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Study of new molecular alterations on philadelphia-negative chronic myeloproliferative neoplasms

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INDEX

INTRODUCTION

MYELOPROLIFERATIVE NEOPLASMS

The term ”myeloproliferative disorders (MPD)” was introduced in 1951 by William Dameshek(1), and was intended to encompass polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myelogenous leukemia (CML), and Di Guglielmo’s syndrome (erythroleukemia). His proposal was based on the observation of similarities in their clinical phenotype, and on the hypothesis that a generalized proliferation of bone marrow cells, due to some unknown stimuli, was the underlying cause. In the 1960, the discovery of the Philadelphia (Ph1)-chromosome as the cytogenetic alteration of CML led to the recognition of that disease as a distinct entity(2). Moreover, also erythroleukemia was kept separated from the others because it was recognized as a variant of acute myeloid leukemia (AML). PV, ET and PMF were so defined as the three “classic” Ph1-negative MPD(3). The 3 forms share several clinical characteristics, such as clonal hematopoiesis, bone marrow hypercellularity, except in advanced stages of myelofibrosis, a propensity to thrombosis and hemorrhage, the tendency to produce extramedullary hematopoiesis and to variably evolve to acute leukemia. Distinct hallmarks include elevated erythrocyte counts in PV, elevated platelet levels in ET, and bone marrow fibrosis in PMF.(4)

Classic MPNs, yet considered rare disorders, are among the most frequent hematologic neoplasms, usually affecting the adult elderly population. A recent study(5), based on the North American Association of Central Cancer Registries (NAACCR) encompassing 82% of total US population, reported an average 2001-2003 annual age-adjusted incidence rate of 2.1 per 100,000 and estimated that there were 6,328 new cases in the total US population in 2004. Furthermore, because of their relatively smooth clinical course, it is likely that many classic MPN cases actually go undetected or are not reported to registries. Advanced age, male sex, and white race were identified as risk factors. Among individuals aged 80 years or older, the rate was as high as 13.3 per 100,000. Familial

clustering of these disorders is known, and supported by a large study recently completed in Sweden.(6) Relatives of patients with MPN had a 5.7 relative risk (RR) of having PV, an RR 7.4 for ET, and an RR of 7.5 for unclassified forms of MPN, together with a borderline increased RR of CML.

The clinical presentation and outcome of familial MPN patients have been suggested to be different from sporadic MPN patients in studies based on small numbers. For example, homozygosity for *JAK2* (V617F) has been reported to identify a higher risk of disease evolution(7). The coexistence of different clinical entities and of *JAK2*V617F-positive and *JAK2*V617F-negative diseases in the same family is noteworthy. However, both this study and another larger study of MPN patients(8) did not find differences in clinical presentation between familial and sporadic cases. The higher risk observed among siblings would suggest a model of recessive inheritance, although whether the phenomenon of anticipation is present or not is debated(6, 8).

CLASSIFICATION OF MYELOPROLIFERATIVE NEOPLASMS

The first systematic attempt to classify MPD and MPD-like clinicopathologic entities was undertaken by the World Health Organization (WHO) committee for the classification of hematologic malignancies(9). According to the 2001 WHO classification system, CML, PV, ET, and PMF were included under the category of "chronic myeloproliferative diseases" (CMPD). The CMPD category also included other "nonclassic" MPD-like disorders such as chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia/hypereosinophilic syndrome (CEL/HES), and "unclassified CMPD." The identification of BCR-ABL as a CML-specific genetic event, in the context of CMPD, has facilitated accurate molecular diagnosis and effective targeted therapy. The lack of knowledge, until recently, on specific genetic defects in the *BCR-ABL*-negative classic CMPDs necessitated that diagnosis rested on a combination of bone marrow histology and a few clinical and laboratory findings to distinguish clonal from reactive myeloproliferation and one CMPD from another(10). In the last years fundamental advances have been done in understanding the molecular pathogenesis of classic *BCR/ABL*-negative CMPD, capped by the discovery of specific molecular abnormalities associated with PV, ET, and PMF. As a result, WHO diagnostic

criteria have been revised, and the term “CMPD” was changed to “myeloproliferative neoplasms (MPN).”(11) The 2008 WHO classification for myeloid neoplasms, which incorporates novel information derived from molecular discoveries in *BCR-ABL* negative “classic” myeloproliferative states and clonal eosinophilic disorders, includes five major entities **(Table 1):** the Acute Myeloid Leukemia (AML) and the Myelodysplastic Syndromes (MDS) with their different subtypes, the Myeloproliferative Neoplasms (MPN), the category of overlapping Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN) and the Myeloid Neoplasms associated with eosinophilia and specific molecular abnormalities. A formal but important modification in the 2008 WHO classification has been the substitution of the attribute “neoplasm” for “disease”. The neoplastic nature of these disorders has been thus formalized , based on information arising from the analysis of the X chromosome inactivation pattern in informative females and other cytogenetic and/or molecular findings in both “classic” and “nonclassic” myeloproliferative disorders(12, 13), and the finding that evolution to AML is part of their natural history(14). Until that point, there has traditionally been a poor interest in these neoplasms by cancer surveillance programs, agencies granting research support, or pharmaceutical companies. The four “classic” MPNs (ie, CML, PV, ET, and PMF) should be distinguished from the other “nonclassic” MPNs, which include chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), systemic mastocytosis (SM), and unclassifiable forms of MPN(15). One important step towards the understanding of these disorders was the discovery of recurrent somatic mutations in two genes, *JAK2* and MPL (16-21).

Table 1. The 2008 World Health Organization Classification for Myeloid Neoplasms.

1. Acute myeloid leukemia (AML) and related precursor neoplasms
2. Myelodysplastic syndromes (MDS)
3. Myeloproliferative neoplasms (MPN)
3.1. Chronic myelogenous leukemia (CML), BCR-ABL1 positive
3.2. Polycythemia vera (PV)
3.3. Essential thrombocythemia (ET)
3.4. Primary myelofibrosis (PMF)
3.5. Chronic neutrophilic leukemia (CNL)
3.6. Chronic eosinophilic leukemia, not otherwise classified (CEL-NOS)
3.7 Hypereosinophilic syndrome
3.8. Mast cells disease
3.9. Myeloproliferative neoplasm, unclassifiable (MPN-u)
4. Myelodysplastic/Myeloproliferative neoplasms (MDS/MPN)
4.1. Chronic myelomonocytic leukemia (CMML)
4.2. Juvenile myelomonocytic leukemia (JMML)
4.3 Atypical chronic myeloid leukemia, BCR-ABL1 negative
4.4. Myelodysplastic/myeloproliferative neoplasm, unclassifiable
4.5. Refractory anemia with ring sideroblasts associated with marked thrombocytosis
5. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB, or FGFR1
5.1. Myeloid neoplasms associated with PDGFRA rearrangement
5.2. Myeloid neoplasms associated with PDGFRB rearrangement
5.3. Myeloid and lymphoid neoplasms with FGFR1 abnormalities (8p11 myeloproliferative syndrome)

DIAGNOSIS

An important new concept introduced by 2008 WHO diagnostic criteria is the integration of histopathologic, clinico-laboratory and molecular information(22); accordingly, tests for *JAK2* or *MPL* mutation have become a standard tool in the diagnostic work up of MPN(23). Detection of one of these mutations unequivocally establishes by itself the presence of a clonal MPN and rules out the possibility of reactive erythrocytosis, thrombocytosis, or myelofibrosis. Unfortunately, they are of no help in distinguishing among the different forms of MPNs, although *JAK2* exon12 mutations have not yet been reported outside PV, and no patient with PV has been found to harbor an *MPL* mutation. In patients with evidence of increased red cell mass, according to WHO criteria, demonstration of *JAK2*V617F mutation allows a diagnosis in greater than 95% of cases, as less than 2% of PV patients harbor *JAK2* exon 12 abnormalities(24). It is debated whether a diagnosis of PV can still be tenable in the absence of *JAK2* mutation(25, 26). The compelling criterion for a diagnosis of ET is a sustained platelet count of greater than 450 x 10^9/L. Notably, this value is lower than the one originally used by the 2001 WHO classification system (600 x 10^9/L),(9) because the latter might have led to inadvertently overlooking classic ET cases with a lower platelet count. This assumption is supported by the discovery of the *JAK2*V617F mutation in some subjects who have a platelet count lower than 600 x 10^9/L.(27) Diagnosis of ET requires exclusion of reactive thrombocytosis(28) as well as of other MPNs that present with thrombocytosis. In particular, exclusion of CML with FISH or PCR analysis for *BCR-ABL* rearrangement is mandatory. Because positivity for *JAK2*V617F or *MPL* mutation cumulatively account for 60% to 70% of ET cases, the assessment of bone marrow morphology remains key to the diagnosis of ET. Bone marrow cellularity in ET is normal or slightly increased, with abundance of large, mature-appearing megakaryocytes devoid of morphological abnormalities and generally dispersed throughout the biopsy. This appearance is distinct from both the panmyelosis typical of PV or the predominant granulocytic hyperplasia with highly bizarre megakaryocytes, often found in abnormally tight clusters, with aberrant nuclear to cytoplasmic ratio and hyperchromatic, bulbous, or irregularly folded nuclei that are found in PMF, even in initial stages without overt fibrosis.(23, 29) Bone marrow histology is required for the diagnosis of PMF. Although advanced reticulin or collagenic fibrosis is typically associated with classic stages of PMF, some degree of reticulin fibrosis

can be found as well as in PV, or more occasionally in ET. Therefore, fibrosis by itself is not synonym for PMF, and diagnosis of PMF can be made even in the absence of overt fibrosis. Also the leukoerythroblastic features of blood smears, with immature myeloid precursors, nucleated red cells, and abnormally shaped erythrocytes (tear-drop cells), is very characteristic, but not diagnostic, of PMF. CML should be ruled out through *BCR-ABL* rearrangement analysis, while finding a positive *JAK2*V617F or *MPL* mutation allows exclusion of reactive forms of myelofibrosis (such as in infectious or inflammatory processes, metastatic cancer, and lymphoid disorders). Anemia, palpable splenomegaly, and raised lactate dehydrogenase levels are additional diagnostic criteria.(11) **(Table 2)**

Table 2. 2008 WHO Diagnostic Criteria for "classic" MPN.

WHO indicates World Health Organization; MPN, myeloproliferative neoplasm; CML, *BCR-ABL1* chronic myelogenous leukemia; PV, polycythemia vera; PMF, primary myelofibrosis; MDS, myelodysplastic syndrome; BM, bone marrow biopsy specimen; Epo, erythropoietin; EEC, endogenous erythroid colonies; LDH, lactate dehydrogenase.

CRITERIA	POLYCYTHEMIA VERA	ESSENTIAL THROMBOCYTHEMIA	PRIMARY MYELOFIBROSIS
Major criteria	**1.** Hgb >18.5 g/dL (men) or >16.5 g/dL (women) **or** Hgb or Hct > 99th percentile of reference range for age, sex, or altitude of residence **or** Hgb >17 g/dL (men) or >15 g/dL (women) if associated with a documented and sustained increase of >2 g/dL from baseline that cannot be attributed to correction of iron deficiency **or** elevated red cell mass >25% above mean normal predicted value **2.** Presence of JAK2V617F or similar mutation	**1.** Sustained platelet count >450 x 109/L **2.** BM showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes. No significant increase or left-shift of neutrophil granulopoiesis or erythropoiesis **3.** Not meeting the WHO criteria for PV, PMF, CML, or MDS or other myeloid neoplasm **4.** Demonstration of JAK2V617F or other clonal marker **or** no evidence of reactive thrombocytosis	**1.** Megakaryocyte proliferation and atypia* accompanied by either reticulin and/or collagen fibrosis **or** In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (ie, pre-fibrotic cellular-phase disease) **2.** Does not meet WHO criteria for CML, PV, MDS, or other myeloid neoplasm **3.** Demonstration of JAK2V617F or other clonal marker **or** no evidence of reactive marrow fibrosis
Minor criteria	**1.** BM showing hypercellularlity for age and trilineage growth (panmyelosis) **2.** Subnormal serum Epo level. **3.** EEC growth.	-	**1.** Leukoerythroblastosis. **2**. Increased serum LDH. **3.** Anemia. **4.** Palpable splenomegaly.
Diagnostic combinations	Both major criteria + 1 minor criterion **or** first major criterion + 2 minor criteria	All 4 criteria must be met.	All 3 major criteria + 2 minor criteria.

