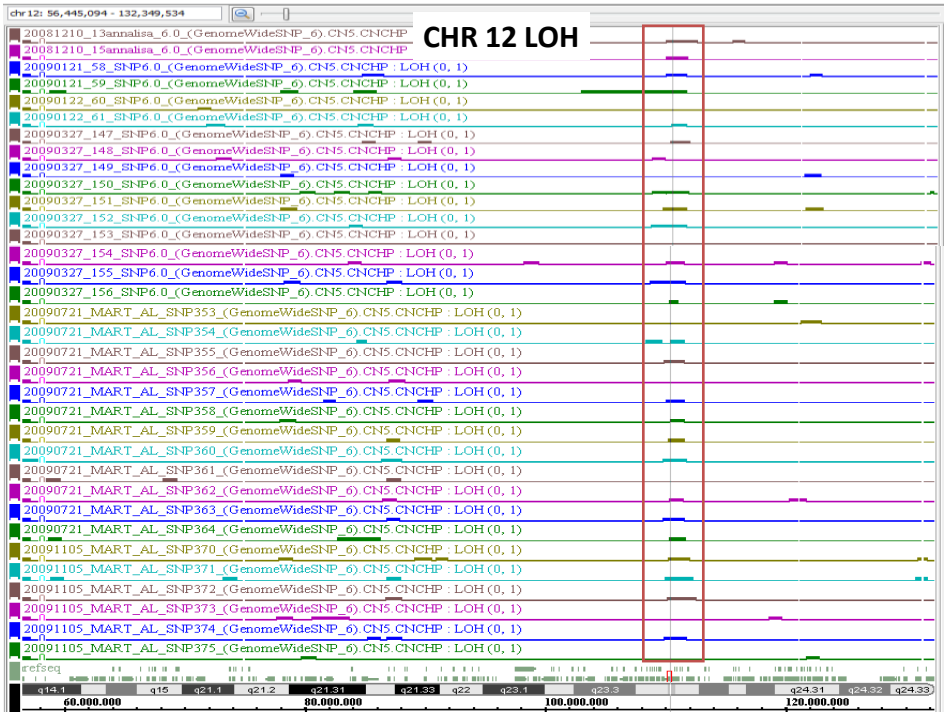


Fig 12 – Chr12q24 LOH

To further investigate this LOH we performed through an external service (Eurofins MWG Operon) deep sequencing of the affected region in a patient with PMF that harbored the longest LOH region. The service consisted in the design of a Roche NimbleGen sequence capture array (385K), enrichment and subsequent sequencing on Roche GS FLX with Titanium series chemistry (Fig 13).

The sequence capture system allows selective enrichment of genomic regions of interest starting from whole genomic DNA. The 385K array captures up to 5 Mbp and contains overlapping high density oligonucleotide probes, 50 to 100 bp long, that enable redundant coverage of the target region.

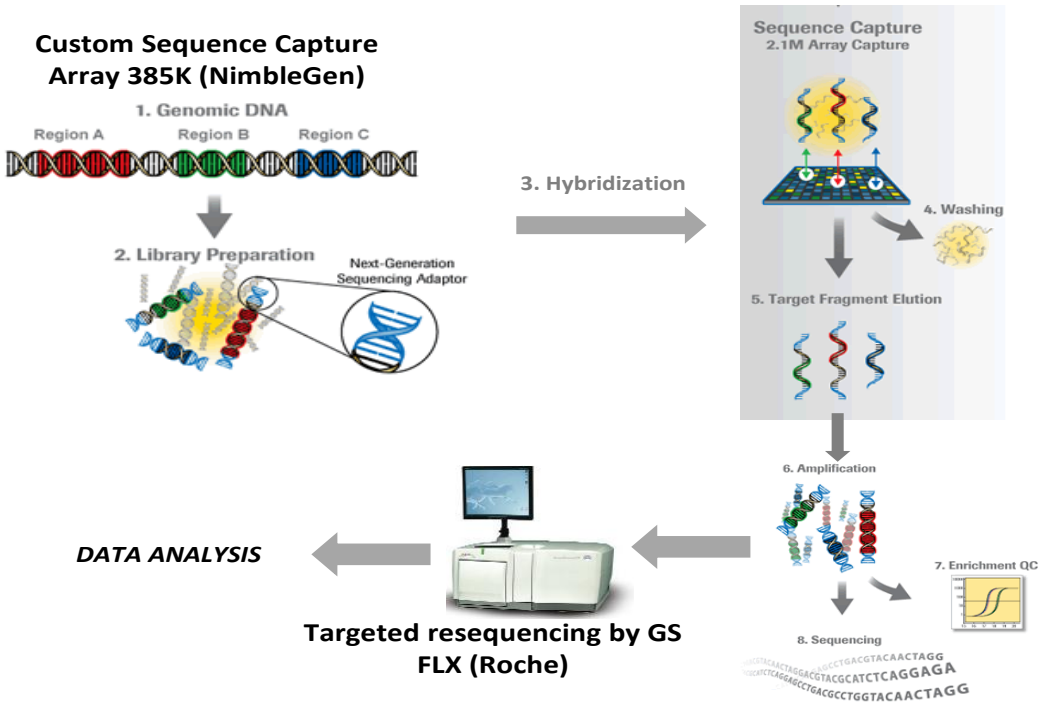


Fig 13 - Deep Sequencing experiment workflow (adapted from NGS brochure, Eurofins MWG Operon)

Genomic DNA (10 to 20 µg) is fragmented and adaptors are linked to the fragments. DNA fragments are then hybridized to the array. After a wash step to remove unbinded DNA, target fragment are eluted from the array. Enriched fragments are amplified by ligation-mediated PCR and subsequently undergo quality control. Then the library containing GS FLX Titanium series chemistry adaptors is verified. Enriched sample are then sequenced on Roche GS FLX that result in hundred thousands of reads. The reads are mapped on the reference sequence and then undergo statistical analysis. For our analysis we considered the High Confidence variations determined by the GS reference mapper application according to flow signal, quality score and difference type information. General rules for HC-variation assessment, as stated by Eurofins MWG/Operon are: (1) there must be at least 3 non-duplicate reads with the difference, (2) there must be both forward and reverse reads showing the difference, (3) if the difference is a single-base overcall or undercall the reads with the difference must form the consensus of the sequenced reads and the signal distribution of the differing reads must vary form the matching reads. For each difference the position on the reference sequence where the variation occurs and type of variation are indicated, as well as total number of reads that fully span the

difference location (depth) and the percentage of different reads versus total reads that fully span variation location (variation frequency).

LNK mutational analysis

Patients enrollment and sample preparation

Mutational analysis of SH2B3 exons 2, 3 and 4 was performed, after informed consent collection, in granulocyte or whole blood DNA obtained as previously described, in 112 subjects with Idiopathic Erythrocytosis from Florence and Vicenza centers.

Exon 2 analysis

Exon 2 was analysed by direct sequencing with a conventional touchdown PCR using forward primer 5'-GCTCCTTCCAGCACTTTCG-3' and reverse primer 5'-CTGGAAAGCCATCACACCTC-3'. 50 ng of DNA were amplified in 25 μL of reaction volume containing 1 U of Ampli-Taq Gold 360 DNA Polymerase, 1X Ampli-Taq Gold 360 Buffer, 1,5 mM of MgCl2, 200μM of dNTPs, 320 nM of each primer and 1,25 μL of molecular grade DMSO (cod D8418, Sigma). Reaction kinetic described in Tab. 7 was designed as a touchdown PCR to improve amplification specificity.

STEP	KINETIC
Activation	95° C 10 minutes
Cycling (10 cycles)	(94° C 30 sec)(67°(-1°/cycle) 30sec)(72° C 30 sec)
Cycling (30 cycles)	(94° C 30 sec)(57° C 30 sec)(72° C 30 sec)
Final extension	72° C 10 minutes

Tab 7 – Kinetic of LNK exon 2 PCR.

Sanger sequencing was performed through an external service. Sequencing output was analysed with Mutational Surveyor software (SoftGenetics, State College PA, USA).

Exons 3 and 4 analysis

Exons 3 and 4 were analysed in a unique amplicon by High Resolution Melting Analysis.

HRMA allows the identification of mutations or polymorphic variants through the analysis of the dissociation curve of a dsDNA that was previously amplified in the presence of fluorescent intercalating dyes, such as LC green, whose absorption and emission spectrum change when they bind DNA compared to the unbinded dye (Fig 14).

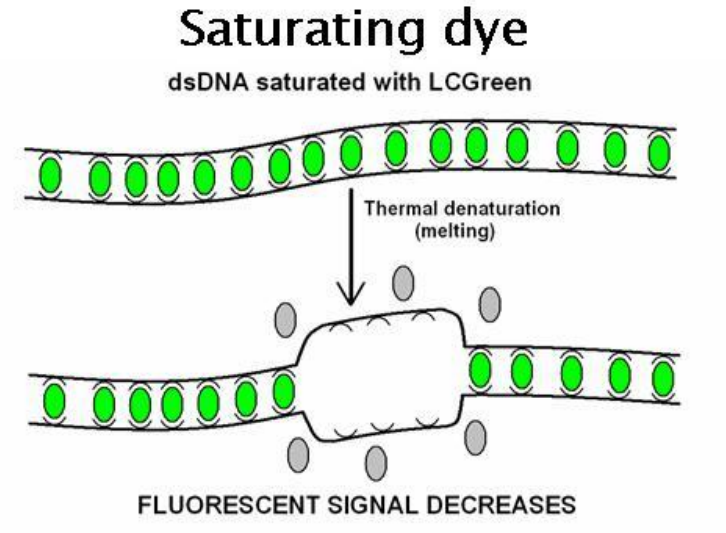


Fig 14 – Fluorescence variation in consequence of LCGreen binding to DNA (image from www.spectron.com)

For each sample 100 ng of DNA were amplified by conventional PCR using forward primer 5'- CCCAGCAACACCATGGATAC-3' and reverse primer 5'- TAAGGATCTGGGCAGGTCAG-3' that generated an amplicon of 475 base lenght. Reaction volume was 25 μL containing 1 U of Taq Gold DNA Polymerase, 1x Taq Gold DNA Polymerase Buffer, 2,5 mM of MgCl2 , 100 μM of dNTPs, 100 nM of each primer and 1X LC green Plus dye (Biochem, Utah). Reaction kinetic after enzyme activation at 95°C for 13 minutes consisted of 35 cycles of (95°C 30sec)(64° C 30 sec)(72° C 30 sec) and final extension at 72° C for 10 minutes.