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

CLINICAL COURSE AND RISK STRATIFICATION

These disorders show a substantial phenotypic mimicry, with signs and symptoms ascribable to the abnormal myeloproliferation (abnormal blood cell counts, splenomegaly, hepatomegaly, extramedullary hematopoiesis, constitutional symptoms) and a tendency to develop thrombotic and hemorrhagic complications. Survival in ET was similar to the sex- and age-standardized European population(30). However, the results of a recent study (30) indicated the importance of a careful distinction between ET and early/prefibrotic PMF and its prognostic relevance. Although inferior to that seen in patients with ET, event-free survival was still relatively long in patients with early/prefibrotic PMF. The last one might mimic ET in the clinical presentations, but laboratory clues for considering the possibility of early/prefibrotic PMF are elevated serum LDH, anemia, leukocytosis, and increased circulating CD34+ cell count. Bone marrow (BM) morphology in ET shows no or only slight increase in age-matched cellularity , no significant increase in granulo- and erythropoiesis, prominent large to giant mature megakaryocytes with hyperlobulated or deeply folded nuclei, dispersed or loosely clustered in the marrow space and no or very rarely minor increase in reticulin . Contrary, early/prefibrotic PMF is characterized by marked increase in age-matched cellularity, pronounced proliferation of granulopoiesis and reduction of erythroid precursors, dense or loose clustering and frequent endosteal translocation of medium sized to giant megakaryocytes showing hyperchromatic, hypolobulated, bulbous, or irregularly folded nuclei and an aberrant nuclear/cytoplasmic ratio and no or no significant increase in reticulin fibers(31). Accordingly, the appearance of such characteristics during the clinical course of ET might be considered as an indication for repeating BM examination to entertain the possibility of overt fibrotic transformation. *JAK2*V617F mutational frequencies were similar between ET and early/prefibrotic PMF, although another recent study had suggested a higher mutant allele burden in the latter(32). True ET presents a less than 1% 10-year risk of leukemic or overt fibrotic transformation. These figures are notably lower than those previously reported in large retrospective studies from independent groups of investigators. For example, in a recent population-based French study of 311 patients with ET, the 10-year risk of leukemic transformation was reported as 8.3%(33); in another multicenter study of 211 patients from China, the 10-year risk of overt fibrotic transformation was reported as 9.7%(34), and the corresponding figure

from a Spanish series was 8.3%(35). Therefore, it is reasonable to assume that data from previous natural history studies in ET are confounded by the inadvertent inclusion of occurrences with early/prefibrotic PMF and do not necessarily apply to patients whose diagnosis is based on strict morphologic criteria. In fact, studies in which an attempt was made to re-review BM histopathology according to WHO criteria, reported disease complication rates were lower (36, 37): 10-year risk of leukemic and overt fibrotic transformation rates were 1.4% and 3.8%, respectively, in one such study from the Mayo Clinic(37) and 2.6% and 3.9%, respectively, in another study from Italy(36). Multivariable analyses in this study confirmed the prognostic relevance of BM histopathology for survival, leukemic transformation, and overt fibrotic progression. BM histopathology–independent risk factors for survival included older age (older than 60 years), leukocytosis (leukocyte count > 11 x 10^9/L), anemia (hemoglobin <12 g/dL), and thrombosis history. Some of these previous studies had also reported a similar adverse survival effect from tobacco use, diabetes mellitus, and male sex. Taking all together the results of all these studies, it is reasonable to conclude that risk factors as anemia, increased serum LDH, leukocytosis and extreme thrombocytosis might be markers of occult early/prefibrotic PMF, what could explain their association with inferior survival or higher risk of disease progression.

Clinical presentation of PV may be recapitulated into 3 main scenarios: diagnosis by chance (most frequent), diagnosis after a thrombotic event and diagnosis resulting from disease-related symptoms. Complete blood cell count (CBC) evaluation has a great relevance as a panmyeloproliferative pattern (erythrocytosis with leukocytosis and/or thrombocytosis) is more consistent with PV than isolated erythrocytosis. Splenomegaly is present in approximately 30% to 40% of PV, mainly when PV is pan-myeloproliferative.(38) Life expectancy of PV patients is reduced compared with that of the general population, as demonstrated in a study including 396 PV patients followed for a median time of 10 years(39) and recently confirmed(40). Mortality rate was known to be age-dependently increased in PV, being 1.6-fold and 3.3-fold higher than in the reference population in patients younger or older than 50 years, respectively(41). Moreover, recent data from 1,263 patients diagnosed according to the 2008 WHO classification have been recently evaluated by the IWG-MRT (42). Advanced age, leukocytosis, leukoerythroblastosis, history of venous thrombosis and abnormal

karyotype were all identified as unfavorable risk factors for survival, while pruritus and thrombocytosis had a favorable impact. Based on Receiver Operating Characteristic (ROC) plots and different hazard ratio-weighted adverse points for age, leukocyte count and venous thrombosis, a novel prognostic model was developed that allows excellent discrimination between high, intermediate and low risk patients; respective median survival were 8.3, 15 and 26 years. Patients' features at diagnosis included pruritus (37%), microvascular disturbances (29%), palpable splenomegaly (37%), abnormal karyotype (13%), and leukoerythroblastosis (6%). Death occurred in 228 cases (18%), leukemic progressions in 42 (3%), and fibrotic transformations in 101 (8%). Post-diagnosis arterial or venous thrombosis occurred in 139 (11%) and 85 (7%) patients, respectively.

Major causes of shortened survival in both PV and ET are represented by thrombotic events and transformation to myelofibrosis or AML, which account for 41% and 13% of total deaths among 1,638 PV patients that were included in the observational arm of the ECLAP study(43, 44). The results of a recent study in patients with polycythemia vera who received a conventional treatment (including phlebotomy, hydroxyurea, or both) show that maintaining a hematocrit target of 45 to 50% was associated with a four times greater rate of death from cardiovascular causes or major thrombosis, as compared with patients maintained at a hematocrit target of less than 45%. The incidence of the primary end point was 1.1 events per 100 patient years in the low-hematocrit group, as compared with 4.4 events per 100 patient-years in the high hematocrit group. Rates of deep-vein thrombosis and cerebral vascular events including strokes and transient ischemic attacks were increased in the high-hematocrit group, confirming the higher incidence of thrombosis observed in the European Collaboration on Low-Dose Aspirin in Polycythemia Vera (ECLAP) study(45-47). The current risk stratification of PV and ET in clinical practice is targeted at the definition of vascular events by advanced age (> 60 years of age) and history of thrombohemorrhagic events(41, 48). In the presence of either of these, a patient is at high-risk, whereas when neither of these is present, the disease is low-risk. The role of generic cardiovascular risk factors, such as hypertension, diabetes, hyperlipidemia, smoking, or genetic alterations of hemostatic factors, is still controversial; however, patients who present with any of these abnormalities are prudentially considered to belong to an intermediate-risk category(48). Studies have demonstrated that leukocytosis is an additional independent risk factor for thrombosis(37, 49, 50).

In different prognostic models powered to predict survival in ET, advanced age, leukocytosis, and anemia have been incorporated as risk factors(33, 45, 51). Recently, a prognostic score named IPSET (International Prognostic Score for Essential Thrombocythemia) was developed: by using three variables, ie age >60 years (point value=2), leukocyte count >11x10^9/L and prior history of thrombosis (point value=1 each), three different risk categories were identified: low risk (0 points), intermediate risk (1-2 points) and high risk ($\geq$3 point), showing median survival "not reached", 24.5 years and 13.8 years, respectively. The same model proved valuable also in younger patients when using age >45 years as an adverse variable. Finally, there is also evidence that *JAK2*V617F mutated status in ET(27, 52-54), and a high V617F allelic burden in both ET(27, 55) and PV(55, 56) are associated with increased risk of thrombosis. No association between the presence of the mutation and outcome was found in ET: although *JAK2* (V617F) is a risk factor for thrombosis, thrombosis accounts only for half of the causes of death; other causes have likely a lower association with the presence of the mutation(54).

Other than thrombosis, hemorrhage, evolution to post-polycythemic or post-thrombocythemic myelofibrosis, and AML transformation represent the most clinically relevant issues in the course of classic MPN.(43, 57, 58)

Transformation to post-polycythemic or postthrombocytemic myelofibrosis represents the natural evolution of PV and ET, occurring usually late in the clinical course. The estimated rate is about 5% after 15 years from diagnosis of PV,(43) whereas data are scanty in ET. Criteria for the diagnosis of evolution to myelofibrosis have recently been proposed by the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT; **Table 3**)(59).

Table 3. Criteria for Establishing the Diagnosis of Evolution to Post-polycythemic or Post-thrombocythemic Myelofibrosis According to IWG-MRT Criteria(59)

CRITERIA FOR POST-POLYCYTHEMIC MYELOFIBROSIS
Required criteria 1. Documentation of a previous diagnosis of polycythemia vera as defined by WHO criteria(22) 2. Bone marrow fibrosis grade 2-3 (according to the European classification(60)) or grade 3-4 (accordingto standard classification(61)) Additional criteria 1. Anemia (below the reference range for appropriate age, sex, and altitude considerations) or sustained loss of either phlebotomy (in the absence of cytoreductive therapy) or cytoreductive treatment requirement for erythrocytosis. 2. A leucoerythroblastic peripheral blood picture 3. Increasing splenomegaly of ≥5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly 4. Development of ≥ 1 of 3 constitutional symptoms: >10% weight loss in 6 months, night sweats, unexplained fever (>37.5°C)
CRITERIA FOR POST-THROMBOCYTHEMIC MYELOFIBROSIS
Required criteria 1. Documentation of a previous diagnosis of essential thrombocythemia as defined by WHO criteria(22) 2. Bone marrow fibrosis grade 2-3 (according to the European classification(60)) or grade 3-4 (according to standard classification(61)) Additional criteria 1. Anemia (below the reference range for appropriate age, sex, and altitude consideration) and a ≥ 20 g/L decrease from baseline hemoglobin level 2. A leucoerythroblastic peripheral blood picture. 3. Increasing splenomegaly of ≥ 5 cm (distance of the tip of the spleen from the left costal margin) or the appearance of a newly palpable splenomegaly 4. Increased LDH (above reference level) 5. Development of ≥ 1 of 3 constitutional symptoms: >10% weight loss in 6 months, night sweats, unexplained fever (>37.5°C)

Diagnosis is made on the basis of meeting all required criteria plus two additional criteria. Survival is very conceivable shortened by the development of myelofibrosis, and may be predicted by hemoglobin level and platelet and leukocyte counts according to a dynamic prognostic model developed in PV patients(62). Evolution to AML occurred in 1.3% of PV patients included in ECLAP study, at a median time of 8.4 years after diagnosis(44). The 10-year cumulative incidence of leukemic transformation in ET was 0.65%, but no risk factors were identify because of the low number of events(54). Survival is dismal, less than 6 months, although recipients of allogeneic HSCT may experience longer remission(63). Advanced age, elevated leukocyte count, and longer disease duration were factors associated with increased risk of leukemic transformation(44). An increased risk of AML was reported in patients who were treated with radioactive phosphorus or chlorambucil in the PVSG trial(64). In addition, sequential or combined use of more than one chemotherapeutic agent, including hydroxyurea (HU), significantly increased the rate of evolution to AML in PV patients in the observational arm of ECLAP study(65).

Life expectancy in PMF is 31% lower than in an age-matched and sex-matched population, with a median survival of 5 years, although younger patients may experience longer survival(41, 66). In the last years a reduction in disease-specific mortality, except in the higher-risk patients, was documented(67). Major causes of death are represented by the sequelae of portal hypertension or hepatic-splenoportal thrombosis, thromboses in various anatomic sites, heart failure due to splenic pooling, infections, pulmonary hypertension, bleeding caused by thrombocytopenia or hemostatic defects, and transformation to AML(68).

Prognostic staging systems for PMF have been developed that allow separation of patients with low-risk and high-risk disease associated with significantly different survival times. Stratification according to risk is of particular importance in younger patients who may potentially exploit the curative efficacy of allogeneic hematopoietic stem cell transplantation (HSCT).Previous studies have identified several adverse prognostic factors for survival, including advanced age, marked anemia, leukocytosis or leukopenia, abnormal karyotype, constitutional symptoms, and presence of circulating blasts(69). Based on some of these variables, different prognostic scoring systems have been proposed(70-72). One of the first used was the “Lille score”, that includes anemia and

abnormal leukocyte count as variables(72); however, it did not clearly separate intermediate- and high-risk patient groups.(69) Then, the Mayo Clinic group tried to improve the Lille score by adding thrombocytopenia (<100x10^9/L) and monocytosis (>1x10^9/L) as additional adverse risk factors(73). This resulted in better, but still suboptimal, separation of intermediate- and high-risk categories. Another scoring system by Cervantes et al,(70)applicable also to younger patients, is based on hemoglobin level and the presence or absence of constitutional symptoms and circulating blasts. However, the value of this system is limited by its ability to identify only 2 risk groups. The currently most used prognostic score was proposed by Cervantes et al on 2009, called IPSS (international prognostic scoring system) based on five parameters: age > 65 years old, constitutional symptoms, Hb < 10 g/dL, WBC count > 25 x 10^9/L and circulating blasts > 1%. (69) Based on these parameters readily available at time of diagnosis, this model identifies four prognostic groups in PMF that are clearly different with regard to survival. The four risk categories are low-risk (no adverse variable), intermediate-1 (one adverse variable), intermediate-2 (two adverse variables) and high-risk (three or more adverse variables) with corresponding survival of 135 months, 95 months, 48 months and 27 months. To make this tool applicable not only at diagnosis but also during the course of disease, the Dynamic International Prognostic Scoring System (DIPSS) was developed that investigated whether the acquisition anytime during follow-up of one or more of the prognostic factors predicts survival. The DIPSS includes the same five variables as the IPSS, but anemia (not therapy-related) is scored as 2-points since acquisition of anemia during disease course influenced survival with an hazard ratio that was double compared to other variables.(74) A further improvement of the DIPSS score is the DIPSS-plus, that incorporates additional variables for improved discrimination of patients' risk categories (75): these are represented by red cell transfusion need, not treatment-related thrombocytopenia, and unfavorable karyotype (ie: complex karyotype or sole or two abnormalities such as +8, -7/7q-, i(17q), inv(3), -5/5q-, 12p-, 11q23). Among 793 patients included in the original study, median survival was 185, 78, 35 and 16 months for low, intermediate-1, intermediate-2 and high risk group, respectively. Finally, a "very high risk" category of patients is characterized by the presence of monosomal karyotype and/or inv(3)/i(17q) abnormalities, or any 2 of the following: circulating blasts > 9%, leukocytes ≥ 40 × 10^9/L, or other unfavorable karyotype. Patients with any of these risk profiles had

median survival of only 9 months compared to 23 months of those in the high-risk category per DIPSS-plus score. Presence of a *JAK2*V617F mutated state independently predicted leukemic transformation in a longitudinal prospective series of PMF patients(76), whereas presence of *MPL*W5151L/K mutation was associated with more severe anemia(77). However, other studies reported a negative prognostic role for survival in PMF for a V617F allele burden in the lowest quartile (ie, $\leq$25%, while noteworthy the overall survival of *JAK2*V617F mutated patients is no different from *JAK2* wild-type) (78, 79), nullizygosity for the *JAK2* 46/1 or "GGCC" constitutional haplotype (80), high plasma levels of some cytokines (interleukin-8, -2R, -12 and -15)(81) a monosomal karyotype (82) and *EZH2* mutated genotype (83); these prognostic variables remain to be validated prospectively and are not routinely assayed a part for karyotypic abnormalities. Risk factors associated with AML progression included ≥3% circulating blast, plt <100x10^9/L and presence of unfavorable karyotype (84); (74, 85, 86)

Another progression event in myelofibrosis often referred to as accelerated phase has not yet received a satisfactory definition, unless one considers accelerated phase as the equivalent of a high-risk condition associated with overwhelming short survival (less than one year); however, it is not unusual finding patients with progressive leukocytosis, thrombocytopenia, anemia, and blast increase (<20%) who can be still satisfactorily managed with conventional cytotoxic agents and/or supportive measures and survive longer than one year eventually dying without evidence of progression to acute leukemia. In a single center study, three characteristics were selected at baseline as putative features of accelerated phase and were associated with poor survival: blasts in blood or bone marrow $\geq$10%, platelets <50 x 10^9/L, and aberrations of chromosome 17 (85). In that study, accelerated phase was a necessary step in the progression to leukemia.

Leukemic (blast) transformation of a previous MPN, that occurs more common after myelofibrosis than PV and ET (87), is defined, according to the 2008 WHO criteria (22), as a persistent elevation in peripheral blood or bone marrow blasts of 20% or more.

MOLECULAR BASIS OF MYLOPROLIFERATIVE NEOPLASMS

Until 2005, the only known molecular alterations were the *BCR/ABL* rearrangement in CML, originated by a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34; q11)(88), the chimeric *FIP1L1-PDGFRA* mRNA in some forms of eosinophilia(89), and *kit* mutations in cases with systemic mastocytosis(90). The first two cases are a paradigm of the importance of recognizing the genetic events at the basis of these disorders, as demonstrated by the development of a specific inhibitor drug, Imatinib, able to induce complete remission in both the diseases. Central to the pathogenesis of CML is the fusion of the Abelson murine leukemia (ABL) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22, which results in expression of an oncoprotein, termed BCR-ABL(88). BCR-ABL is a constitutively active tyrosine kinase that promotes growth and replication through downstream pathways such as RAS, RAF, JUN kinase, MYC and STAT. This influences leukemogenesis by creating a cytokine-independent cell cycle with aberrant apoptotic signals in response to cytokine withdrawal. Small molecule tyrosine kinase inhibitors (TKIs) were developed to exploit the presence of the aberrantly expressed BCR-ABL protein in CML cells. This ‘‘targeted’’ approach was found to dramatically alter the natural history of the disease, improving 10-year overall survival (OS) from 20 to 80–90%(91).

On 2005, three different groups described the Janus kinase 2 mutation (*JAK2*V617F), that is present in the majority of patients with PV and in 50% or fewer of those with ET or PMF(16-19).

In the following 2 years additional mutations were reported such as those in the exon 12 of *JAK2(24)* and in *MPL(20, 21)*. These different mutant alleles all result in a gain of function due to the constitutive activation of tyrosine kinase-dependent cellular signaling pathways, particularly of the JAK-STAT pathway(92, 93).

Members of the Janus kinase family (JAK1, JAK2, JAK3, and tyrosine kinase 2-Tyk2) are named after the Roman god with two faces, meaning ending and beginning, because they contain two symmetrical kinase-like domains: the C-terminal JAK homology 1 (JH1) domain possesses tyrosine kinase function, whereas the immediately adjacent JH2 domain has an important regulatory function. JH2 phosphorylates Ser523 and Tyr570; the

autophosphorylation of Ser523 is the primary event in JH2 activation, and that it is observed in unstimulated conditions. Cytokine-induced receptor dimerization and juxtaposition of the JAKs lead to other regulatory *trans*-phosphorylation events, including phosphorylation of Tyr570. The relatively low catalytic activity of JH2 is in accordance with autophosphorylation of regulatory residues as a physiological function for JH2, whereas JH1 is mainly responsible for phosphorylation of substrate proteins (94-96).

JAK proteins are associated in an inactive state to the cytoplasmic tail of type 1 or type 2 cytokine receptors (eg, erythropoietin receptor, EpoR; thrombopoietin receptor, MPL; granulocyte colony-stimulating factor receptor, G-CSFR; and interferon-gamma receptor, etc). After the engagement of the receptor by corresponding ligand, JAK undergoes a conformational change and becomes activated via phosphorylation of key tyrosine residues. In turn, phosphorylated JAKs mediate phosphorylation of tyrosine residues of the cytoplasmic domain of the receptors and create a docking site for the recruitment of several proteins, ultimately leading to activation of the signal transducer and activator of transcription (STAT), the mitogen-activated protein (MAP) kinase, and the phosphatidylinositol 3-kinase-AKT (PI3K-AKT) pathways(97). Activated STATs dimerize and translocate to the nucleus where they regulate transcription after binding to specific consensus sequences in the promoter regions of several target genes (**Figure 1**).

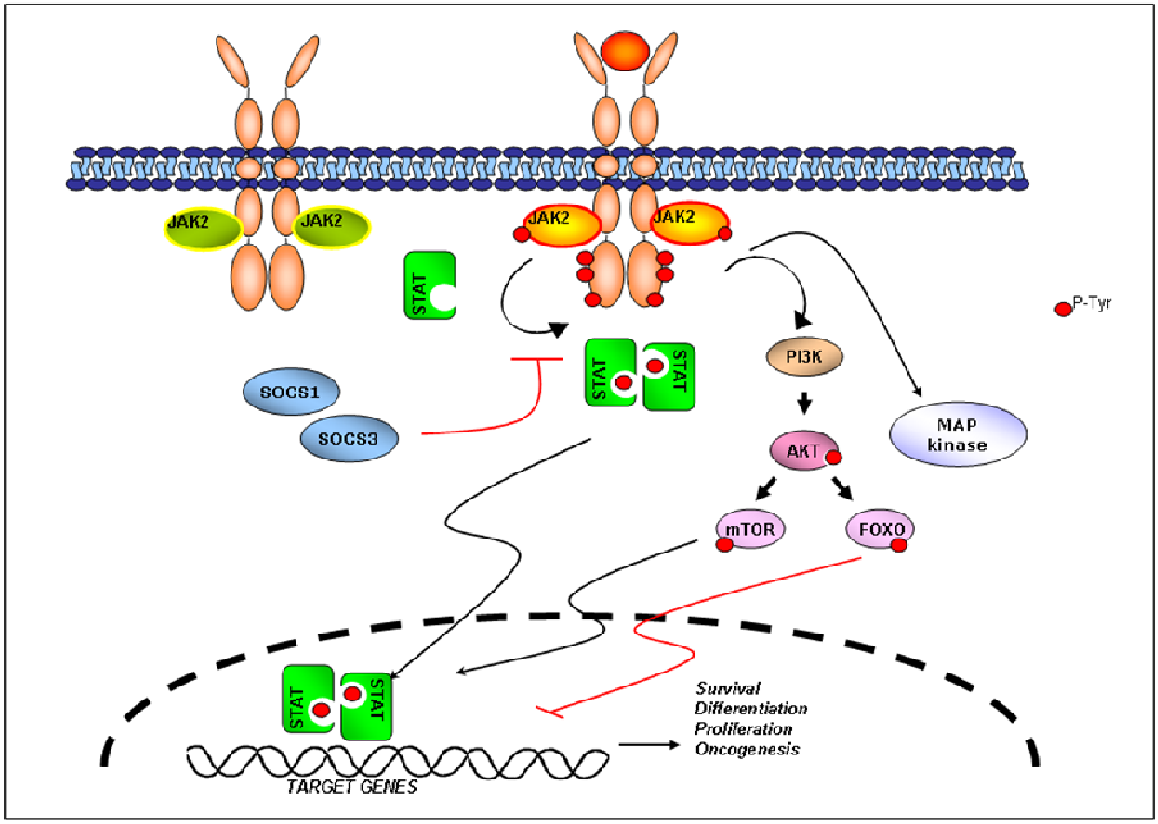


Figure 1. The JAK/STAT pathway, from Vannucchi et al. CA Cancer J Clin. 2009: 59(3):171-91

The entire process is tightly controlled at multiple levels by protein tyrosine phosphatases, suppressors of cytokine signaling (SOCS), and protein inhibitors of activated STAT(98, 99). The *JAK2*V617F mutation is a somatically acquired G to T nucleotide shift at position 1849 in exon 14 that results in a valine to phenylalanine substitution at codon 617; the mutation is located in the JH2 pseudo-kinase domain and is believed to result in the loss of auto-inhibitory control of JAK2 (**Figure 2**).

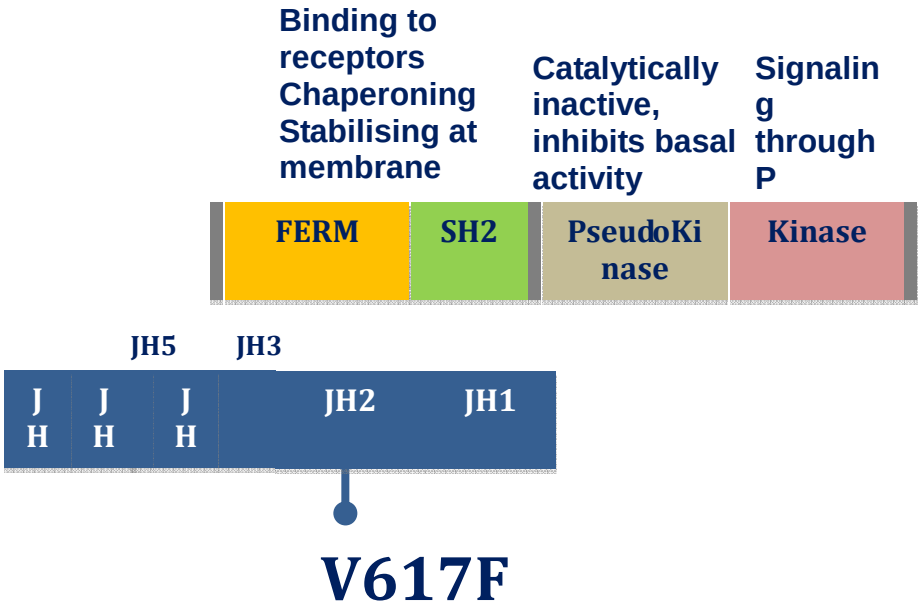


Figure 2. Structure of JAK2, from Vannucchi et al. CA Cancer J Clin. 2009: 59(3):171-91

As a consequence, mutated JAK2 is in a constitutively phosphorylated state, independent from the binding of ligand to its receptor; in fact, when the mutation is introduced into cytokine-dependent cell lines it results in a cytokine-independent growth of the cells and their hypersensitivity to cytokines(17, 18), mimicking the in vitro growth pattern of hematopoietic progenitors from MPN patients. In particular, the gain of function of mutated JAK2 provides a mechanistic explanation for the phenomenon of endogenous erythroid colony formation (EEC)(100), ie, the capacity of erythroid progenitors to spontaneously produce hemoglobinized colonies in vitro in the absence of added erythropoietin, a hallmark of PV and other classic MPNs. Furthermore, transplantation of *JAK2*V617F mutated cells induced a PV-like phenotype in recipient mice(17, 101-104), accompanied by leukocytosis of a different extent and eventually followed by changes suggestive of myelofibrotic transformation. More recently, by manipulating expression levels of the V617F allele, mice with an ET-like phenotype were also generated in the presence of low levels of mutated JAK2(105). Mutational frequency of *JAK2*V617F is estimated to be more than 95% in PV, 60% in ET or PMF, 40% to 50% in refractory anemia with ringed sideroblasts and thrombocytosis (RARS-T)(106, 107), whereas it is very rare in AML or MDS(108, 109). In most patients with PV or PMF, as opposed to a minority of those with ET, the mutation is harbored in a homozygous state, which is

accomplished by mitotic recombination(16-19). In general, the highest V617F allele burden, that is the level of mutated allele relative to normal allele in a cell suspension such as granulocytes, is found in patients with PV followed by PMF and ET(27, 110); however, such variability in the allele burden does not represent a sufficient criterion for distinguishing among different clinical entities, nor does it satisfactorily help to explain the apparent paradox of "one mutant allele-different clinical phenotypes." In fact, how a single V617F mutation can be the basis of different clinical disorders, as in the classic MPN, is still unclear. Conversely, *JAK2*V617F, or other *JAK2* mutations, are likely a necessary component of the PV phenotype because they are detected in virtually all patients with the disease(111) and are sufficient to reproduce the phenotype in mice. In patients with a clinical picture suggestive of PV and who were found to be negative for the *JAK2*V617F mutation, several genetic abnormalities (ie, mutations, deletions, insertions) have been detected in a short region of *JAK2* exon 12 (24, 112). These mutations, which probably account for less than 2% of patients with PV(112), affect autonomous cell proliferation and differentiation in a fashion similar to that of the V617F allele(24).