High Resolution melting Analysis was then performed on Rotor Gene Q 6000 (QIAGEN). In this teqnique LC green that is incorporated in PCR products during

ampification is excited at a wavelenght of 460 nm. Sample fluorescence emission is detected at 510 nm at very close lapses during DNA denaturation. When the double strand opens LC green is released and its fluorescence decreases shaping a sigmoid curve in the graph representing fluorescence variation versus temperature variation. This curve has at least an inflection point that represents amplicon melt temperature. The curve shape and the melting temperature are influenced by amplicon lenght and nucleotide sequence so that a single base variation can change them. Reaction protocol consists of an initial phase of complete DNA denaturation followed by a low temperature renaturation step which promotes heteroduplexes formation. Heteroduplex form in the presence of a sequence variation when a strand harboring the variation renaturates with a strand that does not display the variation so that the dsDNA exhibit a mismatch. The mismatch changes the melting temperature of the molecule so that heteroduplexes differ from the homoduplexes (two strands that perfectly complement either harboring or not the variation) and can be easily detected. Heteroduplexes thus facilitate the identification of homozygous sequence variation.

LNK exons 3-4 were analyzed in a first reaction consisting in 95° C denaturation for 5 minutes, then renaturation at 40° C for 1 minute then HRMA from 84° C and 94° °C with temperature rising by 0.1° C. Since the amplicon generated for this analysis comprised the rs3184504 C/T SNP, three healthy donors DNA representing the three different genotypes of the polymorphism were included in the reaction. Samples that originated a curve overlapping the curves of the two rs3184504 homozygous donors were reanalyzed in a second HRMA. In this late reaction sample were in triplicate, one tube contained 100% of the DNA under examination while the other tubes contained 50% of the sample DNA mixed with 50% of either the rs3184504 C/C or T/T donor DNA. This process facilitate the detection of possible homozygous variant that could mimic the homozygous normal curve.

Mutations were confirmed by direct Sanger sequencing through an external service.

Gene expression assay for 14-3-3 proteins

Gene expression of YWHAZ and YWHAB, encoding 14-3-3 protein zeta and beta respectively, was analysed through Gene Expression Assay on granulocyte RNA of 33 patients with MPN, 23 subjects with idiopathic erythrocytosis and 16 healthy donors (Tab 8)

Diagnosis	Number of Subjects
Healthy Donors	16
PV	8
Idiopathic Erythrocytosis	23
ET JAK2 V617F	5
ET JAK2 wt	8
PMF JAK2 V617F	8
PMF JAK2 wt	4

Tab 8 – 14-3-3 proteins evaluation cohort

Granulocyte fraction was isolated, lysed and washed as described above and before freezing was resuspended in 1 ml TriPure Isolation Reagent (Invitrogen, Life Technologies) that lyses cells and inhibits RNAse. RNA was extracted following manufacturer's protocol. Briefly thawed cells suspension was incubated 5 minutes at room temperature then 0.2 mL of chloroform were added followed by centrifugation at 12000 rpm for 15 minutes at 4° C. This step allows Tripure Isolation Reagent to separate proteins, DNA and RNA in three phases. The upper transparent phase, containing RNA, is recovered and 2µL of glycogen and 0.5 mL of isopropanol are added to favour RNA precipitation. Suspension is incubated at room temperature for 10 minutes after inversion mixing and afterwards centrifuged at 12000 rpm for 10 minutes at 4° C. RNA pellet is then washed with 75% ethanol and centrifuged at 7500 rpm for 5 minutes at 4° C. Finally RNA pellet is dried at room temperature and resuspended in 20 µL of PCR water and quantified by spectrophotometry using Nanodrop (NanoDrop Technologies USA). RNA was converted into cDNA by reverse

transcription PCR using the l'High Capacity cDNA Archive Kit (Applied Biosystems). The reaction mix of 25 µL comprises 1X Reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2mM of $MgCl_2$, 200 mM of dNTPs, random hexamers, RNAse inhibitor and the reverse transcriptase MuLV (Applied Biosystem, Life Technologies). 1 µg of RNA was reverse transcripted according to the following protocol: 22°C for 10 minutes, 42°C 40 minutes, 95°C 5 minutes.

Gene expression was assessed through real time quantitative PCR using the commercially available Gene Expression Assays Hs03044281_g1 (for YWHAZ) and Hs00793604_m1 (for YWHAB) (Applied Biosystem, Life Techonologies). For each sample 100 ng of cDNA were amplified in a reaction volume of 20 µL containing 10 µL of TaqMan PCR Universal Mastermix (Applied Biosystem, Life Technologies), 1 µL of the specific assay FAM-labeled and 1 µL of the RNAse P assay VIC-labeled (Applied Biosystem, Life Technologies) used as housekeeping reference gene. The results were expressed as a difference in the expression level of the target gene compared to the housekeeping for each sample. For each sample the ΔC_t was calculated as the difference between threshold cycle (C_t) of the target gene and the threshold cycle of the reference gene. Then $\Delta\Delta C_t$ was assessed subtracting from the patient's ΔC_t the median ΔC_t of the whole healthy population used as control. The relative quantification value (RQ) was calculated for each sample as $2^{-\Delta\Delta Ct}$.

Statistical analysis was performed using Mann-Whitney, Kruskal-Wallis and contingency table tests on SPSS software version 19.0 (IBM SPSS Statistics).

RESULTS AND DISCUSSION

Study of JAK2 gene in CML

Study of JAK2 46/1 haplotype in CML

In a Swedish registry-based epidemiological study almost 25.000 relatives of 11.039 MPN patients were analyzed demonstrating an increased relative risk for patients first-degree relatives of developing PV (RR=5.7), ET (RR=7.4) and not-otherwise specified MPN (RR=7.5) compatible with a model of autosomal recessive inheritance. An increased relative risk, although not statistically significant, was also evidenced for CML development in MPN relatives (RR=1.9) (Landgren, 2008). We therefore wanted to assess whether JAK2 46/1 haplotype could explain the increased risk of CML in first-degree relatives of MPN patients thus representing a genetic risk factor for CML as it is for Ph negative MPNs. Genotyping of the 46/1 haplotype tagging SNP rs12343867 was performed in a cohort of 84 patients with CML. As shown in Tab 9 we evidenced that in CML patients the distribution of the C allele of rs12343867, that stands for 46/1 haplotype, is 0.25 thus clearly overlapping that of healthy controls.

Diagnosis	N° of subjects	N° of allele	rs12343867 T allele	rs12343867 C allele (46/1 haplotype)	46/1 haplotype frequency	P value
CML	84	168	125	43	0.2559	1.0
DONORS	235	470	351	119	0.2531	

Tab 9 - 46/1 haplotype frequency in CML

These findings excluded that 46/1 haplotype could be a genetic risk factor for CML predisposition as it is for classical Ph-negative MPNs.

Concomitant occurrence of BCR/ABL and JAK2 V617F mutations in CML

In a recent study evaluating the occurrence of mutations commonly associated with Ph-negative MPNs and Myelodisplastic Neoplasms in 54 patients with CML, cases of TET2, CBL, ASXL1 and IDH1 alteration were reported in 14% of accelerated phase and 40% of blast phase. On the contrary, chronic phase patients did not harbor any additional mutation other than BCR/ABL rearrangement, including JAK2 V617F mutation (Makishima, 2011). Nevertheless isolated cases of concomitant BCR/ABL and JAK2 V617F mutation were reported in literature (Hussein, 2008). We evaluated the frequency of this double mutation occurrence in a large cohort of 314 CML patients. We identified 8 subjects (2.55%) carrying both BCR/ABL rearrangement and JAK2 V617F mutation whose main characteristic are reported in table 10.

Pt	Sex	Age (Y)	% JAK2 V617F	CML phase/duration (Months)	Features at CML diagnosis				
					Karyotype	BCR/ABL transcript	WBC 10^9/L	HB g/dL	PLT, 10^9/L
1	F	82	61	CP / 9	46,XX,t(9;22)(q34;q11)	b3a2	46	11.8	462
2	F	58	9	CP / 75	46,XX,t(9;22)(q34;q11)	b3a2	136.6	11.1	383
3	F	67	39	CP / 80	n/a	n/a	29.9	11.6	355
4	M	52	15	CP / 140	n/a	e8a3	8.5	14.9	773
5	M	71	19	CP / 38	n/a	b3a2	n/a	n/a	n/a
6	F	52	25	CP / 35	46,XX,t(9;22)(q34;q11)	b2a2	147.7	9.9	2250
7	F	71	13	CP / 75	46,XX,t(9;22)(q34;q11) 15/15	b3a2	24.5	13.7	200
8	F	69	15	CP / 102	46,XX,t(9;22)(q34;q11) 10/10	b3a2	13,2	13.8	302

Tab 10 – Characteristics of mutated patients

All double mutated patients were in chronic phase of CML. Patient 1 had a previous diagnosis of PV treated with hydroxyurea (HU) for 10 years. Due to

progressive leukocytosis despite cytoreductive therapy, she underwent diagnostic test for CML that evidenced BCR/ABL transcript. She was treated with Imatinib soon shifted to Dasatinib due to lack of achievement of a complete cytogenetic response (CcyR). At the time of CML diagnosis, JAK2 V617F burden was 1-2%, around the detection limit, while 9 months later at the time of sampling it had raised up to 61%. Patient 4 was diagnosed with CML and initially treated with interferon that induced hematologic and cytogenetic response. He presented marked thrombocytosis that persisted after he shifted to imatinib treatment despite achievement of a major molecular response (MMR). This course seems to support the hypothesis of a proliferative competition between BCR/ABL and V617F mutated clones, as suggested by previous report (Bocchia, 2007). None of the other patients had any feature of Ph-negative MPN. Patient 2 received first HU then shifted to Imatinib therapy that leaded her to CcyR; at the time of sample collection for JAK2 quantification she had lost MMR and karyotype evidenced chr 8 trisomy. Patient 3 was treated with imatinib in first line then with dasatinib due to loss of response; at the time of sampling she had lost MMR. Patients 5 and 6 were treated with HU and then with imatinib and achieved CcyR and MMR. Patient 7 was treated with imatinib as first line then with dasatinib due to failure in MMR attainment; at the time of sampling she was in CcyR. Patient 8 was treated with combination of imatinib and pegylated interferon then with imatinib alone; at sampling she was in MMR without therapy. The role of JAK2 V617F mutated clone in these patients is unclear since it did not appear to confer any specific feature of Ph-negative MPN. The prevailing clinical phenotype is indeed that of a typical CML. Nevertheless this study demonstrated that concomitant occurrence of BCR/ABL rearrangement and JAK2 V617F mutation is a rare but reproducible phenomenon, that should be searched for in selected cases due to the important therapeutic implications.

Study of chromosome 12q24

Genotyping of 12q24 SNPs

The analysis of the three SNPs rs3184504, rs11065987 and rs11066301, part of the newly identified haplotype that spans the chr12q24 region revealed an association with Myeloproliferative Neoplasms. For each polymorphism, the minor allele was previously shown to be part of the haplotype configuration that associates with increased platelet count and higher risk of CAD in the normal population (Soranzo, 2009). As expected the results are comparable for three variants. For the rs11065987 variant the distribution of the three genotypes is significantly different from that of healthy donors, particularly regarding the frequency of minor allele homozygous G/G genotype (30% Vs 15%; p=0.017). When dividing MPN patients according to JAK2 mutational status we evidenced that the difference is mainly provided by the group of JAK2 V617F MPN that display lower frequency of A/A and A/G genotype and a double frequency of G/G genotype compared to donors (32% Vs 15%; p=0.009). Genotype distribution in JAK2-mutations negative MPN did not significantly differ from that of the healthy controls although there was a slight difference in genotype distribution in the group of JAK2 WT ET (p=0.023). When considering separately the three diseases we found no significant difference for ET and PMF compared to controls while on the contrary PV patients display an increased frequency of both A/G and G/G genotype (p=0.027). The group of post-PV secondary myelofibrosis demonstrates the greater difference in respect to genotype distribution in healthy group (p=0.001) with the highest increase of G/G genotype (48%) (Tab 11)

Diagnosis	N. of patients	rs11065987 A/A	rs11065987 A/G	rs11065987 G/G (minor allele)	*p value*
DONORS	95	25 sbj (26%)	56 sbj (59%)	14 sbj (15%)	
MPN	**210**	**42 pts (20%)**	**103 pts (50%)**	**62 pts (30%)**	**p=0.017**
MPN JAK2 V617F	**157**	**31 pts (20%)**	**75 pts (48%)**	**50 pts (32%)**	**p=0.009**
MPN JAK2 WT	**53**	**11 pts (21.5%)**	**28 pts (55%)**	**12 pts (23.5%)**	**p=ns**
PV	64	10 pts (16%)	33 pts (52%)	20 pts (32%)	p=0.027
PPV-MF	27	4 pts (15%)	10 pts (37%)	13 pts (48%)	p=0.001
ET	**66**	**14 pts (22%)**	**31 pts (48%)**	**19 pts (30%)**	**p=ns**
ET V617F	40	7 pts (18%)	23 pts (57%)	10 pts (25%)	p=ns
ET WT	26	7 pts (29%)	8 pts (33%)	9 pts (38%)	p=0.023
PMF	**40**	**14 pts (35%)**	**19 pts (47.5%)**	**7 pts (17.5%)**	p=ns
PMF V617F	26	10 PTS (38%)	9 PTS (35%)	7 PTS (27%)	p=ns
PMF WT	14	4 pts (29%)	10 pts (71%)	0 pts (0%)	p=ns
PET-MF WT	13	0 pts (0%)	10 pts (77%)	3 pts (23%)	p=ns

Tab 11 – rs11065987 genotype frequencies

For rs11066301 variant that is located in PTPN11 gene we observed a similar result. The group of MPN patients display significantly different distribution of the genotypes compared to healthy controls (p=0.03) with a particular increase in minor allele G/G genotype (28% Vs 15%). This difference is mainly accounted for by the group of JAK2 V617F MPN where both the A/A and A/G genotypes are decreased while the G/G genotype is significantly increased (33% Vs 15%; p=0.006). The group of JAK2-mutations negative MPN does not differ from the group of donors. Even for this variant the group of PV significantly differs from donors (p=0.011) as well as post-PV myelofibrosis (p=0.004) that display the highest frequency of G/G homozygous genotype (44% Vs 15%). The groups of ET and PMF do not differ from donors with the exception of JAK2 V617F PMF (p=0.03) (Tab 12)

Diagnosis	N. of patients	rs11066301 A/A	rs11066301 A/G	rs11066301 G/G (minor allele)	*p value*
DONORS	95	25 sbj (26%)	56 sbj (59%)	14 sbj (15%)	
MPN	**210**	**40 pts (19%)**	**111 pts (53%)**	**59 pts (28%)**	**p=0.03**
MPN JAK2 V617F	**157**	**31 pts (20%)**	**74 pts (47%)**	**52 pts (33%)**	**p=0.006**
MPN JAK2 WT	**53**	**9 pts (17%)**	**37 pts (70%)**	**7 pts (13%)**	**p=ns**
PV	64	10 pts (16%)	32 pts (50%)	22 pts (34%)	p=0.011
PPV-MF	27	5 pts (19%)	10 pts (37%)	12 pts (44%)	p=0.004
ET	**66**	**12 pts (18%)**	**38 pts (58%)**	**16 pts (24%)**	p=ns
ET V617F	40	6 pts (15%)	24 pts (60%)	10 pts (25%)	p=ns
ET WT	26	6 pts (23%)	14 pts (54%)	6 pts (23%)	p=ns
PMF	**40**	**13 pts (32.5%)**	**19 pts (47.5%)**	**8 pts (20%)**	p=ns
PMF V617F	26	10 pts (38%)	8 pts (31%)	8 pts (31%)	p=0.03
PMF WT	14	3 pts (21%)	11 pts (79%)	0 pts (0%)	p=ns
PET-MF WT	13	0%	12 pts (92%)	1 pts (8%)	p=ns

Tab 12 – rs11066301 genotype frequencies

Also T allele of rs3184504 SNP, located in LNK gene, is similarly associated with the global group MPN (p=0.007) where the main difference is for the homozygous T/T genotype (34% Vs 16%). According to JAK2 mutational status only the JAK2 V617F MPN group significantly differ from donors (p=0.006). However, also in the group of JAK2 WT MPN there is an increase frequency of the minor allele homozygous genotype (30% Vs 16%; p=ns). As for the other two SNPs the difference in genotype distribution is harboured by the groups of PV (p=0.004) and PPV-MF (p=0.003). PPV-MF have the greatest rate of T/T genotype in comparison to donors (48% Vs 16%). ET patients group displays a borderline significant difference compared to controls (p=0.05) that is caused by the group of JAK2-mutations negative ET (p=0.002). Also JAK2 V617F PMF group differs from donors (p=0.04) (Tab13).

Diagnosis	N. of patients	rs3184504 C/C	rs3184504 C/T	rs3184504 T/T (minor allele)	*p value*
DONORS	95	16 sbj (18%)	60 sbj (66%)	15 sbj (16%)	
MPN	**210**	**35 pts (17%)**	**102 pts (49%)**	**70 pts (34%)**	**p=0.007**
MPN JAK2 V617F	**157**	**25 pts (16%)**	**75 pts (49%)**	**54 pts (35%)**	**p=0.006**
MPN JAK2 WT	**53**	**10 pts (19%)**	**27 pts (51%)**	**16 pts (30%)**	**p=ns**
PV	64	6 pts (9%)	32 pts (51%)	25 pts (40%)	p=0.004
PPV-MF	27	4 pts (16%)	9 pts (36%)	12 pts (48%)	p=0.003
ET	**66**	**13 pts (20%)**	**32 pts (48%)**	**21 pts (32%)**	**p=0.05**
ET V617F	40	7 pts (17.5%)	24 pts (60%)	9 pts (22.5%)	p=ns
ET WT	26	6 PTS (23%)	8 PTS (31%)	12 pts (46%)	p=0.002
PMF	**40**	**12 pts (30%)**	**20 pts (50%)**	**8 pts (20%)**	p=ns
PMF V617F	26	8 pts (31%)	10 pts (38%)	8 pts (31%)	p=0.04
PMF WT	14	4 PTS (29%)	10 PTS (71%)	0 PTS (0%)	p=ns
PET-MF WT	13	0 pts (0%)	9 pts (69%)	4 pts (31%)	p=ns

Tab 13 – rs3184504 genotype frequencies

When considering the minor allele frequencies of the three variants the association remains for the global group of MPN patients and particularly for JAK2 V617F MPN (Tab 14). This association for all variants is caused by the groups of PV and post-PV myelofibrosis. The extent of the association might be higher, in a larger cohort, compared to that we revealed considering that the minor allele frequencies in our control group were higher compared to the HapMap CEU population (Tab 15.)

Diagnosis	N. of patients	rs11065987 G allele freq	*p value*	rs11066301 G allele freq	*p value*	*rs3184504 T allele freq*	*p value*
DONORS	95	0.44		0.44		0.49	
MPN	**210**	**0.54**	**p= 0.018 OR=1.53**	**0.54**	**p=0.022 OR=1.5**	**0.58**	**p =0.014 OR=1.56**
MPN JAK2 V617F	**157**	**0.56**	**p=0.01 OR=1.6**	**0.56**	**p=0.008 OR=1.65**	**0.59**	**p=0.0095 OR=1.6**
MPN WT	**53**	**0.5**	**p=ns**	**0.48**	**p=ns**	**0.56**	**p=ns**
PV	64	0.58	p=0.02 OR=1.7	0.59	p=0.0087 OR=1.8	0.65	p=0.0026 OR=2.07
PPV-MF	27	0.67	p=0.005 OR=2.5	0.63	p=0.02 OR=2.1	0.66	p=0.025 OR=2.157
ET	**66**	**0.54**	**p=ns**	**0.53**	**p= ns**	**0.56**	**p= ns**
ET V617F	40	0.54	p=ns	0.55	p= ns	0.53	p= ns
ET WT	26	0.54	**p=ns**	0.5	p= ns	0.62	p= ns
PMF	**40**	**0.41**	**p=ns**	**0.44**	**p= ns**	**0.45**	**p= ns**
PMF V617F	26	0.44	p=ns	0.46	p= ns	0.5	p= ns
PMF WT	14	0.36	p=ns	0.39	p= ns	0.36	p= ns
PET-MF WT	13	0.62	p=ns	0.54	p= ns	0.65	p= ns

Tab 14 – Minor allele frequencies of the three SNPs

	rs11065987 G allele freq	rs11066301 G allele freq	rs3184504 T allele freq
HapMap - CEU	0.34	0.35	0.41

Tab 15 – Minor allele frequencies of the three SNPs in the HapMap population

The minor allele of of each SNP is significantly higher in MPN patients when compared to healthy donors thus indicating that this haplotype could represent a new genetic risk factor for MPN. When dividing patients according to JAK2 mutational status it becomes clear that the association is provided by the group of V617F mutated MPN while JAK2 wild type MPN do not significantly differ from donors. Furthermore the frequency of the minor allele of each SNP is increased in PV and PPV-MF patients with this latter group harbouring the highest frequency of the minor alleles for the three SNPs. Considering that 95% of PV and PPV-myelofibrosis patients are JAK2 V617F mutated, these data somehow suggest an association between chr12q24 haplotype

and JAK2 mutation. This association may be perhaps explained with the same “fertile ground hypothesis” that has been made for JAK2 46/1 haplotype to explain its association with MPL mutations. It looks indeed possible that 12q24 haplotype is related to a functional variant still unknown that associates with JAK2 V617F mutation favours MPN development.

Since the SNPs of the 12q24 haplotype have been variably associated with hematological parameters in general population we assessed whether their presence could affect these or others features in PV patients. Clinical features of our PV cohort are reported in Tab 16. All values are at the time of diagnosis.

Main Clinical Features of the PV group	
Gender (M)	40/64
Age at diagnosis (yrs)	59 (35-90)
WBC count (10^9/L)	10.4 (±4.3)
Hb (g/dL)	17.5 (±1.79)
Ht (%)	54.0 (±5.6)
MCV (fL)	81.9 (±10.3)
Plt (10^9/L)	516 (± 221)
LDH (UI/L)	395 (± 171)
EPO (mU/mL)	7.5 (±6.3)

Tab 16 – Clinical features of PV group

PV patients were divided according to the presence of zero, one or both association allele for each SNP to assess whether there is a difference in clinical features according to the genotype and eventually how much each allele contributes to this difference.