Another recurrent molecular abnormality of MPN is represented by somatic mutations at codon 515 of *MPL*(20, 21), which, as is the case with *JAK2*V617F, involve early myeloid and lymphoid progenitors(113-115). MPL (named after myeloproliferative leukemia virus oncogene homolog) is the receptor for the cytokine thrombopoietin (Tpo) and is highly expressed in early hematopoietic progenitors and in cells of the megakaryocytic lineage(116). The two most common *MPL* mutations, which are located in the cytoplasmic juxtamembrane portion, are represented by W515L (a tryptophan to leucine substitution) and W515K (a tryptophan to lysine substitution). They have been detected in 5% to 11% of patients with PMF(20, 21, 77) and in up to 9% of *JAK2*V617F-negative cases of ET.(117, 118) Other unusual MPL mutations (eg *MPL*W515S, W5151A, and *MPL*S505N, initially discovered in association with inherited familial thrombocytosis) have also been reported.(119) *MPL*W515L induced both cytokine-independent growth and Tpo hypersensitivity in cell lines, resulting in constitutively activated JAK-STAT/ERK/Akt signaling pathways,(120) and caused a PMF-like disease in mice. (20) At variance with the *JAK2*V617F transplantation model, the disease induced by *MPL*W515L was characterized by a rapidly fatal course, marked thrombocytosis, leukocytosis, hepatosplenomegaly, and

bone marrow fibrosis, all reminiscent of PMF(20). Interestingly in some patients, multiple *MPL* mutations or the coexistence with *JAK2*V617F allele were described.(77, 118, 121)

Regardless, there is evidence to suggest that neither *JAK2*V617F nor MPL mutations may be the initial clonogenic event in MPN and that a "pre-*JAK2*" mutated cell may exist(122, 123). *JAK2*V617F mutation is integral to the classic MPN, but its exact hierarchical position in pathogenesis and its role in phenotypic variability remain to be clarified. In support of this is also the observation that leukemic blasts in patients who evolve to AML from a pre-existing *JAK2*V617F-positive MPN are often negative for the *JAK2*V617F mutation(124, 125).

Numerous reports have been published investigating chromosomal abnormalities by conventional cytogenetic analysis(126-129). Abnormal cytogenetics was described to be frequent in PMF, whereas less prevalent in the other two disease entities, PV and ET(130). Some aberrations have been recurrently observed, most notably, deletions of chromosome 20q, 13q, 12p, trisomy 8 and 9, gains of 9p and various translocations(131-134). The karyotyping methods used usually detects large-scale genomic changes, and those studies did not succeed in the identification of target genes. Microsatellite studies on chromosome 9 identified acquired uniparental disomy of the short arm (9pUPD) as a common defect in MPN(135). In depth investigation of this region resulted in the identification of the V617F mutation in the Janus kinase 2 gene (*JAK2*)(19, 136-138). Studies on other members of the JAK2 pathway led to the identification of different mutations of the thrombopoietin receptor *MPL*, which were often associated with 1pUPDs(21, 139, 140). After high resolution DNA microarrays became available, it has been possible to study cytogenetic changes in great detail. Consequently, new target genes have been identified, such as *TET2*, associated with deletions and UPDs of chromosome 4q(141, 142), or *CBL*, associated with aberrations of chromosome 11q(143, 144).

Other mutations affecting the intracellular signaling of JAK-STAT

CBL. CBL (Casitas B-lineage Lymphoma) is an adapter protein that has been shown to possess E3 ubiquitin ligase activity;it serves to negatively regulate JAK2 signaling through

ubiquitination and subsequent internalization of growth factor receptors. Because of these factors, CBL has been considered to be a tumor suppressor gene that acts to suppress JAK2 signaling. In MPN cells, mutations that inactivate or cause truncation of CBL result in enhanced downstream signaling through JAK/STAT and modulation of cell cycle, proliferation, and apoptosis-related proteins. (145, 146) CBL mutations are most frequently observed in a distinct group of myeloid disorders, myelodysplastic syndromes-myeloproliferative neoplasms (MDS/MPN); this subgroup of hematological malignancies includes the chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML) and juvenile myelomonocytic leukemia (JMML). Most CBL mutations are missense mutations or small deletions around the linker region. A remarkable feature of patients with Cbl mutations is that the wild-type CBL allele is frequently lost in leukemic clones and is replaced with the mutant allele by acquired uniparental isodisomy (aUPD)(143, 144, 147).

SOCS. Suppressor of cytokine signaling (SOCS) proteins are also negative regulators of JAK signaling, the expression of which is inactivated by mutation or through CpG island hypermethylation of the SOCS1 or SOCS3 promoter in MPN cells, leading to excessive cytokine signaling(148, 149). Although mutations in the SOCS genes have been described in MPN, their occurrence is rare, and their role in the pathogenesis of MPN is controversial.

LNK. LNK, (also called Src homology 2 B3), is a plasma membrane-bound adapter protein with several domains including an SH2 domain that can bind to MPL and JAK2 and negatively regulate JAK/STAT signaling in response to Epo receptor or MPL signaling(99, 149). LNK mutations have been detected in 6% to 13% of chronic phase and blast phases of MPN and occur within a hot spot in the pleckstrin homology domain of exon 2.(99, 150) LNK mutations in JAK2V617F-negative MPNare associated with myeloid progenitor expansion and cytokine responsive pSTAT5 and pSTAT3 expression, which phenocopies activating mutations in JAK2(99, 151).

Mutations affecting the epigenetic regulation

TET2. The TET2 gene is located on chromosome 4q24, a common break point involved in translocations in myeloid neoplasms.TET2 functions to convert 5-methylcytosine to 5-hydroxymethycytosine (5hmC) and has been found to be mutated in 7% to 16% of patients with JAK2V617F MPN(146, 148, 149, 152). Mutations in TET2 compromise its catalytic activity, resulting in lower levels of 5-hmC in the genomic DNA as well as global hypomethylation of DNA(152). TET2 mutations have been observed in both JAK2V617F-positive and JAK2V617Fnegative MPN; however, because of the diverse array of the identified mutations, they provide limited prognostic relevance(142, 153).

EZH2. The enhancer of zeste (EZH2) gene encodes a histone methyltransferase that along with suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) comprises the polycomb repressive complex (PRC) 2. The PRC2 complex functions to epigenetically silence gene expression through trimethylation of lysine (K) 27 on histone H3, a repressive histone mark(154).34,35 Mutations in EZH2 have been observed throughout the coding sequence but predominate in the C terminal SET domain and negatively affect the histone methyltransferase activity of the PRC2 for lysine (K) 27 on histone H3.36,37 EZH2 mutations have been identified in 5.9% of patients with PMF, 1.2% of post-PV-MF, and 9.4% of post-ET-MF and were found to coexist in w40% of JAK2V617F-positive and w22% of ASXL1 mutation-positive patients with MF.37 Patients coexpressing these mutations cluster into the International Prognostic Scoring System (IPSS) high-risk category, have shorter leukemia-free survival and significantly shorter overall survival (OS) compared with EZH2 wild-type (WT) patients.Patients with PMF and mutated EZH2 showed higher leukocyte and blast counts and presented with larger spleens at diagnosis than those with WT EZH2(155).

ASXL1. ASXL1 mutations have been identified in 55% of PMF and 22% of post-PV/ET-MF patients(156). Mutations in ASXL1 are most commonly located in exon 12 and result in a missense or a frameshift, resulting in a loss of function for ASXL1 and decreased ASXL1 expression(157, 158). Loss of ASXL1 function leads to a genome-wide loss of

trimethylation on lysine 27 of histone H3 and increased gene expression at loci with bivalent chromatin marks. ASXL1 mutations have also been observed to coexist with JAK2V617F in approximately 48% of PMF and post-PV/ET-MF patients. Similar to EZH2 mutations, patients with coexistence of ASXL1 mutations and JAK2V617F cluster into the IPSS high-risk category, experience significantly shorter LFS, and significantly shorter OS. In addition, ASXL1 mutant patients had a greater than 2-fold higher incidence of leukemia (34.9% vs 15.2%) than ASXL1 wild-type patients. Furthermore, LFS in double ASXL1/EZH2-mutated PMF patients was significantly shorter than in patients with single ASXL1 or EZH2 mutations (25 vs 138 vs 153 months)(159).

IDH1/2. Similar to other myeloid malignancies, isocitrate dehydrogenase 1 (IDH1) and IDH2 mutations have been identified in MPNs(146, 160, 161). Unlike WT IDH1 and IDH2, which catalyze the conversion of isocitrate to a-ketoglutarate (a-KG), mutant IDH1 and IDH2 proteins possess neomorphic enzyme activity, resulting in the production of the oncometabolite, 2-hydroxyglutarate (2-HG)(162). This enzymatic activity and the resulting 2-HG lead to impairment of TET2 catalytic function and global increases in DNA hypermethylation(163-165). Recently, 2-HG produced by IDH mutant cells was also shown to competitively inhibit a-KG-dependent dioxygenases, including the histone demethylation activity of a family of Jumonji-C domain histone demethylases(165). This situation led to marked increases in repressive histone methylation marks, suggesting that IDH mutations preferentially affect repressive histone methylation marks in addition to increasing DNA methylation in the transformed cells(165). Mutations in IDH1/2 are believed to be independent predictors of leukemic transformation because they are frequently identified in blast-phase MPN but not chronic-phase MPN. This finding suggests that IDH mutations may collaborate with JAK2V617F in transformation to leukemia. A recent study showed that IDH-mutated MPN patients showed significantly shorter LFS and OS than patients with mutated JAK2(166).

DNMT3A. DNMT3A belongs to a family of DNA methyltransferases that includes DNMT1 and DNMT3B, which catalyze the conversion of cytosine to 5-methylcytosine(167, 168).

DNMT3A mutations have been identified in MPN, although they are most commonly found in cases of MDS and acute myeloid leukemia (AML) at position R882, which lies in the methyltransferase domain(169). Previous studies have shown that loss of DNMT3A in HSCs resulted in altered DNA methylation patterns at different loci(168). DNMT3A mutations in myeloid malignancies lead to an impairment of its DNA methyltransferase activity, which may improve the response to treatment with DNA hypomethylating agents(168).

Mutations associated with leukemic transformation

IKZF1 molecular alteration in this gene, named IKAROS family zink finger 1, that belongs to the IKAROS transcriptor factor family, are very rare in chronic forms of MPN, but they has been found in about 19% of blast phases(146). IKZF1 is believed to modulate expression of lineage-specific genes through a mechanism that involves chromatin remodeling and results in effective lymphoid development and tumor suppression. IKZF1 mutations are prevalent in ALL, including blast-phase CML or BCR-ABL1-positive ALL, suggesting a pathogenetic contribution to leukemic transformation(170).

TP53 TP53 is a transcription factor that responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. TP53 is kept inactive essentially through the actions of the ubiquitin ligase MDM2. TP53 mutations are very rare in the chronic phase of the disease, and were found in approximately 20% of leukemias secondary to MPN(171). Mutated TP53 in secondary AML is a strong independent prognostic factor of poor survival. The importance of TP53 in the leukemogenesis of secondary AML is further supported by the finding that gains of 1q32.1 harboring the MDM4 were found exclusively in secondary AML(172).

RUNX1 The RUNX1 gene, known also as acute myeloid leukemia 1 gene (AML1), encodes a Runt-related transcription factor, which is part of the RUNX gene family. Its functions include a primary role in the development of all hematopoietic cell types, and it can produce oncogenic transformation to acute myelogenous leukemia. In MPNs, RUNX mutations has been observed in 5 out of 18 secondary AML, whereas no mutation was detected in patients at chronic phase. Ding et al. showed that RUNX1 point mutations

may have a leukemogenic potential in JAK2V617+ stem cells or in pre-JAK2 stem cells, and they may promote leukemic transformation(173).

Descriptions of sporadic mutations in **NRAS/KRAS** and deletions of **NF1** have been reported(172).

An hypermutable *JAK2* haplotype: 46/1 or "GGCC""

A germline constitutive JAK2 haplotype, called GGCC or 46/1, was described as a susceptibility factor for BCR-ABL negative MPNs (174). Over 80% of all the JAK2V617F mutations in MPN occur on this specific JAK2 haplotype. The mechanisms linking the haplotype block and the acquired MPN remain unclear. Two hypotheses have been proposed: the "hypermutability" of the chromosome region that could facilitate the arising of typical MPN somatic mutations(175), or the "fertile ground hypothesis" that could confer selective advantage to cells carrying the 46/1 haplotype (176). 46/1 is a strong predisposition factor for development of JAK2V617F-associated MPNs.

THERAPY

The "standard of care" for patients with MPNs is based on a few prospective clinical trials that (i) addressed the randomized, double-blind and placebo-controlled use of low-dose aspirin in 518 patients with PV without clear indication and contraindication to the drug (46) and (ii) assessed efficacy of hydroxyurea in high-risk patients with ET against non-myelosuppressive therapy (n=114) (177) or (iii) anagrelide (n=809) (178); results of a prospective clinical trial that clarify the target hematocrit level to be maintained in PV were recently published (47). In the "pre-JAK2 inhibitor" era, no controlled trial was performed in PMF (see below). Thus, indications for treatment are largely based on experts' recommendations. At this regard, a recent initiative from the European Leukemia Net organization resulted in the production of a consensus document of management recommendations that kept into consideration most recent advances in terms of diagnosis and risk assessment (179). This 21-member panel of experts drew a list of "goals

of therapy" for patients with MPNs that are reported in **Table 4**; regrettably, most of them represent still "unmet clinical needs".

Table 4. Targets for therapy, ie "clinical needs", in patients with MPNs according to the European Leukemia Net (ELN) management recommendations (179).