Statistical analysis revealed that minor allele of rs11065987 and rs3184504 do not associate with platelet count in PV patients. This association occurs for rs11066301 but in contrast with literature data showing that G allele is associated with increase in platelet count, in our cohort a single copy of the minor allele determines lower platelet count while a second copy of the minor allele did not further contribute (Tab 18 and Fig 15).

rs11065987	***p value***			
	All genotypes one Vs each other	A/A Vs A/G	A/G Vs G/G	A/A Vs G/G
PLT	ns	ns	ns	ns
Hb	ns	ns	ns	ns
Htc	ns	ns	ns	ns
EPO	0.034	0.012	ns	0.032

Tab 17 – p values of association between rs11065987 and clinical features

rs11066301	***p value***			
	All genotypes one Vs each other	A/A Vs A/G	A/G Vs G/G	A/A Vs G/G
PLT	0.031	0.019	ns	ns
Hb	ns	ns	ns	ns
Htc	0,038	ns	0.018	ns
EPO	0.007	0.003	ns	0.006

Tab 18 – p values of association between rs11066301 and clinical features

rs3184504	***p value***			
	All genotypes one Vs each other	C/C Vs C/T	C/T Vs T/T	C/C Vs T/T
PLT	ns	ns	ns	ns
Hb	ns	ns	ns	ns
Htc	ns	ns	ns	ns
EPO	Ns (0.069)	0.03	ns	0.028

Tab 19 – p values of association between rs3184504 and clinical features

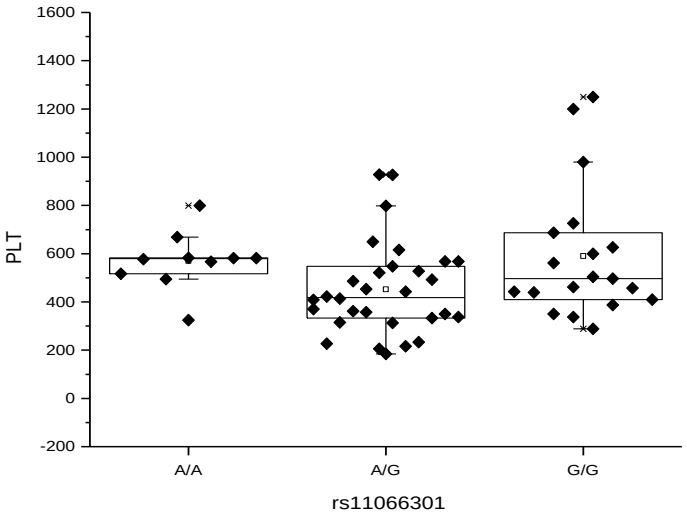


Fig 15 – Association between rs11066301 and platelet count

In contrast to the data reported in literature we found that minor allele of rs11066301 and not rs11065987 was associated with Hematocrit (Fig 16).

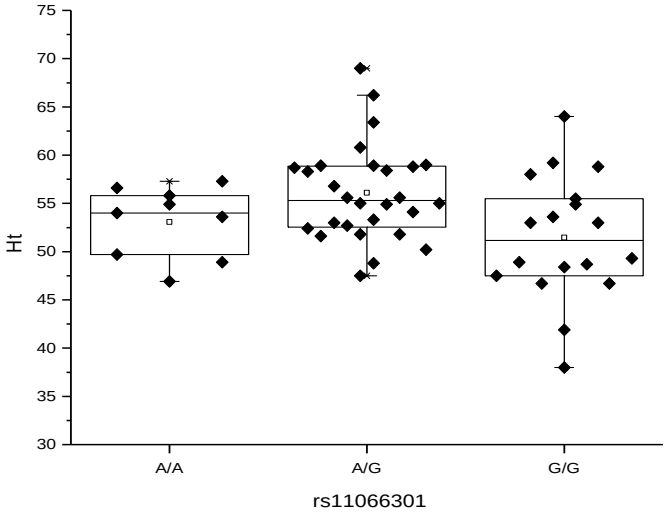


Fig 16 – Association between rs11066301 and hematocrit

We evidenced for all the three SNPs that a single copy of the minor allele determines significantly decrease in serum EPO value while the second copy doesn't add difference (Tab 17/18/19, Fig 17/18/19). To our knowledge such association has not been previously reported.

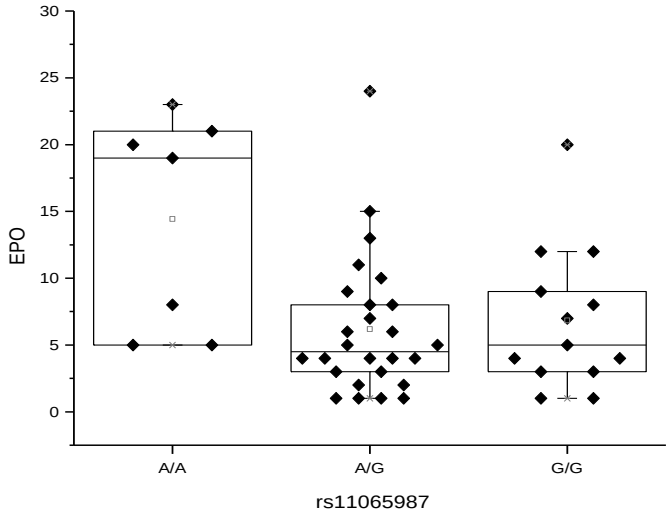


Fig 17 – Association between rs11065987 and EPO

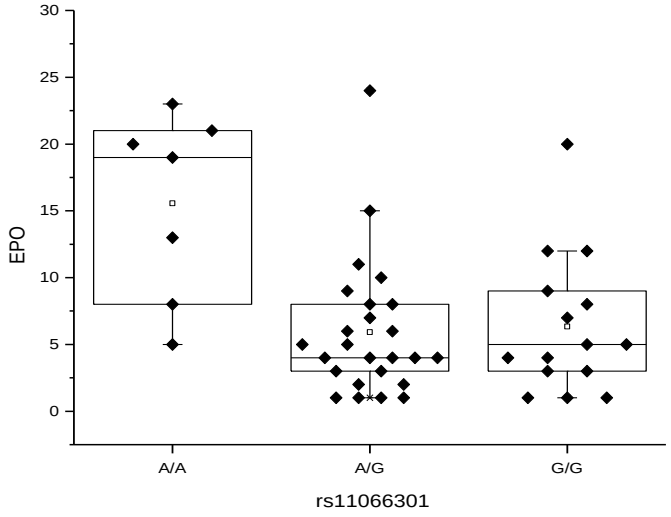


Fig 18 – Association between rs11066301 and EPO

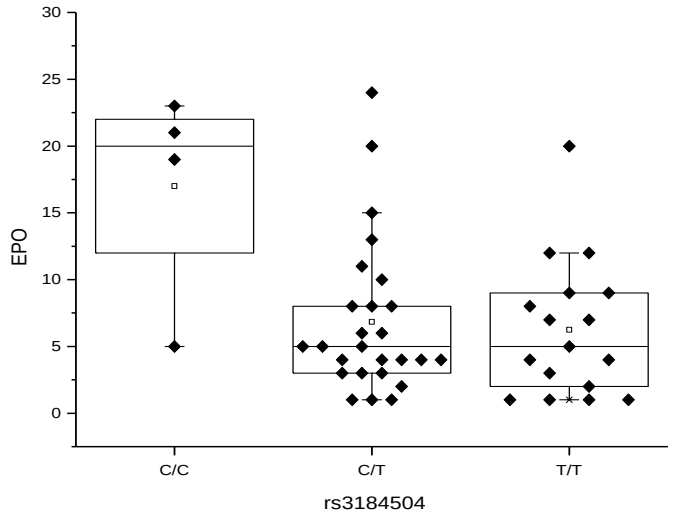


Fig 19 – Association between rs3184504 and EPO

In PV patients genotype of rs11065987, rs11066301 and rs3184504 doesn't correlate with increased incidence of major thrombosis, thrombosis district, microvascular alterations. Subsequently we analysed the post-PV myelofibrosis patients cohort (Tab 20) to asses if the associations found for PV were also confirmed in this group.

Main Clinical Features of the PPV-MF group	
Gender (M)	13/27
Age at diagnosis (yrs)	61 (48-83)
WBC count (10^9/L)	15.8 (± 11.1)
Hb (g/dL)	13.1 (± 2.2)
Ht (%)	43.2 (± 6.1)
MCV (fL)	79.4 (± 14.4)
Plt (10^9/L)	414 (± 333)
LDH (UI/L)	576 (± 328)

Tab 20 – Clinical features of PPV-MF patients

rs11065987	***p value***			
	All genotypes one Vs each other	A/A Vs A/G	A/G Vs G/G	A/A Vs G/G
PLT	ns	ns	ns	ns
Hb	0.007	Ns (0.062)	0.003	ns
Htc	0.01	0.013	0.008	ns

Tab 21 – p values of association between rs11065987 and clinical features

rs11066301	***p value***			
	All genotypes one Vs each other	A/A Vs A/G	A/G Vs G/G	A/A Vs G/G
PLT	ns	Ns	ns	Ns
Hb	Ns (0.066)	Ns	0.033	Ns
Htc	0.032	ns	0.015	ns

Tab 22 – p values of association between rs11066301 and clinical features

rs3184504	***p value***			
	All genotypes one Vs each other	C/C Vs C/T	C/T Vs T/T	C/C Vs T/T
PLT	ns	Ns	ns	Ns
Hb	ns	Ns	0.034	Ns
Htc	ns	ns	ns	ns

Tab 23 – p values of association between rs3184504 and clinical features

In the group of post-PV secondary myelofibrosis we did not find any association of the minor allele of the three SNPs with platelet count (tab 21/22/23). On the contrary we evidenced that minor allele of both rs11065987 and rs11066301 are associated with increased hemoglobin and hematocrit values (tab 21/22 and fig 20/21/22/23). Interestingly this association is present only for the heterozygous genotype while the homozygous patients display lower values, more similar to that of homozygous for ancestral allele that indeed do not differ significantly. A possible explanation for this phenomenon is that patients homozygous for the association allele, due to increased

Hb and Ht values, underwent more massive phlebotomy prior to diagnosis establishment.