Polycythemia Vera and Essential Thrombocythemia
Prevention of thrombosis and hemorrhages
Control of signs of myeloproliferation (erythrocytosis, leukocytosis, thrombocytosis)
Control of symptomatic splenomegaly
Management of profound constitutional symptoms
Prevention of evolution to PPV/PET-MF and of leukemia transformation
Management of disease-associated thrombo-hemorrhagic events, and of risky situations (surgery, pregnancy, …)
Primary Myelofibrosis
Shortened survival
Increased risk of leukemic transformation
Management of severe anemia, often requiring frequent RBC transfusions, and severe thrombocytopenia or neutropenia in some patients
Control of marked hepato-splenomegaly to prevent/reduce symptoms of early saziety, severe abdominal discomfort, changes in bowel habits, painful splenic infarcts, portal hypertension leading to ascites and variceal bleeding, compromised mobility and movement, and cachexia
Management of non hepatosplenic extramedullary hematopoiesis and its complications (cord compression, ascites, pulmonary hypertension, pleural effusion, lymphoadenopathy, skin tumors)
Prevention of thrombo-hemorrhagic complications
Control of marked leukocytosis or thrombocytosis
Management of profound constitutional symptoms including fatigue, weight loss, cachexia, pruritus, night sweats, low-grade fever, and bone and joint pain

There is one major point to consider: while prevention of complications associated with the disease and/or therapy is the goal of treatment in PV and ET, cure and/or prolongation of survival is the major objective in myelofibrosis. Thus, if prolongation of survival or cure is not possible, palliation from symptoms, aimed at improving the overall quality of life, constitutes the main goal of therapy in these subjects.

Quality of life (QoL) in MPN patients is compromised by a spectrum of constitutional symptoms and manifestations due to the enlarged spleen that globally affected more than 70% of the 1,179 patients enrolled in an Internet-based survey conducted at Mayo Clinic (180). Other manifestations included fatigue (80%)%, night sweats (50%), bone pain (45%), pruritus (52%; more common in PV than ET, 65% versus 39%) and weight loss (13%). In addition, a proportion of patients treated with conventional cytotoxic drugs, interferon, and/or phlebotomy experience side effects such as unwanted myelosuppression, pulmonary, mucous and skin toxicities (most frequent for hydroxyurea), flu-like syndrome, depression, exacerbation of autoimmune manifestations (interferon) or intolerance to the procedure and symptomatic iron deficiency in those treated with intensive phlebotomy regimens. The impact of these symptoms on the QoL is captured by the Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF), a 27-item instrument that complements and extends the SAF previously developed specifically for Myelofibrosis (MSAF) (181, 182). The MPN-SAF has been validated in 402 patients with the three MPNs in different languages (English, Italian, Swedish), proving to be reliable, reproducible, and overall well correlated with results of the EORTC (European Organization for Research and Treatment of Cancer)-QOL C30 questionnaire (183). A simplified and more friendly version, the “Total Symptom Score” (TSS), that includes only 10 variables (fatigue, early satiety, abdominal discomfort, inactivity, concentration, night sweats, pruritus, bone pain, fever and weight loss) has also been devised (184). The usefulness of serial administration of the MSAF as a tool for assessing changes in symptomatic burden correlated with treatment has been addresses in the framework of a phase I/II trial with INCB018424/Ruxolitinib, a JAK1 and JAK2 inhibitor (see below) in 87 patients with myelofibrosis. The form proved to be sensitive to symptomatic changes that on turn were highly correlated with more objective clinical endpoints, such as improvements in both weight loss and performance status (as assessed by a 6-minute walk test). Therefore, MPN-SAF and similar instruments represent valuable tools for

assessing the achievement of one major goal of therapy in myelofibrosis and other MPNs, ie the improvement of quality of life; as such, they have been included in any of the trials with novel drugs. Finally, it is likely that these instruments can be of help for the routine assessment of patients' symptoms.

Polycythemia vera and essential thrombocythemia. Taking into an account that improvement of survival cannot represent a reasonable therapeutic end-point for these disorders, since they present only a modest reduction of survival compared to control population, and that the main reason for mortality and morbidity are thrombotic complications, the rationale for treatment is represented by prevention of cardiovascular events, according to the risk stratification criteria outlined in **Table 5**.

Table 5. Risk stratification criteria in PV and ET are based on age and history of cardiovascular events.

Risk category	Age >60 or history of thrombosis	Cardiovascular risk factors (*)
Low	NO	NO
Intermediate	NO	YES
High	YES	----

(*) cardiovascular risk factors are represented by diabetes, hypertension, smoking, hypercholesterolemia.

Low-risk patients with PV are managed with phlebotomy plus low-dose aspirin, while ET patients may be followed without any intervention or aspirin only. The recommended hematocrit level was set at less of 45%, and even lower (42%) for women (185, 186). However, in the ECLAP trial no difference in the rate of thrombosis or death was found between patients who had their hematocrit maintained under 45% or above 45% and up to 55% and received concomitantly low-dose aspirin (45). Debate about the optimal hematocrit target level has been resolved by the results of a large prospective Italian trial

(CYTO-PV), that compares two different hematocrit target levels (≤45% and 45% to 50%). The results of this study in patients with polycythemia vera who were receiving conventional treatment (including phlebotomy, hydroxyurea, or both) show that maintaining a hematocrit target of 45 to 50% was associated with four times the rate of death from cardiovascular causes or major thrombosis, compared with maintaining a hematocrit target of less than 45%. The incidence of the primary end point was 1.1 events per 100 patient years in the low-hematocrit group, as compared with 4.4 events per 100 patient-years in the high hematocrit group. Rates of deep-vein thrombosis and cerebral vascular events including strokes and transient ischemic attacks were increased in the high-hematocrit group, confirming the higher incidence of thrombosis observed in the European Collaboration on Low-Dose Aspirin in Polycythemia Vera (ECLAP) study(47). The use of low-dose aspirin (81 to 100 mg daily) in PV is based on the result of the ECLAP trial; no similar study exists in ET, and the indication for treatment in this condition is simply mutuated. A retrospective study of 198 low-risk ET patients treated with antiplatelet drugs (93% low-dose) as monotherapy compared to 102 similar subjects followed with observation only showed no difference in the overall rate of thrombosis, while the risk of venous thrombosis was 4.0-fold increased in *JAK2*V617F-positive patients and 2.5-fold higher in those with generic cardiovascular risk factors who did not received antiplatelet medication. Therefore, this study suggests that aspirin prophylaxis might be worthwhile, although accounting for a slightly increased risk of hemorrhages, in selected categories of low-risk ET patients, while for the others observation only may be adequate enough (187).

Cytoreductive therapy is indicated for PV and ET patients at high-risk of thrombosis occurrence (if aged only) or recurrence (if with prior thrombotic history). However, cytoreduction may eventually be required for reasons other than increased thrombotic risk that include: poor tolerance to phlebotomies or frequent phlebotomy requirement (PV); symptoms due to progressively enlarging spleen; uncontrolled leukocytosis or thrombocytosis; severe constitutional symptoms; severe pruritus; aspirin-insensitive manifestations of microvascular involvement. Cytotoxic drugs currently employed as first-line therapy are hydroxyurea and interferon-α, the choice depending also on the age of patient, comorbility and the availability of interferon that varies in different countries.

Busulphan may be used as first line drug in elderly subjects, if better tolerated and/or preferred.

The dose of hydroxyurea is usually titrated based on the target level of hematocrit and/or platelet count; a set of criteria for definition of clinicohematologic response in PV and ET has been devised by the ELN mainly for use in the settings of clinical trials more than for clinical practice (**Table 6**).

Table 6. European Leukemia Net criteria for assessment of clinicohematologic response in patients with PV and ET (188).

	Polycythemia Vera	**Essential Thrombocythemia**
<u>Clinico-hematologic response</u>		
Complete Response	Ht lower than 45% without phlebotomy, AND Platelet count ≤ 400 x10^9/L, AND WBC count ≤ 10 x 10^9/L, AND Normal spleen size on imaging, AND No disease related symptoms	Platelet count ≤400 x10^9/L, AND No disease-related symptoms, AND Normal spleen size on imaging, AND White blood cell count ≤10 x10^9/L
Partial Response	In patients who do not fulfill the criteria for complete response: Ht lower than 45% without phlebotomy, OR Response in 3 or more of the other criteria	In patients who do not fulfill the criteria for complete response: Platelet count ≤600 x10^9/L OR decrease >50% from baseline
No Response	Any response that does not satisfy partial response	Any response that does not satisfy partial response
<u>Molecular response</u>		
Complete response	Reduction of any specific molecular abnormality to undetectable levels	
Partial Response (Applies only to patients with a baseline value of mutant allele burden greater than 10%)	A reduction equal to or greater than 50% from baseline value in patients with less than 50% mutant allele burden at baseline, ***<u>OR</u>*** A reduction equal to or greater than 25% from baseline value in patients with more than 50% mutant allele burden at baseline.	
No Response	Any response that does not satisfy partial response	
<u>Histologic response</u>		
Bone marrow histological remission	Presence of age adjusted normocellularity and no reticulin fibrosis	Absence of megakaryocyte Hyperplasia

They involve three sets of response categories, namely clinical-hematological, molecular, and histological response (188). At present, clinical-hematological criteria only are employed for monitoring the response to conventional cytoreductive therapy, since no drug, with the exception of interferon, has produced evidence of changes in histopathology or molecular status (189). Therefore, sequential monitoring of molecular response can be recommended only within clinical trials. However, the impact of response ranking according to ELN criteria on hard clinical endpoints such as thrombosis and death in ET has been questioned by recent studies, showing that ELN-coded responses were not associated with occurrence of thrombosis; indeed, platelet count resulted of no prime relevance, unlike age, leukocytosis and thrombotic history, in the definition of ELN responses (190). Similarly, achieving ELN response (complete or partial) or hematocrit response among 261 PV patients treated with hydroxyurea did not result in better survival or less thrombosis and bleeding, whereas having no response in leukocyte or platelet count was associated with higher risk of death and thrombosis and bleeding, respectively. Furthermore, resistance to HU was associated with higher risk of death and transformation (191).

Some patients may present or develop refractoriness to maximized hydroxyurea dosage and/or develop severe toxicities that force to reduce the dosage or stop the treatment shifting to second line therapies. A standardized definition of clinical resistance and intolerance to hydroxyurea in PV and ET has been developed by the ELN (192, 193). *Resistance/refractoriness* is defined as the unsatisfactory control of hematocrit, leukocyte or platelet counts, failure to reduce splenomegaly by at least 50% or relieve splenomegaly-associated symptoms; *toxicity* may be due to excessive unwanted myelosuppression (neutropenia, anemia, thrombocytopenia) or unacceptable non-hematologic toxicities (mucocutaneous, gastrointestinal, fever, pneumonitis). These criteria are used for evaluating the opportunity to move patients to second-line therapies and have also been employed for enrollment in clinical trials with novel drugs.

Interferon produces hematocrit control in 50–94% of PV patients (194) and control of thrombocytosis in 70–80% of ET patients (reviewed in (195)); unfortunately, more than one third of the patients receiving conventional formulations of interferon develop severe side effects leading to discontinuation. More recently, pegylated preparations of INF- α

have been used in PV (40 patients) (196) or in PV and ET (40 and 39 subjects, respectively) (197) with lower rate of discontinuation (≅20%). Hematologic remission occurred in >80%, associated with progressive decrease of *JAK2* V617F allele burden.

Second-line therapies in hydroxyurea-resistant/refractory patients (192, 193) are represented by interferon-α, busulfan, pipobroman, or anagrelide in case of ET; on turn, hydroxyurea may be tried in those who were intolerant to interferon as first line therapy. Radiophosphorus is now very rarely employed in older PV patients; in studies of the Polycythemia Vera Study Group (PVSG) this treatment was associated with an increased rate of leukemia (198). Busulphan is preferred in older patients, having care of careful titration because of its potent myelosuppressive effects. Anagrelide is approved in Europe as second line for hydroxyurea resistant or intolerant patients with ET; in the United States of America it is also approved as first-line drug. Anagrelide was compared to hydroxyurea in the PT- trial, on the top of low-dose aspirin, in high-risk ET patients; results indicated superiority of hydroxyurea due to a significant lower risk of arterial thrombosis, major hemorrhages and fibrotic transformation, while anagrelide proved superior against venous thrombosis (178). Side effects of anagrelide include headache, flushing, cardiopalm and arrhythmias (199). Since anagrelide has anti-platelet activity, the concomitant use of aspirin should be evaluated case by case.

Due to the paucity of alternative therapeutic approaches in patients who are resistant, refractory or intolerant to hydroxyurea a number of recent trials have evaluated the role of novel drugs. The JAK1 and JAK2 inhibitor Ruxolitinib was used in a phase 2 trial that enrolled 39 subjects with ET and 34 PV satisfying the ELN criteria for hydroxyurea intolerance/refractoriness (200). The overall response rate was 97% (50% complete and 47% partial) in PV and 90% (26% complete and 74% partial) in ET. Almost all PV patients achieved control of hematocrit and stopped phlebotomies, 68% experienced a complete resolution of splenomegaly, and more than 70% normalized blood count. Among ET patients, forty-nine percent achieved a normal platelet count. Treatment was very well tolerated with no emergent toxicity. The histone deacethylase (HDAC) inhibitor ITF2357 (Givinostat) was administered to 12 PV and 1 ET patients in a phase 2 study (201). Preclinical studies showed that Givinostat induced a specific down modulation of phosphorylated JAK2V617F and inhibition of its downstream signaling without

significantly affecting wild-type JAK2 or STAT (202). The drug resulted well tolerated, although most patients experienced grade 2 gastrointestinal toxicity. Overall 1 complete, 6 partial and 4 no responses (ELN criteria) were documented; splenomegaly improved in 75% and most patients experienced improvement of constitutional symptoms and pruritus. It is remarkable that both JAK2 and HDAC inhibitors have striking effects on pruritus; indeed, intractable pruritus, typically aquagenic is complained by most PV patients and represents a disabling condition. Pruritus is poorly responsive to conventional treatments, while remissions after interferon are more common. The pathogenetic mechanisms of pruritus in MPNs are still largely unknown, but abnormal activation of *JAK2* V617F mutated basophils and mast cells has been advocated (203, 204).