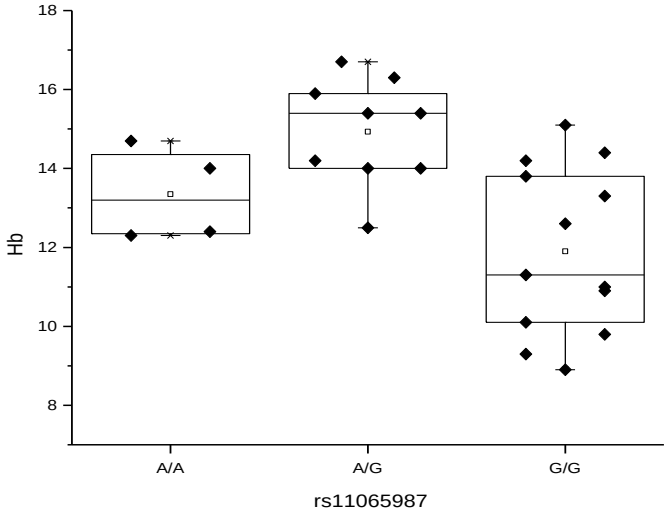


Fig 20 – Association between rs11065987 and hemoglobin

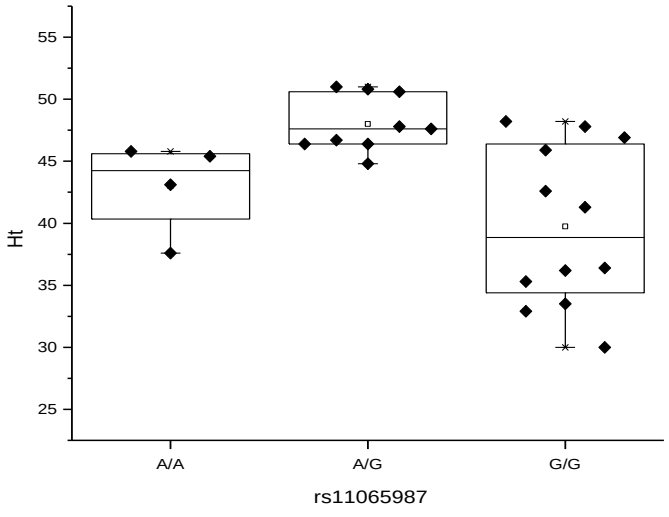


Fig 21 – Association between rs11065987 and hematocrit

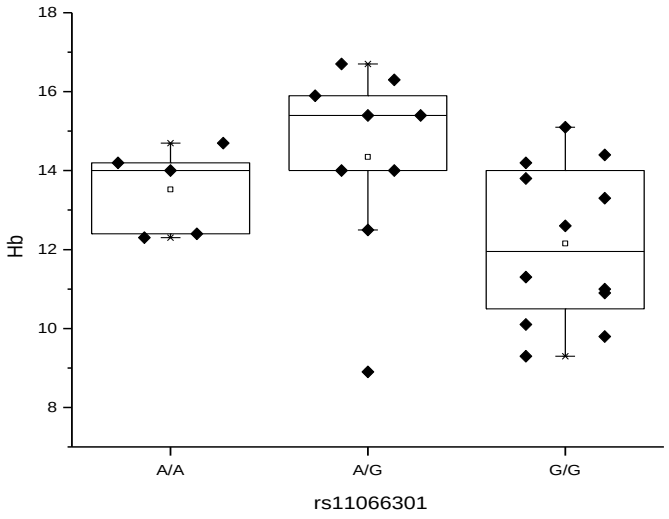


Fig 22 – Association between rs11066301 and hemoglobin

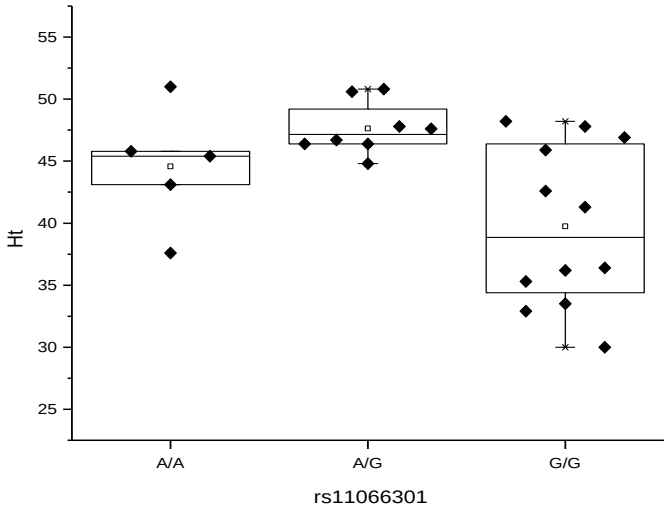


Fig 23 – Association between rs11066301 and hematocrit

Deep sequencing of chr12q24 region

The LOH that we evidenced in the 12q24 region, although it was small, contained several potential candidate genes that may have a role in MPN pathogenesis. The deep sequencing of this target region made in a PMF patient presenting the largest LOH signal, returned us a list of alterations compared to the reference sequence. LNK mutations in exons 2, 3 and 4 were previously excluded and indeed were not evidenced with deep sequencing. We decided to investigate the High Confidence variations. Alteration were filtered according to depth of sequencing and frequency of variation. All the known SNPs present either in NCBI SNP database or in 1000 genome project database were excluded from the analysis. Then variants located in intergenic regions as well as intronic variants were excluded. Subsequently, we attempted to confirm by Sanger sequencing the remaining alterations, starting from those with higher depth and frequency that we regarded as more informative. Such filtered variations were present in CUX2, C12ORF51, RPL6 and PTPN11. We screened all of them but none was confirmed by direct sequencing with the except of an heterozygous insertion of an adenine in position 111708456 of CUX2 gene. This alteration was confirmed by bidirectional sequencing but we regarded as it is not meaningful because it is located in the last position of a processed transcript that does not originate a protein product. PTPN11 gene was largely affected from variation so we sequenced a large part of the gene (exons 2/3/4/5/6/9/10/11/12/13/15) but like the other genes we could not confirm any of the listed variation. Is possible that since some of the listed variations are present at low levels they may be under the detection limit of Sanger sequencing so we plan to further analyse them with other methods such as High Resolution Melting Analysis or COLD-PCR. However further analysis of this region in a larger group of patients may possibly clarify its role in MPN pathogenesis.

LNK mutational analysis

LNK mutational analysis of exons 2, 3 and 4, encoding the PH domain of the protein, revealed 4 different mutations in 6 patients (5.3%) of the 112 analyzed. Three patients exhibited the previously described heterozygous 622G>C substitution in exon 2 leading to E208Q mutation (Fig 24).

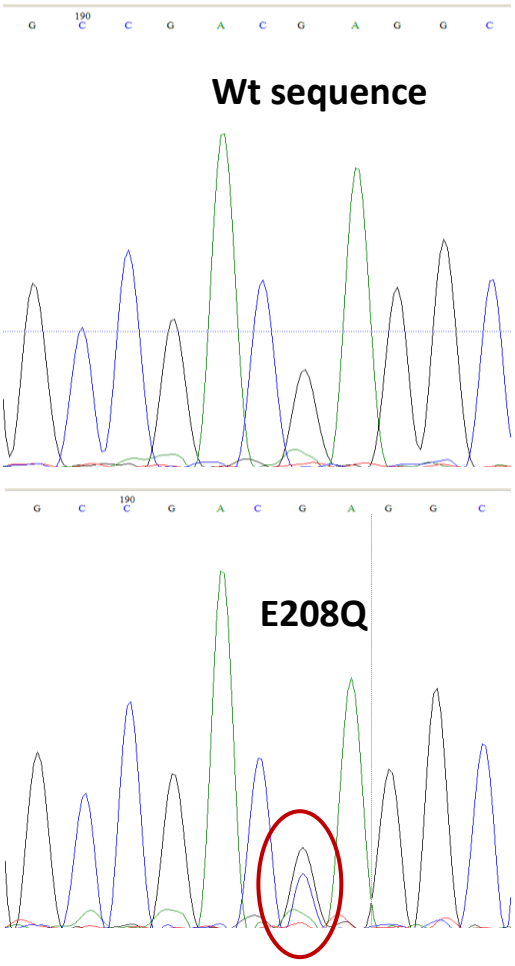


Fig 24 - E208Q mutated and corresponding wt sequence

This mutation was previously evidenced in other studies confirmed as somatic (Oh, 2010). Other two patients displayed mutations in exon 2. One consists in a heterozygous 464C>T substitution leading to P155L variation (Fig 25 left), indexed in 1000genomes Project variation database as ESP_12_111856413, with a MAF<0.01. This variant is predicted to be deleterious with SIFT software and probably damaging with PolyPhen (Fig 26). The other exon 2 mutation is a heterozygous 639C>A change causing S213R substitution (Fig 25 right). This mutation occurs at the same position of

SNP rs111360561 (S213S), for whom the A variant, displayed by our patient is predicted to be deleterious with SIFT and probably damaging with PolyPhen (Fig 26).

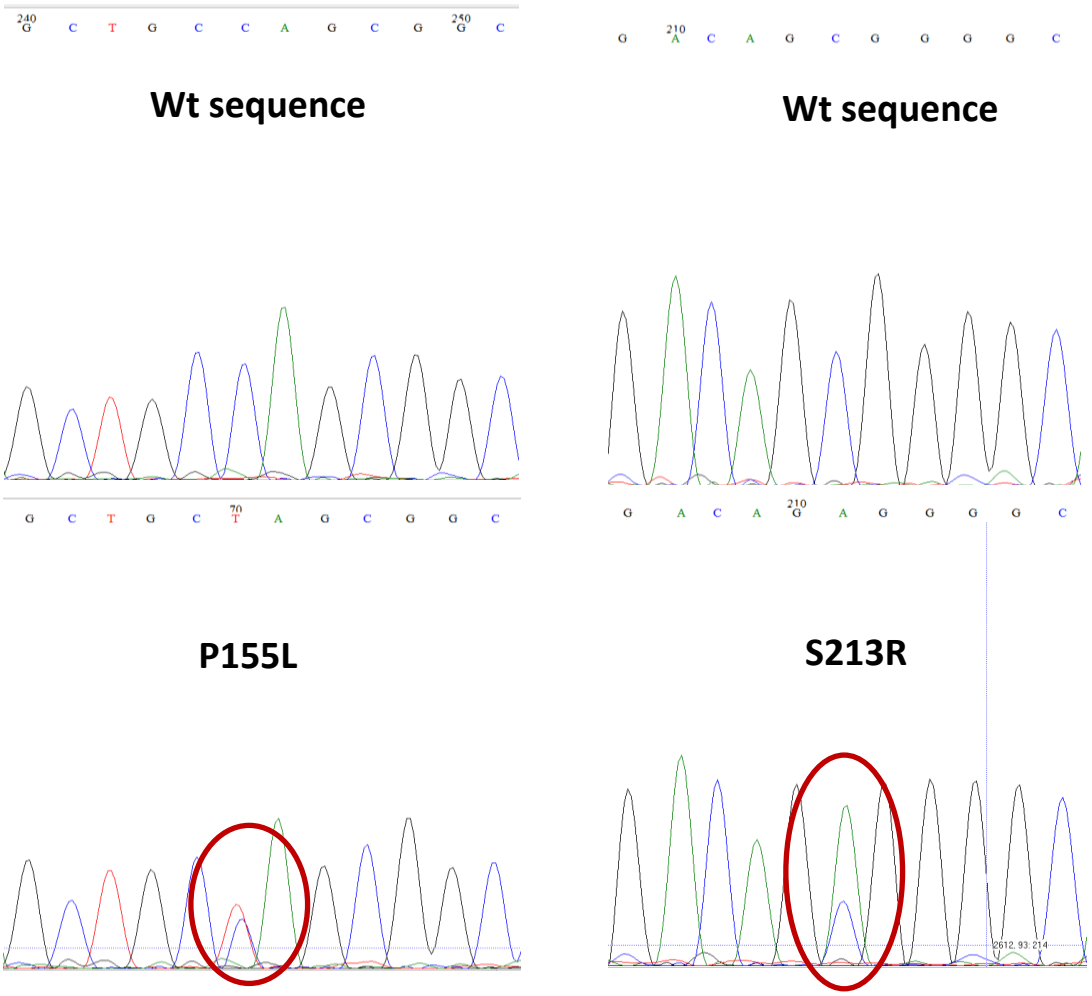


Fig 25 - P155L (left) and S213 (right) mutated and corresponding reference wt sequence

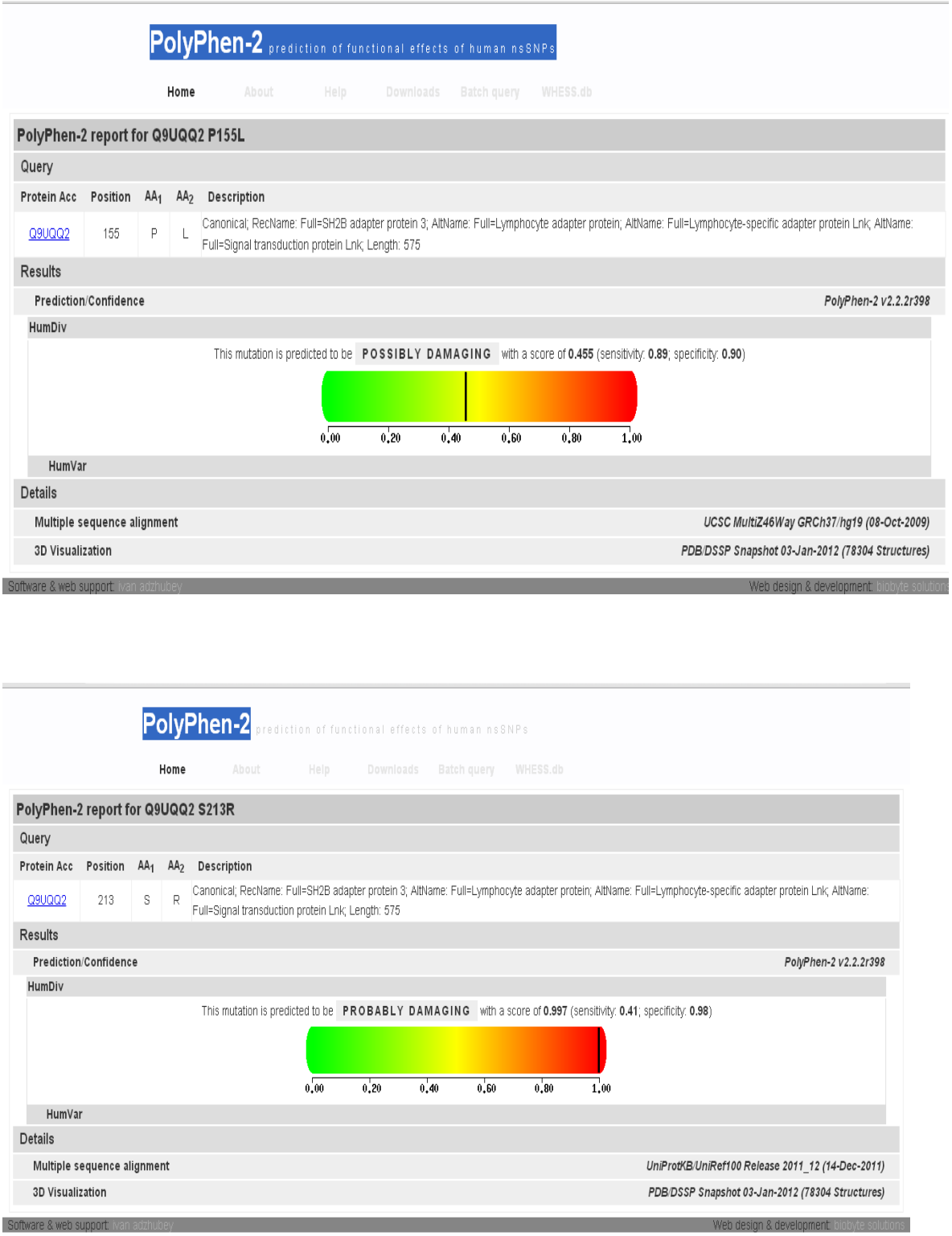


Fig 26 - Polyphen analysis of P155L (upper panel) and S213R (lower panel) mutations

The sixth patient harbored a mutation in exon 3 that was identified through HRMA. As described above, the amplicon generated for exons 3-4 HRM analysis encompasses the SNP rs3184504 so healthy donors DNA for the three genotypes was included in the reaction. HRMA profile of the mutated patient clearly differed from the curves of the

donors as shown both in the normalized graph of fluorescence versus temperature rising and in the difference graph were all the curves are normalized versus the rs3184504 T/T genotype (Fig 27). Subsequent direct sequencing confirmed the presence of a 820A>G substitution leading to T274A mutation (Fig 28).

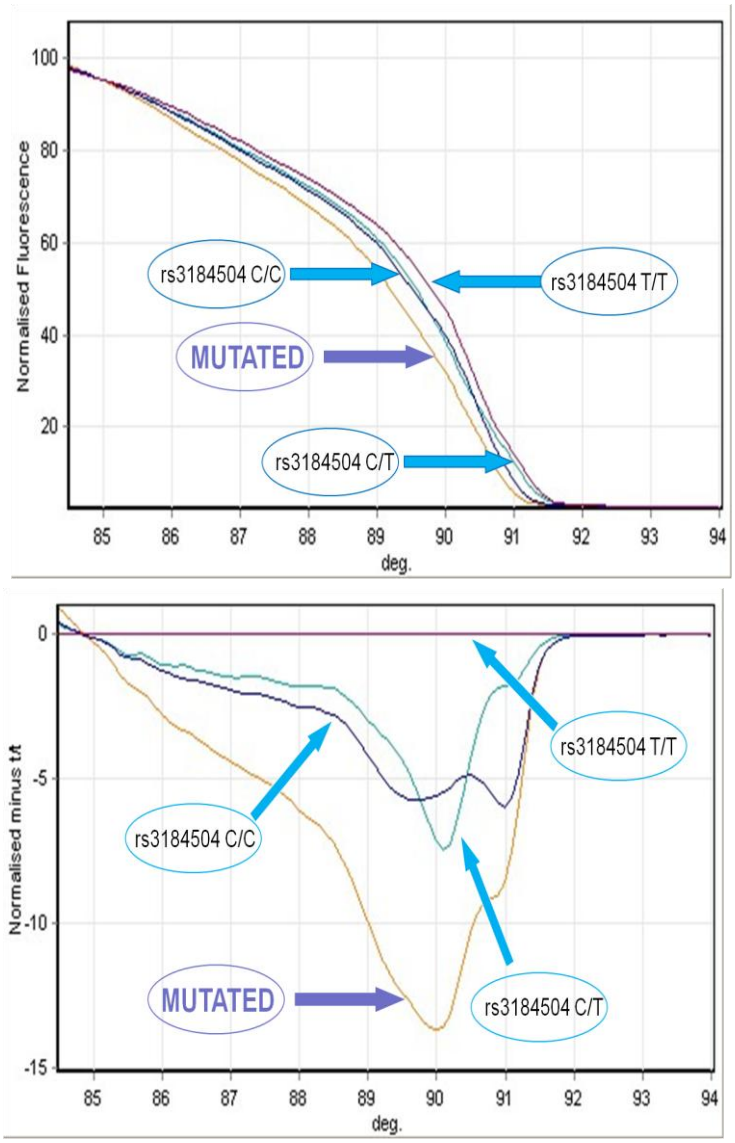


Fig 27 – HRMA profile of the mutated patients and three donors corresponding to the 3 SNP genotype (upper panel) and difference graph where the curves are normalized versus rs3184504 T/T genotype (lower panel)

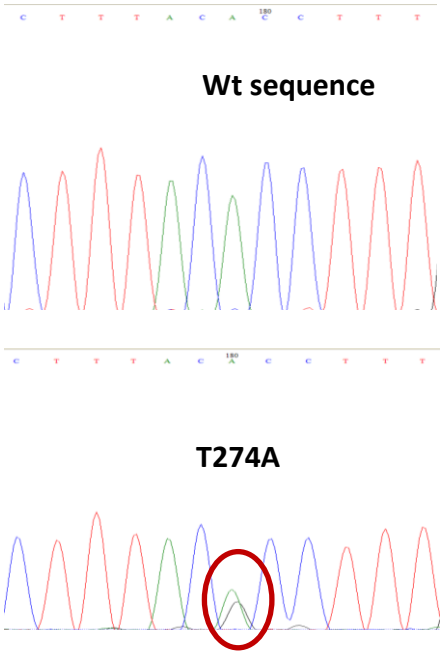


Fig 28 – T274A mutated and corresponding wt sequence

This mutation is not listed in the NCBI dbSNP or in the 1000genomes Project database and it's predicted to be damaging with SIFT and probably damaging with a score of 1.00 with PolyPhen (Fig 29)

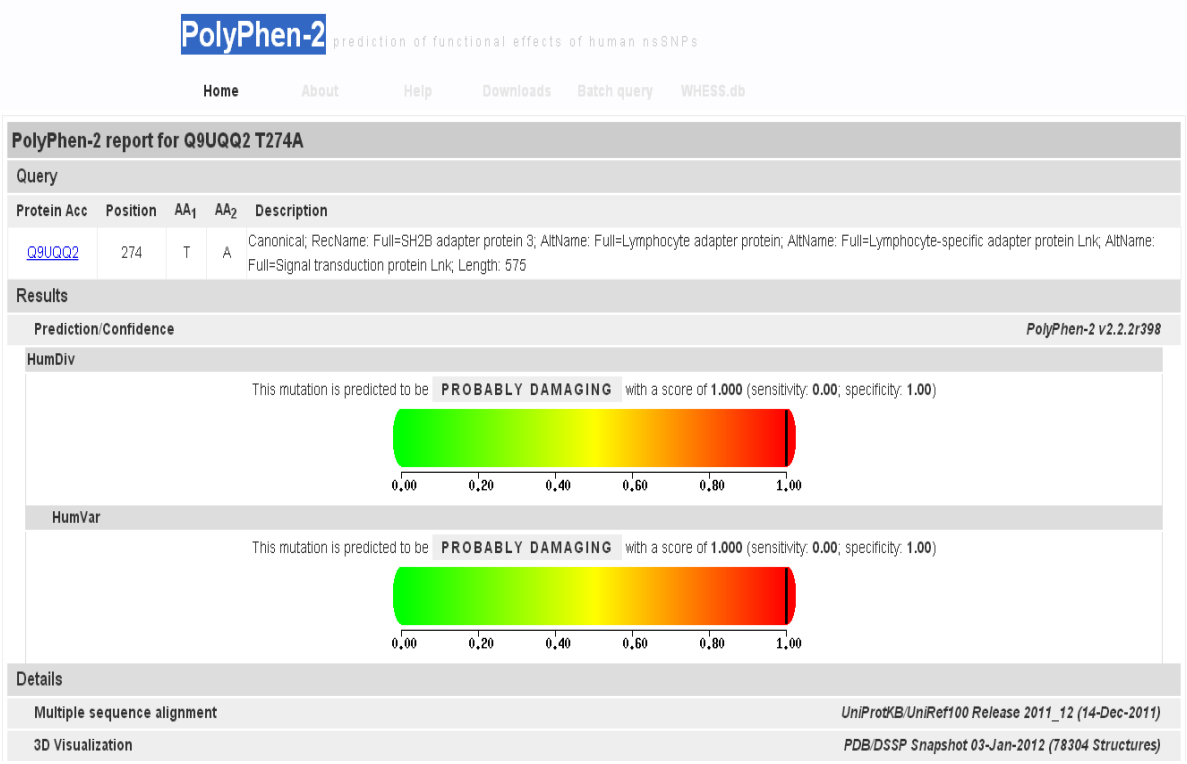


Fig 29 - Polyphen analysis of T274A mutation

We did not find mutations in exon 4. Unfortunately we could not test germline DNA to confirm newly identified mutations as somatic.