Finally, there is no substantiated evidence from a number of retrospective studies, concerning the supposed leukemogenic potential of hydroxyurea, while the sequential/combined use of multiple chemotherapeutic agents is known to associate with a higher than expected rate of leukemic transformation (44). A long-term analysis of a French study that randomly assigned 285 PV patients younger than 65 yr to hydroxyurea versus pipobroman showed a statistically significant reduction of median survival and a higher cumulative incidence of acute myeloid leukemia/myelodysplasia in the pipobroman arm compared to hydroxyurea (205).

Myelofibrosis. Patients with MF, including both PMF and PPV-/PET-MF, have to face a number of clinical problems, listed in **Table 4**, that are well reflected by the variety of therapeutic approaches being used, often in variable sequence and combinations, but most often with frustrating results. Indeed, therapy in MF is a personalized affair: which are the most compelling needs of the patient, the performance status, the potential benefits and anticipated risks of treatment, and also the patient's preference, should be determined and they ultimately drive therapeutic choices. The most frequent reasons why patients with MF needs treatment are represented by anemia, splenomegaly and/or hepatomegaly, symptomatic foci of non hepatosplenic hematopoiesis, uncontrolled leukocytosis or thrombocytosis, thrombo-hemorrhagic complications and severe, debilitating constitutional symptoms. On the other hand, patients who have no or modest

symptoms can be managed with a “watch-and-wait” strategy, postponing a therapeutic decision until the appearance of hematologic abnormalities or worsening of clinical manifestations. In high-risk patients, strict follow-up is especially intended to define the optimal timing for SCT, if feasible.

Anemia. Low-dose prednisone, erythropoiesis-stimulating agents (ESA), androgens or danazol are commonly used for the treatment of anemia (206, 207), with responses not exceeding 20-40%, often of brief duration. A therapeutic trial with ESA is usually justified in patients with moderate, non-transfusion dependent anemia, low (<125 U/L) serum erythropoietin level and modestly enlarged spleen; if no response occurs within a couple of months, there is no reason to continue. Thalidomide (50 mg/day) is used in association with tapering prednisone; response rates are 20% to 40% (208), but the treatment is often poorly tolerated with >50% early discontinuation rate. Prophylaxis of thrombosis with aspirin is recommended in patients with a platelet count >50x10^9/L. Lenalidomide is first line therapy in case of an anemic patient with del(5q31) abnormality: improvement and eventually resolution of anemia, occasionally associated with evidence of molecular remission, is common (209). On the other hand, outside the del(5q) condition, lenalidomide showed little benefit in improving anemia (19%) or reducing splenomegaly (10%) and caused grade 3/4 myelosuppression and non-hematologic toxicity in 88% and 45%, respectively, of the patients in a multicenter ECOG phase 2 trial (210). Partially conflicting results were obtained in another trial that documented 30% and 42% responses in anemia and splenomegaly, respectively, while the toxicity profile was remarkable (211).

There is interest in the possible role of Pomalidomide, an immunomodulating agent with better activity and lower toxicity compared to thalidomide and lenalidomide, for the treatment of myelofibrosis-associated anemia, according to the results of a phase 2 randomized double-blind trial (212). Intriguing, the drug was almost ineffective in *JAK2* wild-type subjects enrolled in another trial (213). A phase 3, multicenter, double-blind, placebo-controlled study in transfusion-dependent MF patients is ongoing. In addition, preliminary results of a phase 1/2 trial indicated an unique activity of the JAK2 inhibitor CYT387 against anemia (214): of the 22 subjects evaluable for anemia 63% qualified for a

response in anemia (IWG-MRT criteria), with 5 subjects becoming transfusion independent. Final data of this study are awaited.

Splenomegaly and systemic symptoms. Approaches for treatment of symptomatic splenomegaly (ie, local pain, early satiety, bloating, splenic infarctions, compression of abdominal organs, portal hypertension, cytopenias) are medical (hydroxyurea, busulphan; cladribine intravenously) or surgical. A reduction in spleen size of 25% and 50% was observed in 35% and 17% of the patients receiving hydroxyurea in a single center retrospective study (215); these usually occurred in subjects with non-massive (<10 cm) splenomegaly and preferentially in those who were *JAK2*V617F mutated (206). However, none of the 73 patients in the "best available treatment" arm of COMFORT-II study (see below), of whom 60% received hydroxyurea, achieved a $\geq$35% spleen volume reduction (that equals a 50% reduction in palpable spleen) (216). Splenectomy still carries a definite risk of peri-surgical complications in up to 30-50% of the patients and are fatal in 5-10%. Later complications include bleeding, thrombosis, infections and a "myeloproliferative" reaction that can be controlled with hydroxyurea. Platelet count should be lowered to $\cong 200 \times 10^9$/L during the operative period to reduce the risk of thrombosis; prophylactic full-dose heparin should be maintained for at least 4-6 weeks (217). Thrombosis occurs frequently in the splancnic veins. Since progressive hepatomegaly may follow splenectomy, a markedly enlarged liver is a contraindication to splenectomy. Current data do not convincingly support an increased rate of leukemic transformation after spleen removal. All this notwithstanding, splenectomy remains a valuable alternative for symptomatic splenomegaly when medical therapy is ineffective or not tolerated, or in case of splenomegaly-associated anemia with reported improvement in up to 50% of cases. Responses are lower ($\cong$25-30%) in case of thrombocytopenia; isolated thrombocytopenia is not an indication to splenectomy since it might herald an impending leukemic transformation (217). The use of radiotherapy for the enlarged spleen is reserved to very selected cases, due to poor efficacy and the risk of local inflammatory complications and long-lasting cytopenias. Recently an effective response in patients with accelerate phase disease, including clearance of circulating blasts and basophilia, has been observed in few cases of high risk MF treated with low dose splenic radiotherapy (218)

The severe constitutional symptoms which affect a large majority of MF patients (180) represent a truly “unmet clinical need” if considering conventional therapies. Low-dose prednisone may produce some feeling of well-being, but the effect is usually modest and transient. Responses to hydroxyurea are commonly modest at best and transient, as supported by results of the randomized COMFOR-II study indicating that none of the patients in the “best available therapy” arm had any significant improvement in symptoms as objectively measured by the EORTC QLQ-C30 or FACT-Lym score (216).

The approach to symptomatic splenomegaly and severe constitutional manifestations has been revolutioned by the results of clinical trials with JAK2 inhibitors. Ruxolitinib (INCB018424) is a JAK1 and JAK2 inhibitor that was recently granted approval by FDA as first-line drug for treatment of patients with intermediate or high-risk myelofibrosis, including primary myelofibrosis, post-polycythemia vera and post-essential thrombocythemia myelofibrosis. In a phase 1/2 trial, about half of the 153 patients with MF had a $\geq$50% reduction of splenomegaly and rapid improvement of constitutional symptoms, cachexia and exercise tolerance (219). Thrombocytosis and leukocytosis normalized in similar proportion of the patients. Treatment was well tolerated (non-hematological grade 1/2 toxicity in <10% of the patients) with dose-limiting toxicity represented by reversible thrombocytopenia. Anemia, an on-target effect of the drug owing the absolute requirement of functional JAK/STAT pathway for erythropoiesis, represented the most common side-effect of treatment; although it was easily manageable with dose adjustment, some patients required transfusion. This study also documented a dramatic normalization of inflammatory cytokines that correlated with symptomatic improvement (219). Two phase 3 multicenter studies with ruxolitinib as single therapy in MF have been completed; of note, they also represent the first controlled studies ever performed in this clinical settings. The first study, COMFORT-I (COntrolled MyeloFibrosis study with Oral JAk Inhibitor Tretment), assessed the activity of ruxolitinib against placebo in 309 patients (220), while COMFORT-II compared the activity of ruxolitinib against “best available therapy” (BAT) at investigator’s choice in 219 patients randomized 2:1 to the active arm (216). The primary study endpoint of $\geq$35% spleen volume reduction from baseline, as measured by MRI (or CT scan), at 24 (COMFORT-I) or 48 weeks (COMFORT-II) was reached by 41.9% and 28.5% of the patients, respectively, compared to 0.7% and 0% in control arms (P<.0001). Clinically meaningful

improvements in Quality of Life measurements and MF-associated symptoms, compared with placebo and BAT, were observed in each of the studies. These conclusions derived from a broad array of validated assessment tools, that included MFSAF and TSS (in COMFORT-I) and EORTC QLQ-C30 and Fact-Lym in COMFORT-II. Toxicity was confirmed to be modest and mainly represented by dose-dependent worsening of anemia. A similar spectrum of activity was reported for other JAK2 inhibitors such as TG101348 (221), CYT387 (222), SB1518, with differences in respective spectrum of non-hematologic toxicities. Everolimus, an inhibitor of the mammalian target of rapamycin (mTOR)/AKT pathway, that is abnormally activated in MPN cells, produced improvement of splenomegaly and complete resolution of systemic symptoms and pruritus in 50%, 60% and 80%, respectively, of the 30 patients enrolled in a phase I/II trial (223). Improvement of symptoms and spleen enlargement were preliminary reported in studies using the HDAC inhibitor LBH589 (panobinostat) (224).

In summary, ruxolitinib, as the funding member of an expanding family of JAK2 inhibitors, has demonstrated marked efficacy against splenomegaly and constitutional symptoms in patients with primary and secondary forms of myelofibrosis, representing an unforeseen and exciting progress over conventional therapies. Some additional points warrant consideration. The first is that ruxolitinib was equally effective in patients harboring or not the JAK2V617F mutation, indirectly suggesting that autonomously activated JAK/STAT is present in most MF patients probably as the consequence of other still unknown mutations. Second, efficacy of the drug was maintained over time in most patients, as shown by results in COMFORT studies, but patients who interrupt treatment experience a gradual return of symptoms and of spleen enlargement within a few days to weeks; upon re-initiation of ruxolitinib treatment, symptoms and splenomegaly improve again. However, in the light of reports that patients who discontinued the drug experienced acute relapse of their symptoms and splenomegaly, occasionally requiring hospitalization (225), it is more prudent to adopt a tapering schedule rather than abrupt discontinuation. Finally, initial results from COMFORT-I suggested a survival advantage for ruxolitinib-treated patients; after a median follow up of 51 weeks, 13 (8.4%) deaths were recorded in the ruxolitinib group compared with 24 (15.7%) in placebo group (226). Undoubtedly, further data are needed before one can derive definite conclusions about an impact of anti-JAK2 therapy on overall survival.

Extramedullary hematopoiesis. In case of asymptomatic localization of hematopoietic tissue patients are usually left untreated; symptomatic manifestations are preferentially treated with low-dose, fractionated (up to 1 Gy in 10 fractions) radiation in involved fields. Single-fraction (100 cGy) whole-lung radiotherapy is promptly effective in case of lung involvement. Occasionally, surgical removal of extra-hepatosplenic foci of hematopoiesis is needed.

Allogeneic SCT is the only proven curative treatment approach in myelofibrosis; demonstration that achieving a *JAK2*V617F negativity after SCT associates with complete hematologic and cytogenetic remission and a low incidence of relapse indicates that cure is achievable through elimination of the mutated clone (227). However, SCT still has high mortality and morbidity; one-year mortality is approximately 30% and 10-20% with conventional and reduced-intensity regimens, respectively, and 5-year survival is 40-50%. By comparison, in a retrospective analysis of young (<60 years), high-/intermediate-risk PMF patients (the usual candidate to SCT) who did not undergo the procedure, the 3-year survival ranged from 55% to 77%, overall comparable to the results obtained with SCT (228). Therefore, the risk associated with SCT is justified in patients with projected survival of less than 5 years, as are those in the intermediate-2/high risk category. Furthermore, patients who acquire unfavorable cytogenetic aberrations (particularly of chr 17) and/or manifest signs of disease progression (increasing blasts, worsening of thrombocytopenia) that could imply impending transformation to acute leukemia and expected survival of one year (85) should be subjected to SCT without further delay. Improvements in candidate selection and refinements of conditioning and immunosuppressive regimens could be certainly key for attracting more patients to SCT procedure; on the other side, should the evidence of survival benefit with ruxolitinib be substantiated, it can be expected that use of SCT will be even more conservatively reserved to patients with highly unfavorable characteristics.