We evaluated if the presence of LNK was associated with any clinical feature. Our patients group comprised mainly males (103/112), with median age at diagnosis of 56 years (14-79), average WBC count of 7.8 10^9/L (± 2.7), Hb value of 17.9 g/dL (± 1.38), average Ht value of 52.6% (± 4), average platelet count of 235 10^9/L (± 109) and average EPO value of 10.4 mU/mL (± 6.6). All patients were confirmed as negative for JAK2 V617F and exon 12 mutations. Table 24 reports the main clinical characteristics of patients harboring LNK mutations.

Pt	LNK mutation	Age/Sex	WBC (10^9/L)	Hb (g/dL)	Ht (%)	MCV (fL)	PLT (10^9/L)	EPO (mU/mL)	JAK2
# 1	E208Q	79 (F)	4.81	15.9	47.6	93	136	20.5	WT
# 2	E208Q	52 (M)	9.57	20.9	63.3	101	210	15.1	WT
# 3	E208Q	52 (M)	6.2	17.3	51.9	88	215	11.3	WT
# 4	P155L	59 (M)	6.76	16.7	52.7	91	187	11.4	WT
# 5	S213R	66 (M)	11.30	17	50.4	94	160	4	WT
#6	T274A	74 (M)	5.30	18.1	53.9	89	181	18	WT

Tab 24 – Clinical features of LNK mutated patients

When evaluating if LNK mutation was associated with any clinical feature we found that mutated patients display significantly lower platelet count compared to non-mutated cases (181.5 Vs 238.4, p=0.04) (Fig 30). Since LNK is a negative regulator of TPO signalling through MPL and its mutations have been shown to disrupt protein function this association is quite surprising. Nevertheless it is somehow consistent with our findings on minor allele of rs11066301 being associated at least in PV patients with decreased platelet count. Although our data are in contrast with literature supporting a role for these SNP in increasing platelets in general population, both strongly suggest that 12q24 region is involved in regulation of platelet production.

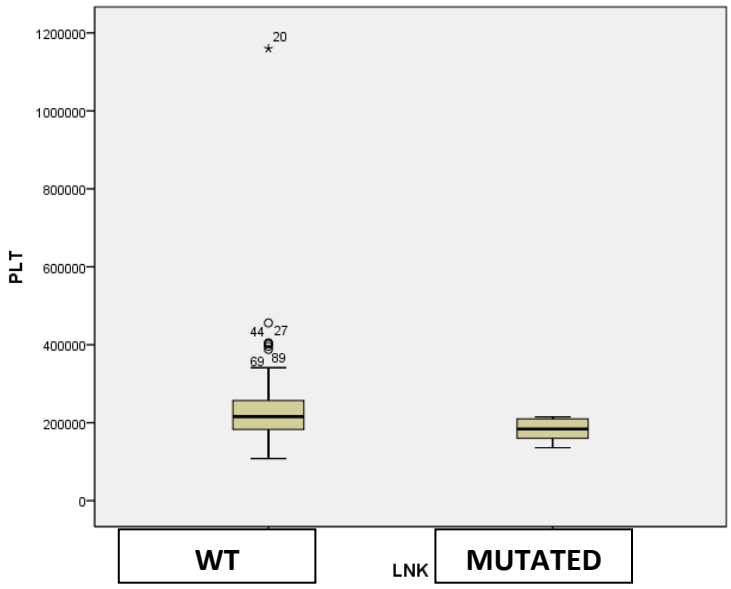


Fig 30 – Plot showing association of LNK mutation with decreased platelet count

We failed to identify any statistically significant association of LNK mutation with other clinical parameters such as WBC, Hb, Ht, EPO MCV, LDH, CD34+ cells count and red cell mass. Likewise we couldn't find any difference in LNK mutated group compared to non-mutated, regarding the incidence of splenomegaly, constitutional symptoms, thrombotic or hemorragic events and microvascular involvement. This lack of association, except for platelet count, is probably due to the small number of mutated cases identified. Further studies are needed to confirm the role of LNK mutations in reducing platelet count. Nevertheless our data confirm in a larger cohort previous reports of LNK mutations in idiopathic erythrocytosis and contribute to clarify their role in affecting disease phenotype. LNK mutational analysis might thus be useful to reveal underlying clonal disease in patients that are negative for others more frequent mutations occurring in PV.

Study of 14-3-3 proteins

Hyperexpression of 14-3-3 Beta and 14-3-3 Zeta has been reported in cases of solid cancer such as breast and lung cancer. To support the possible role of these proteins in the pathogenesis of myeloproliferative neoplasms we made two hypotheses. YWHAB that encodes for 14-3-3 beta is located in 20q13.12 region. Gain of 20q13 has been reported in 19% of chronic lymphocytic leukemia (Rodrìguez, 2012). For 14-3-3 Beta we hypotized that its hyperexpression, possibly due to copy number variation or altered methylation pattern, may affect JAK2/STAT pathway through enhanced inhibition of LNK. In this scenario LNK inhibition of JAK2 would be strongly decreased resulting in proliferative advantage in the absence of JAK2 activating mutations. YWHAZ that encodes for 14-3-3 zeta is located in 8q23 region. Hyperexpression of YWHAZ could be as well caused by copy number variation such as chromosome 8 trisomy that is frequent in MPN and gain of 8q that has been reported in AML (Batanian, 2001). A possible role for 14-3-3 zeta hyperexpression in MPN pathogenesis might be explained as for 14-3-3 beta, with enhanced inhibition of LNK. Nevertheless since 14-3-3 Zeta is a target of mir-451 we may also hypothesize that its hyperexpression could be caused by downregulation of mir-451. Down-regulation of mir-451 has been associated with worse prognosis in cases of gastric cancer (Bandres, 2009) and with relapse in head and neck carcinoma (Hui, 2010). Increased levels of mir-451 have been demonstrated during normal erythroid differentiation (Bruchova, 2007) while downregulation of this microRNA has been involved in childhood pre-B-ALL (Ju, 2009). Furthermore mir-451 targets the 3' UTR of BCR/ABL transcript and can impair BCR/ABL protein production (Iraci, 2009). In another study mir-451 was found to be downregulated in CML at the time of diagnosis (Dg) and in case of hematological relapse (Hr) compared to cases in major molecular response (MMR) or suboptimal response (SR). In CML levels of BCR/ABL transcript inversely correlates with mir-451 expression in Dg, Hr and MMR, but not in SR. In vitro cultures demonstrated that mir451 is downregulated in leukocytes of CML compared to donors leukocytes and that its levels increase after culturing with Imatinib in CML sample but not in healthy donor samples. These data suggest that there may exist a reciprocal regulatory loop between mir-451 and BCR/ABL (Lopotova, 2011). As previously reported in mice lacking mir-451, 14-3-3 zeta accumulates thus enhancing relocalization of FoxO3

outside the nucleus (Yu, 2010). Thereafter we hypothesized that also in myeloproliferative neoplasms hyperexpression of 14-3-3 zeta may lead to impairment of FoxO3 that is a proapoptotic transcriptional factor thus being responsible for decreased apoptosis rate and increased survival of malignant cells. A recent study demonstrated that FoxO3 -/- mice develop a myeloproliferative disorder with increased WBC, augmented circulating neutrophils and monocytes and reduction of lymphocytes. Moreover FoxO3 -/- mice display splenomegaly and extramedullary hematopoiesis. Progenitor compartment is increased in bone marrow, spleen and peripheral blood and is hypersensitive to cytokines. Furthermore FoxO3 -/- hematopoietic progenitors exhibited increased amount of intracellular ROS that lead to enhanced AKT/mTOR pathway activation in response to IL-3 and Epo. Interestingly FoxO3 -/- mice display significant reduction of LNK expression, due to ROS accumulation, that in turn enhances Akt/mTOR signalling (Yalcin, 2010).

In an explorative cohort, gene expression assay of YWHAZ (encoding 14-3-3 zeta) and YWHAB (14-3-3 beta) did not reveal statistically significant differences in the expression of both these genes in any of the patients group compared to healthy controls. Nevertheless we are planning further studies on larger number of patients to address our hypothesis. Our interest particularly concerns JAK2-mutations negative cases of ET and PMF as well as idiopathic erythrocytosis. While in ET and PMF altered 14-3-3 proteins expression may be responsible for phenotype modulation, in case of idiopathic erythrocytosis we speculate that altered expression of 14-3-3 proteins may contribute, either through deregulation of LNK/JAK2 pathway or through enhanced survival due to reduced FoxO3 activity, to the pathogenesis of a clonal disease that may be otherwise undetected.

CONCLUSIONS

The study of abnormalities affecting the JAK2 gene in patients with Chronic Myelogenous Leukemia dealed with two aspects: the evaluation of JAK2 46/1 haplotype as a possible risk factor for CML development and the assessment, on a large cohort, of the previously reported concomitant occurrence of JAK2 V617F mutation with BCR/ABL rearrangement. In our cohort of CML patients 8 of 314 (2.55%) were found to harbor JAK2 V617F mutation in addition to BCR/ABL fusion gene. One of the patients had a previous ten years history of PV treated with HU before the establishment of CML diagnosis. Another patient was diagnosed with CML and achieved hematologic and cytogenetic response under interferon treatment. Due to persistent thrombocytosis he shifted to Imatinib that led him to MMR but did not affect thrombocytosis. This behavior is consistent with the hypothesis of two mutated clones that competitively proliferate. All the other patients had no features of Ph-negative MPN. The role of JAK2 V617F in CML patients is still unclear since it does not seem to influence disease phenotype by giving specific features of a Ph-negative MPN. Nevertheless it could be appropriate to evaluate JAK2 mutational status in selected cases with proven CML. The occurrence of double JAK2 and BCR/ABL mutated cases and the reported increased risk of MPN relatives to develop CML, led us to hypothesize that CML and Philadelphia negative MPN could share common genetic predisposition factors. We attempted to evaluate if the recently discovered JAK2 46/1 haplotype may be a risk factor for CML development as it is for MPN. We therefore screened 84 CML patients by genotyping 46/1-tagging SNP rs12343867. We evidenced that 46/1 haplotype distribution in CML patients is similar to that of healthy controls. Our findings thus exclude a role for 46/1 haplotype in CML predisposition.

A recent study of GWA evidenced an haplotype on chromosome 12 in the q24 region associated in the general population with increased risk of CAD and increased platelet count. Since the most common cause of death in MPN patients are thrombotic events we asked whether the frequency of this haplotype could be higher in these patients. We decided to analyze three SNPs: rs11065987, located in an intergenic region between ATXN2 and SH2B3 gene, rs11066301, located in PTPN11 and rs3184504 located in SH2B3. 12q24 haplotype has been previously shown to associate

also with Ht and Hb values while rs11065987 is associated with Ht and rs3184504 has been shown to influence eosinophil count. Furthermore our interest in this region increased after a SNP array analysis performed by us in a group of MPN patients evidenced a small LOH region on chr12q24. Moreover some of the genes located in this region may have a possible role in hematopoiesis. We analyzed 210 patients with MPN and 95 healthy controls. As expected, results are comparable for the three SNPs and show their significant association with MPN diagnosis. Minor allele frequency of each SNP was indeed significantly higher in MPN compared with donors (rs11065987 MAF 0.54 Vs 0.44 p=0.018; rs11066301 MAF 0.54 Vs 0.44 p=0.022; rs3184504 MAF 0.58 Vs 0.49 p=0.014). This association is accounted for by the group of JAK2 V617F MPN and in particular by PV (rs11065987 MAF 0.58 Vs 0.44 p=0.02; rs11066301 MAF 0.59 Vs 0.44 p=0.0087; rs3184504 MAF 0.65 Vs 0.49 p=0.0026) and PPV-MF group (rs11065987 MAF 0.67 Vs 0.44 p=0.005; rs11066301 MAF 0.63 Vs 0.44 p=0.02; rs3184504 MAF 0.66 Vs 0.49 p=0.025). These data suggest that 12q24 may be a genetic risk factor for MPN development. The association is provided by PV and PPV-MF patients and since they are V617F positive in 95% of cases we suggest that this haplotype associates with JAK2 mutation. A possible explanation for such association might be the same “fertile ground hypothesis” that supported the association of JAK2 46/1 haplotype with MPL mutations, that is a relation between 12q24 haplotype and an unknown functional variant that together with V617F mutation increases MPN susceptibility. We then attempted to verify if 12q24 haplotype influenced any clinical feature of PV or PPV-MF patients. Patients were divided according to the presence of zero, one or both minor allele for the three SNPs. In PV statistical analysis revealed lack of association with platelet count for minor allele of rs11065987 and rs3184504. This association occurs for rs11066301 but, in contrast to literature data showing minor allele to be associated with increased platelet count, our data demonstrate that a single copy of minor allele significantly decreases PLT count. Regarding hematocrit we found that minor allele of rs11066301 and not rs11065987 as reported in literature is associated with Ht value. We also evidenced a new association of the minor allele of each SNP with lower EPO value. The same analysis was done in PPV-MF patients. In this group we did not find any association between minor allele of the three SNPs and platelet count. We evidenced that minor allele of rs11065987 and rs11066301 are

associated with increased Ht and Hb values. Interestingly this association is present just for the heterozygous genotype. A possible explanation for this phenomenon is that patients homozygous for the association allele, due to increased Hb and Ht values, underwent more massive phlebotomy prior to diagnosis establishment. To further evaluate this region we performed a deep sequencing of the patient harboring the largest LOH of the 12q24 region evidenced by SNP array. We analyzed the High Confidence variation and excluded the known SNPs listed in NCBI SNP database or 1000 Genome Project variation database and variations located in intergenic or non-coding regions. Subsequently, we attempted to confirm by Sanger sequencing the remaining alterations that were in CUX2, C12ORF51, RPL6 and PTPN11 genes. We failed to confirm any of these variations with the except of an heterozygous insertion in CUX2 gene that we regarded as not meaningful because it is located in the last position of a processed transcript that does not originate a protein product. PTPN11 gene, encoding a tyrosine phosphatase that is frequently mutated in juvenile myelomonocitic leukemia and in rare cases of myelodisplastic syndromes and acute myeloid leukemia, was largely affected by variation. Like other genes we could not confirm any of its variations. Is possible that since some of the listed variations are present at low levels they may be under the detection limit of Sanger sequencing so we plan to further analyse them with other methods such as High Resolution Melting Analysis or COLD-PCR.

The 12q24 region contains SH2B3 (LNK) gene that encodes for an adaptor protein. LNK is highly expressed in hematopoietic stem and progenitor cells where it acts as negative regulator of cytokine signalling. LNK has been shown to negatively regulate the TPO/MPL pathway thus affecting megakaryopoiesis and platelets production. The inhibition occurs through the binding, via SH2 domain, of the three main effectors of downstream MPL signalling: JAK2/STAT, MAPK and AKT. LNK similarly regulates erythropoiesis by inhibiting downstream EPO receptor signalling. Recently LNK mutations have been reported in patients with Myeloproliferative Neoplasms, mainly affecting PH and SH2 domains thus determining loss of protein function. LNK mutations were also found in patients with Idiopathic Erythrocytosis. We analyzed LNK exons 2, 3 and 4, encoding the PH domain, in 112 patients with Idiopathic Erythrocytosis and we evidenced 4 different mutations in 6 patients (5.3%). Three

patients exhibited the previously described E208Q mutation, in exon 2, that in a previous study was shown to strongly impair LNK function. Other two patients display mutations in exon 2. One is the P155L variation, indexed in 1000genomes Project variation database as ESP_12_111856413, with a MAF<0.01 that is predicted to be deleterious with SIFT software and probably damaging with PolyPhen. The other exon 2 mutation is the S213R substitution that occurs at the same position of SNP rs111360561 (S213S), for whom the A variant, displayed by our patient is predicted to be deleterious with SIFT and probably damaging with PolyPhen. The sixth patient harbors a mutation in exon 3, evidenced by High Resolution Melting Analysis and subsequently confirmed by direct sequencing as T274A. This mutation is not listed in the NCBI dbSNP or in the 1000genomes Project database and it is predicted to be damaging with SIFT and probably damaging with a score of 1.00 with PolyPhen. When evaluating the possible impact of LNK mutation on clinical features we evidenced that mutated patients display significantly lower platelet count compared to non-mutated cases (181.5 Vs 238.4, p=0.04). This association is quite surprising considering that LNK is a negative regulator of TPO signalling through MPL and its mutations have been shown to disrupt protein function. Nevertheless this observation is consistent with our findings on minor allele of rs11066301 being associated at least in PV patients with decreased platelet count. Although our data are in contrast with literature supporting a role for these SNP in increasing platelets in general population, both these findings strongly suggest that 12q24 region is involved in regulation of platelet production. We did not observe any other association between LNK mutation and clinical phenotype but we hypotize that this lack of association is probably due to the small number of mutated cases identified. Further studies are needed to confirm the role of LNK mutations in reducing platelet count. Our data confirm in a larger cohort previous reports of LNK mutations in idiopathic erythrocytosis and clarify their role in affecting disease phenotype. LNK mutational analysis might thus be useful to reveal underlying clonal disease in patients that are negative for others more frequent mutations occurring in PV.

14-3-3 proteins are a family of seven isoforms that bind several molecules such as kinases, phosphatase, receptors and transcriptional factors thus been implicated in many biological processes. With the except of 14-3-3 sigma, the other isoforms are

suggested to be oncogenes. Indeed up regulation of 14-3-3 proteins has been reported in solid cancer and often correlated with poor prognosis. 14-3-3 zeta mRNA has been demonstrated to be a target of mir-451 that is involved in erythroid differentiation. Mice lacking mir-451 display enhanced expression of 14-3-3 zeta that in turn determines relocalization of transcriptional factor FoxO3 in the cytoplasm thus avoiding its pro-apoptotic activity. Recently LNK has been shown to be a target of 14-3-3 proteins in mice. 14-3-3 inhibit LNK activity on JAK2 by preventing LNK/JAK2 interaction partially through LNK sequestration in the cytoplasm. We analyzed the expression of 14-3-3 zeta and beta in a small cohort of MPN patient; we made two hypotheses concerning possible role of these proteins in the pathogenesis of myeloproliferative neoplasms. Since 14-3-3 beta is encoded by YWHAB gene located on chromosome 20q13.12, we hypothesized that hyperexpression of 14-3-3 beta, possibly due to copy number variation or altered methylation pattern, may affect JAK2/STAT pathway through enhanced inhibition of LNK that would result in decreased inhibition of JAK2. This alteration would confer a strong proliferative advantage in the absence of JAK2 activating mutations. 14-3-3 zeta maps on chromosome 8q23. A possible role for 14-3-3 zeta hyperexpression in MPN pathogenesis might be explained as for 14-3-3 beta, with enhanced inhibition of LNK. Nonetheless since 14-3-3 zeta is a target of mir-451 we may also hypothesize that its hyperexpression could be a consequence of mir-451 downregulation. Downregulation of mir-451 was reported in hematological neoplasms such as childhood ALL and CML. In our hypothesis reduced expression of mir-451 would lead to accumulation of 14-3-3 zeta that would consequently determine enhanced relocalization of FoxO3 outside the nucleus. This mechanism would be responsible for reduced apoptosis and enhanced survival of malignant cell. However, n our explorative cohort of MPN patients we did not reveal statistically significant increase in the expression of 14-3-3 zeta and beta. Nevertheless we intend to conduct further study to possibly support our hypothesis. Our interest concerns JAK2 mutations negative ET and PMF cases in which 14-3-3 upregulation may modulate disease phenotype and Idiopathic Erythrocytosis for whom altered expression of these proteins may contribute to the pathogenesis of an otherwise undetected clonal disease.

In conclusion, the information provided in this work add to the growing body of

knowledge about the inherited genetic background on which myeloproliferative neoplasms, that are characterized by an incredible somatic genetic complexity., arise. These information could be of value in understanding the mechanisms underlying the heterogeneous clinical presentation and clinical course, and by identify predisposition and phenotype-modifiying alleles could be of importance for patient counseling and management.

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PUBLICATIONS

- Pieri L, **Spolverini A**, Scappini B, Occhini U, Birtolo S, Bosi A, Albano F, Fava C, Vannucchi AM*Concomitant occurence of BCR_ABL and JAK2V617F mutation* Blood. 2011 Sep 22;118(12):3445-6.
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