AIM OF THE STUDY

The “classic” Philadelpha-negative chronic myeloproliferative neoplasms include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). They share common characteristics; although increased erythrocyte mass is a phenotypic hallmark in PV, thrombocythemia is a key feature of ET, and bone marrow fibrosis is the diagnostic prerequisite of PMF, the last two phenotypes are often seen among all three MPNs. In addition, MPN has an inherent tendency toward thrombosis and bleeding, and less frequently, toward leukemic transformation. In MPN different mutations have been identify as implicated in disease pathogenesis. Some, such as *JAK2* or *MPL* mutations, contribute to defining the MPN phenotype, while mutations in *ASXL1*(158) and *IDH1/2*(229) or deletions of *IKZF1*(230) are associated with transformation to post-MPN AML. Functional studies of JAK2V617F, JAK2-ex12 and MPLW515L/K in cell lines and in animal models have established their oncogenic character and proven their disease causing potential. However, about 40% of patients with ET or PMF lack a specific molecular feature, including JAK2V617F and MPL mutations. Both bone marrow transplant models (20, 24, 101-103, 136) and transgenic mice(105, 231, 232) expressing these mutations showed that they are capable of inducing myeloproliferative phenotypes in mice resembling human MPN. Animals expressing JAK2-V617F in bone marrow transplant models display erythrocytosis associated with with mild leukocytosis in C57Bl/6. However, the mice developed erythrocytosis with dramatically increased leukocyte count and bone marrow fibrosis on a different genetic background (Balb/c) (101). This observation provided evidence that the genetic background can modify the phenotype induced by JAK2-V617F. In a transgenic animal model, variable expression level of the JAK2- V617F transgene induced thrombocythemia in low expressing animals, whereas polycythemia was present in animals with high transgene expression(105). Although the animal models provided some clues as to how a single aminoacid substitution in JAK2 can result in three different phenotypes, this question has not yet been answered. It remains to be seen whether the genetic makeup of patients and/or the expression level of the mutant protein determine which MPN entity the patient will develop upon the acquisition of JAK2-V617F. When the abundance of the mutant JAK2 allele was compared with clonality, deter mine through X chromosome inactivation analysis, in MPN patients in granulocytes and platelets, a number of ET, PMF and PV

patients with monoclonal myeloid cells displayed significantly low abundance of the mutant JAK2 allele, suggesting that only a small proportion of clonal cells carried the JAK2-V617F mutation(233, 234). These studies provided evidence that somatic mutations precede the acquisition of JAK2-V617F and thus experimentally proved that the 'multi-hit' model of MPN pathogenesis is in place at least in a subset of MPN patients. Presence of del20q has been reported either as a mutation establishing clonal hematopoiesis before the acquisition of JAK2-V617F(235) or, in a different recent study, del20q-positive progenitors have been observed as a minor clone arising from JAK2-V617F-positive cells(236). Many other clonal features have been reported, such as cases of patients homozygous for JAK2-V617F that have a population of cells with uniparental disomy 9p (9pUPD), some patients with JAK2-ex12 that acquire 9pUPD; a patient with polycythemia vera with both JAK2-V617F and JAK2-ex12 mutations was reported in whom the two mutations represented two clones of cells(237). A few recent studies have assessed cytogenetic profiles of MPN patients in chronic phase and in transformation to AML, however the number of studied samples has been rather limited(238, 239).

Therefore, the picture of clonal and genetic variability of MPN is still to be completed and better understood. The aim of this study is to provide further information on this field. To this end I took advantadge of the use of high-resolution SNP arrays to detect chromosomal aberrations that could be correlated with clinical parameters such as post-MPN AML transformation. In the second part, my research focused on some specific alterations to better understand if they could be related to molecular defects potentially implicated in the pathogenesis of these disorders.

METHODS

PATIENT SAMPLES

We collected 79 peripheral blood samples from MPN patients (**Table 7**) after written informed consent.

Table 7. Florence's patients characteristics.

	PV	PMF	PPV-MF	PET-MF	CP pre-AML MF	AML phase
n	33	19	20	1	6	6
M/F	19/14	12/7	11/9	0/1	3/3	3/3
JAK2 status						
WT	1 (3%)	3 (16%)	0	1 (100%)	2 (33%)	5 (83%)
Homozygous mutated	26 (79%)	13 (68%)	19 (95%)	0	4 (67%)	1 (17%)
Heterozygous mutated	6 (18%)	3 (16%)	1 (5%)	0	0	0

CP=chronic phase

To improve the statistical significance of the study, we merged our cases with that of the Medical University of Vienna, Austria and the University of Pavia, Italy, for a total of 444 samples. Of them, only 408, representing 398 patients, were considered evaluable. The patients were diagnosed as chronic-phase PV (n = 162), chronic-phase ET (n = 80), chronic-phase PMF (n = 79), or post-MPN AML (n = 29) at the time of sampling (Figure 1A). The remaining 58 patients showed clear signs of disease progression although they did not fulfill the clinical criteria for post-MPN AML diagnosis. Diagnoses for these patients included post-PV or post-ET (secondary) myelofibrosis, as well as the clinical stage of accelerated phase (AP). This group of 58 patients will be referred to as sMF/AP**(Figure 3A).** The whole cohort included 199 male and 209 female samples. Of the total 408 samples, 297 (72.8%) harbored mutations in the *JAK2* gene **(Figure 3B).**

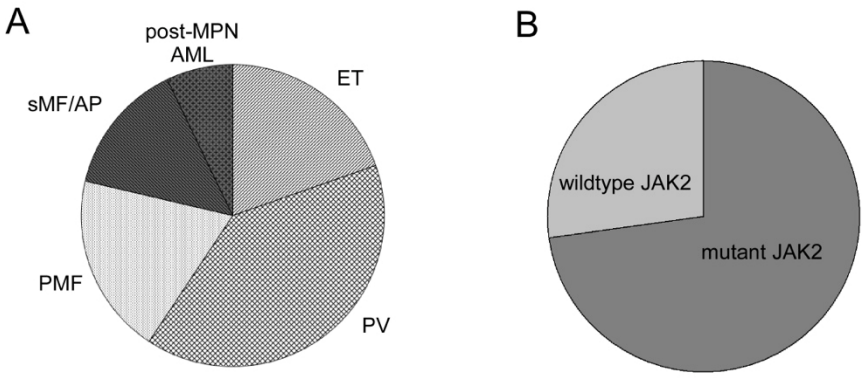


Figure 3. (A) Diagnosis distribution. (B) Fraction of the patients with mutant or wild type *JAK2*.(240)

For each patient we collected approximately 30 ml of peripheral blood to collect mononuclear cells, granulocytes and the cell subpopulations CD34 +. For some patients about 5 mL of bone marrow blood were also collected.

Collection of mononuclear cells

The blood sample was layered on a density gradient (Lymphoprep, Ficoll Hypaque) and centrifuged at 1600 rpm for 35 min at 20° C. Mononuclear cells were collected and washed in sterile PBS at 4° C and centrifuged at 1200 rpm for 10 minutes at 8° C. This procedure was repeated twice. After aspirating the supernatant and resuspended the pellet carefully, the cells were counted using a Burker chamber.

Collection of granulocytes

After centrifugation on a density gradient and the collection of mononuclear cell fraction, the granulocyte layer localized above the erythrocyte pellet was collected. Granulocytes were then resuspended in 40 ml of NaCl 0.2% for the erythrocyte lysis. After centrifugation at 1500 rpm for 10 minutes, the supernatant was eliminated by obtaining the granulocyte pellet, which underwent a second lysis in 40 ml 0.2% NaCl and new

centrifugation at 1500 rpm for 10 minutes. The pellet was resuspended in 2 ml of PBS, transferred in two eppendorf and centrifuged at 3000 rpm for 5 minutes. One aliquot was preserved in lysis solution of guanidine isothiocyanate (Trizol, GibcoBRL, Italy) for the extraction of RNA, while a second aliquot was frozen dry for the extraction of DNA.

Extraction of DNA from granulocytes

Genomic DNA was extracted from granulocytes with QIAmp DNA Blood Kit (Qiagen, Germany). The granulocyte pellet was resuspended in 200 µl of PBS and were added 20 µl of Proteinase K and 200 µl of Buffer AL. After incubation at 56 ° for 10 minutes were added 200 µl of ethanol and the mixture was transferred to Qiagen column and centrifuged first at 6000 rpm for 2 minutes and then at 12,000 rpm for 1 minute. After transferring the column to a new tube were added 500 µl of Buffer AW1. After centrifugation at 8000 rpm for 1 minute were added 500 µl of buffer AW2. After centrifugation at 12,000 rpm for 4 minutes were added 100 l of Buffer AE, incubating at room temperature for 5 minutes. Finally after centrifugation at 12,000 for 1 minute the eluate obtained was transferred into a sterile eppendorff, and the DNA collected quantified by spectrophotometric absorption at 260 nm (NanoDrop, Willmington, USA).

Extration of RNA from Trizol

Briefly the granulocyte pellet resuspended in Trizol, is incubated at room temperature for 2-3 minutes, after adding 0.2 ml of chloroform and centrifuged at 12000 for 15 min at 4 ° C. We obtain three phases: one pink, one white and one interface. The upper aqueous phase was recovered and was added with to 2 µl of glycogen and 0.5 ml of isopropanol to precipitate the RNA. Incubated at room temperature for 10 min after stirring for inversion, then centrifuged at 12000 rpm for 10 min at 4° C. The RNA pellet was then washed with 75% ethanol and finally centrifuged at 7500 rpm for 5 min at 4° C. The pellet was dried for 5 min and finally resuspended in 20 µl of sterile water (Eurobio). The RNA obtained was quantified by spectrophotometric assay (NanoDrop, Willmington, USA).

Reverse Transcription PCR (RT-PCR)

The technique of reverse transcription allows to obtain cDNA starting from RNA in a reaction that involves the use of buffers, nucleotides, random hexamers, RNAse inhibitors and reverse transcriptase enzyme MMLV-RT (Applied Biosystems) reaction catalyst. The reaction mix was made in a final volume of 20 µL in the presence of 10x reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 200 mM dNTP, 2 mM MgCl2, RNase Inhibitors, following the scheme of amplification : 10 min at 22 ° C, 40 min at 42 ° C 5 min at 95 ° C.

Karyotype analysis

The karyotype is the complete set of all chromosomes detected during the metaphase of a cell. In that phase, the chromosomes are in their most compact state, and then easy to observe under a microscope after appropriate staining. The chromosomes are arranged in descending order according to the size and layout of the centromere. The human karyotype shows 46 chromosomes: 22 pairs of autosomes and one pair of heterosomes (X, Y). When the different chromosomes are colored in a uniform manner, it is difficult to distinguish them based only on their size and shape, and for this reason was developed a number of techniques that color some chromosomal regions or bands more intensively than others. The study of the banding karyotype allows to determine at first glance any macroscopic abnormalities of chromosomes, due, for example, to duplications, deletions, translocations. The karyotype analysis was performed using conventional techniques at the SOD of Cytogenetics of Careggi Hospital, Florence, using cells of bone marrow and peripheral blood lymphocytes.

FISH analysis

The FISH technique allows direct visualization of genetic alterations on interphase nuclei and metaphase chromosomes. A DNA probe is labeled with a fluorescent compound and hybridized o cells immobilized on a glass slide (in situ). The choice of FISH probes to be used depends on the experimental design and the locus of interest must be known. 1 mL of peripheral blood of the patient was fixed with fixative solution containing methyl

alcohol and acetic acid in a 3:1 ratio. A drop of sample was dropped on a glass slide, previously cooled to -20 ° C, The slides were then dehydrated in ethanol for 1 min prior to 70%, then 90% ethanol and absolute ethanol (1 min each), subsequently incubated for 3 minutes in solution 70% formamide / 2X SSC and then again dehydrated. After 10 min of denaturation at 75° C and after another 40 minutes at 37° C, the probe was applied to the sample. At the end of the hybridization stage, of 24-48 h at 37 ° C, the slides were washed first at 72° C for 2 minutes in solution 0.4x SSC / 0.1% NP-40, then at room temperature for 1 minute in solution 2X SSC / 0.1% NP40, all strictly in the dark. After the addition of 10uL of DAPI slides were evaluated under the microscope.

Microarray analysis

Patient DNA was processed and hybridized to Genome-Wide Human SNP 6.0 arrays (Affymetrix) according to the manufacturer's instructions, in collaboration with the Department of Hematology and Oncological Sciences L. and A. Seragnoli, University of Bologna, Italy. The raw data of 444 samples were processed using Genotyping Console Version 3.0.2 software (Affymetrix) by the group of Dr. Robert Kralovics of the Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria. The data were first analyzed for quality, and samples showing high background signal were excluded. In addition, several mononuclear cell samples showing deletions at the TCR locus on chromosome 14 in > 50% of the cells, which is suggestive of a significant number of T cells in the sample, were excluded from further analysis. In total, we excluded 36 samples and used the remaining 408 (representing 398 patients) for further analysis. For 10 patients, we had 2 samples each from 2 different disease stages. Chromosomal aberrations such as deletions, gains, and UPDs were annotated after loss of heterozygosity and copy number regions were identified. The criteria for UPDs were a terminal location (at the ends of chromosomal arms) and a size of at least 1 Mb. Terminal UPDs in patients with numerous extensive (> 10Mb) interstitial runs of homozygosity were not included. In general, we cannot exclude that some chromosomal aberrations were germline, because we did not test constitutional DNA for all samples. We also did not annotate aberrations that mapped to known copy number variation loci according to the Database of Genomic Variants (DGV version 5, human reference genome assembly hg18). The microarray raw

data (CEL files) and the processed data (CNCHP files) are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-608.

Whole exome sequencing

Five tumor samples and two matched control samples were analyzed by whole exome sequencing. Genomic DNA libraries were generated either by using the NEBNext DNA Sample Prep Reagent Set 1 (New England Biolabs, Ipswich, MA) or the TruSeq DNA Sample Prep-Kit v2 (Illumina, San Diego, CA). Whole exome enrichment was performed using the Sure Select Human All Exon Kit (Agilent, Santa Clara, CA) or the TruSeq 1 Exome Enrichment Kit (Illumina). The exome - enriched libraries were hybridized to Illumina flowcells V1 or V3 and sequenced using the Illumina HiSeq 2000 instrument. The sequence reads were aligned against the human reference genome (hg18) using BWA v0.5.930(241). Subsequently, the aligned samples were post processed using GATK v1.5(242) following their best practices guidelines (v3). Briefly, this comprises marking PCR-duplicate reads, recalibrating the base quality scores and local realignment around insertions/deletions (indels). Variant discovery was performed on the post-processed alignment files using GATK's Unified Genotyper(243). The final variant lists were generated using GATK's Variant Quality Score Recalibrator using the suggested filtering parameters.

Single-gene mutational analysis

Primers for PCR not taken from other publications were designed using the *Primer 3* tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) or the *ExonPrimer* tool (http://ihg.gsf.de/ihg/ExonPrimer.html) . *CBL* exons 8 and 9 which were taken from a publication by Sanada et al(244).

PCRs were performed using the AmpliTaq Gold DNA Polymerase with Gold Buffer and MgCl2 solution (Applied Biosystems / Life Technologies, Paisley, UK) or the AmpliTaq Gold 360 Mastermix (Applied Biosystems) *JAK2* mutational status was determined using allele-specific PCR for the JAK2-V617F mutation, as described previously(235). Exon sequencing

of runt-related transcription factor 1 (*RUNX1*), tumor protein p53 (*TP53*), *IDH1*, *IDH2,* cut-like homeobox 1 (*CUX1*), and SH2B adaptor protein 2 (*SH2B2*) was performed using the BigDye Terminator version 3.1 cycle-sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis was performed using Sequencher Version 4.9 software (Gene Codes). Nucleophosmin (*NPM1*) exon 12 was analyzed for duplications. The exon was amplified with dye-labeled primers, and PCR product length differences were detected on the Genetic Analyzer. The data were analyzed using Gene Mapper Version 4.0 software (Applied Biosystems). We also screened for fms-related tyrosine kinase 3 internal tandem duplications (FLT3-ITD) and FLT3-D835 mutations, as described previously(43).

Table 8. Primer sequences and PCR conditions for single gene mutational analysis.

gene	exon	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	PCR conditions
RUNX1	1	GTAGGGCTAGAGGGGTGAGG	AAGACAGGGAACTGGCAGG	95°C – 5 min. *10x touch down (-1°C/cycle):* *94°C – 30 sec.* *67-57°C – 30 sec* *72°C – 30 sec.*
	2	AACCACGTGCATAAGGAACAG	GCAGAAACAGCCTTAATTATTTGG	
	3	CCCTGAACGTGTATGTTGGTC	CAAGCTAGGAAGACCGACCC	
	4	CATTGCTATTCCTCTGCAACC	AAGACAGACCGAGTTTCTAGGG	
	5	GTAACTTGTGCTGAAGGGCTG	AGGTTGAACCCAAGGAATCTG	
	6	GAAAGACAAGAAAAGCCCCAG	AGTTGGTCTGGGAAGGTGTG	
	7	TGTGACATATTTGAACAAGGGC	TTTTACTCAATAATGTTCTGCCAAC	
	7 a	GGAAGGGAAGGGAAATCTTG	ATTACAGACCCACATTCTGCC	
	8-1	GTCCTCTTGGGAGTAGCATCC	CACCACGTCGCTCTGGTTC	
	8-2	AGCGCAGGGAGGCCCGTTCCA	GGGATCCCGGCGGGCTTGTCGC	
TP53	1-2	TCTCAGACACTGGCATGGTG	TGGGTGAAAAGAGCAGTCAG	*26x:* *94°C – 30 sec.* *57°C – 30 sec* *72°C – 30 sec.* *72°C – 10*
	3	CGTTCTGGTAAGGACAAGGG	GAAGAGGAATCCCAAAGTTCC	
	4-5	GCATGTTTGTTTCTTTGCTGC	CATGGGGTTATAGGGAGGTC	
	6	GTGCTGGGCACCTGTAGTC	AGCAGTAAGGAGATTCCCCG	
	7-8	TGGTTGGGAGTAGATGGAGC	GCCCCAATTGCAGGTAAAAC	
	9	TGCCGTTTTCTTCTTGACTGT	GCAGGCTAGGCTAAGCTATGA	
	10	TGCATGTTGCTTTTGTACCG	AGCTGCCTTTGACCATGAAG	
	11	ATTTGAATTCCCGTTGTCCC	GCAAGCAAGGGTTCAAAGAC	

IDH1	4	GGTGTACTCAGAGCCTTCGC	AATTTCATACCTTGCTTAATGGG	*min.*
IDH2	4	TCATGAAGAATTTTAGGACCCC	CATTTCTGCCTCTTTGTGGC	

KDM1B	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**	**T° ann.**	**Amplificated fragment (bp)**
EX 6	CTAACCTGCCTCTCGTTTTG	AAACAGCATTTTCATCCATGC	60	272
EX 7	AAGATGACCAAGTGGTGGAG	CAACCTTGACAGAATGATCAACAC	60	306
EX 8	CCTGAGACTATATTTTATAAGCAACCC	TCAAAGGCACAATTAACCCC	60	140
EX 9	TCTGCATGTACATTTCTTGTCTG	GTTCCTCAGGGAGGGGAG	60	328
EX 10	TGGATTTGGAAGTTACAGCTTG	CCTTTTCAACATGGTCCTCC	60	294
EX 11	TTTAAATGGATTGTTTTCCTGC	AGCGTTTGTCCTGATCAATG	60	312

FOXB1	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**	**T° ann.**	**Amplificated fragment (bp)**
EX 1.1	CTCTCCCTTCTCGCTCAGTC	GCGAACCTGGGCTCTTG	64	368
EX 1.2	CCTCCGGAGCCCTAACAC	CATGGATGCACAGAGACACC	64	493
EX 2.1	CTGTTACTCGGTCTGGCTGA	GGTCGGACTTAAGCACCTTG	60	514
EX 2.2	ATGGACCGCTTCCCCTACTA	TTAATGGGCACGGGGATG	62	662
EX 2.3	GCGCGGGAATACAAGATG	GCGAGTTCGAGAGCAAGG	58	334

EFTUD1	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	T° ann.	Amplificated fragment (bp)
EX 1	CTTTAGCCTCTGGGCCATTT	TGAGACATACAGAGACACATCTCC	60	215
EX 2	GGTATGATCCATGACCCCTTT	CCTTTTGGATGGCTGAAGAA	60	225
EX 3	TTCTTTGGGAAGTGAAGATGC	GATGCAAAGCTGTGTTTCGT	60	165
EX 4	CCTGGACAACTGAAAATGTGTG	CCCCTCTTTCCTCACCCTTA	60	229
EX 5	TCAAATCATGAGTTTTACATTTTTCTT	CCCAGCTCAGTAACTTCCTTACA	60	248
EX 6	GAATGACGCTCTGCCATTTC	TGCAAAGAGATTTAATCAGATGC	60	284
EX 7	AACTCTGCAGTGGGAAAGGA	AAGGGCTTTTCATTTCTCTTCTC	60	231
EX 8	CATGAACATTTGGTCTTCTTTCA	ACCAACAACCAAAAGGGACA	60	181
EX 9	TCAGGAACTTAAGCCCGTATG	TGCCTAAAGATGCCTAAAGGT	60	150
EX 10	TCCCCTCTCCAATCTTGTGT	TCTTGGTTACCATAACGCATTG	60	243
EX 11	TGGCTCAAGGTTTTCAGTCC	GTCTAGAGCAGCCTGGATGG	60	228
EX 12	TTGATGATGCCATCCATGTT	CTAGCCCAGCCCAACTTTC	60	241
EX 13	CAATGCTTGCTGTTTCCCTAC	TGCAACAGAGCCTACTTAGGAA	60	247
EX 14	TTCTTGGCACTAATTATCTTTTTGT	TCAGACCCTCGAGAAACAAT	60	249
EX 15	TTTATGTTGTATCTCAGCCATTTTT	CAAATTTTGCATTGTGTGAAGTC	60	163
EX 16	ATGCTTCCAAATGCCTCCTA	CAGCATCACCCTACCTAGCA	60	203
EX 17	TTGATGCTGCTGTTTTTCTGA	AAACATATGCTTTTAAAAATAAGGTCA	60	242
EX 18	AGCCTTGGGAATCACACATC	TCAAGTGTTTTAACAAATGGAACCT	60	241
EX 19.1	TTCCCTTCAGTAGCCAGAGC	GGGCATTTCATTTTTCTCCA	60	350
EX 19.2	GCTCTCAATCAATGGGATGTT	TTTTGTGTGTTTTGTGTACATGC	60	249
EX 20.1	TTTAACCTTCTGTTATTTCTCTTGAA	TCTGGGTGACTTCTTCTGGA	60	300
EX 20.2	TAAACTTGCCACGCTCAGTG	GAGTTCTGAAAATCTTCACTTTTATTG	60	300
EX 20.3	ATTTGGCCCAAGAAAATGTG	TTCCACCAGAACAGGTTTCA	60	300

EX 20.4	ATCTGGCAAAAGAGGGACA	GCATTTCTCACTATCTTTACCTTTTC	60	298
EX 21	CATGTCTTGGTGTGGCTCAA	GGAATGAATGGGAACTTGGA	60	277
EX 22	CCTTGCACATTCTGTCATCTTC	GGAAAAGAATCCACCAGTAGTAGG	60	247

GNAL	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**	**T° ann.**	**Amplificated fragment (bp)**
EX 1.1	AATGCAAAATGACCCTCTGG	AGACTGTAGCACAGACCCATGT	Touchdown 64°	245
EX 1.2	CCCACATGGGTCTGTGCTAC	CTTCTCCTTCGGCTTGTCTG	Taq gold 360 58°	546
EX 1.3	CAGACAAGCCGAAGGAGAA	GAACCATTCCACCCACAGTT	Taq gold 360 58°	368
EX 2	TGGAGGTAAAGACAGGAGGC	GAGGATCATGCATATTCTAGCAG	60	136
EX 3	AGAGTTGTTGTGCTGCTTGG	CTGTAGGGGTCAGGGAGCTG	Taq gold 360 64°	387
EX 4.1	CCCCGTCTCCGTTCATTG	TCAGGAAGTTAGGAATGTGCG	60	272
EX 4.2	GACCATCTCTTTCAGCAGCA	CTCGCGTCGTTCTTTTTCAT	60	189
EX 4.3	ACAGCAAGACGACGGAAGAC	ATTTCCTACACGCGGGTTC	Taq gold 360 55° 40 cicli	300
EX 5	GGTGAGAGATGGCAGCG	CGTCTGGGAGAGAGACGAAG	60	190
EX 6	CAATTGATTGGAATCAGTGCTAAG	GAATGAAGAGCACTTGACCTTTC	60	169
EX 7	CATACATGGAGCCTAAATGTTTATC	GCATTTAGAGAGCTGGATTTCG	60	301
EX 8	TGCAGACTTCCACAAAGAAAATC	CAGTACTGAGAAGGCAGCATAATC	60	308

EX 9.1	TGACACAGGATCTGAGAGGC	CTGAACCATGTCCCAGGTG	60	288
EX 9.2	GAGAAATGATGGTTCAGGCAG	CACTGGAGCCCAGGAGTTC	62	292
EX 10	CCATCCTTGCTGTACTTGGG	AGGGCAGTACAAACAATTTGC	60	293
EX 11	CGGGCTTTACCTTGAAGAAG	GACATCATGTGGCCCTGG	60	183
EX 12	AAGCACGTTTGCCATTGTC	TGCTGAGTGTTAGAATTCACTCC	60	175
EX 13	TCGAGTAGCTGCTGGTGTTG	AGGTGTGAGCCACAACGC	Touchdown 62°	297
EX 14	AATTTAGCAACTTCTATGTTAGAGGC	TCTTTGATTCATCAGAGGTATGTG	60	305
EX 15	GCAGTCTAACGTTGAAATTCCTTC	AAACAAAAGATTAAGAAGGGAGG	60	214
EX 16	TCCTTCCCCAGAGTACATGC	CTGGCAGTCAGGCAGTCC	60	261

SETD1B	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**	**T° ann.**	**Amplificated fragment (bp)**
EX4	GGGGGATGTTGTGTTTTCC	GAAAGGGTCGGGCTGACA	60	390
EX16	AGACCGGGGCTCACCTCT	CCACAAAAGGGGGTCAGTCT	60	232
EX17	CAGACTGACCCCCTTTTGTG	TATAACCCTTGGCCAACCTG	60	298
EX18	ACCAGGGGCTCATTCTCC	CAGGGCTACGGCTGACAT	60	250

GTF2H3	**Forward primer (5' -> 3')**	**Reverse primer (5' -> 3')**	**T° ann.**	**Amplificated fragment (bp)**
EX11	GAAGGGTGGGCTTCATGTTA	TGGCTGAAAAACAACACAGC	60	290
EX12	TGTTTTTCAGCCACCCTATTG	CTGAAATTACAGAGAGGAAAACATT	60	165
EX13	TGAGATTGTGTGGGTGGCTA	TCTTCCCAACAAAGAATCTGCT	60	212

Agarose Gel Electrophoresis

The agarose gel electrophoresis is the standard method used to separate, identify and purify DNA fragments ranging in size from a few hundred bases to 20 kb. Agarose is a polymer of carbohydrate extracted from seaweed. When melted and gelled it forms a matrix, with a porosity dependent on agarose concentration. The method take advantage of the negative charge of DNA, that in an electric field migrate toward the positive pole. The porous matrix of the gel retards the migration of the DNA, allowing small fragments move more quickly than larger ones. As a result, the DNA fragments will separate in the matrix depending on the molecular weight. Most commonly used gel concentrations are the 0.8 to 2%, that can separate fragments from about 50 bp to about 20,000 bp. The electrophoresis buffer is a saline solution that serves both to conduct the electric current and to control the pH during electrophoresis. A visual marker (Loding Buffer) allows to follow the progress of the electrophoresis. The agarose is dissolved using buffered solutions; TAE and TBE buffers are the most commonly used, and are constituted by Tris, which allows maintaining a constant value of pH of the solution, and EDTA which chelates divalent cations, such as magnesium. With "A or B" is indicated boric or acetic acid that provide the appropriate ionic strength to the buffer. DNA fragments can be observed by staining with fluorescent molecules and use of a transilluminator.

Sanger sequencing, PCR subcloning

Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit and the 3130xl Genomic Analyzer (Applied Biosystems / Life Technologies, Paisley, UK). Sequence analysis was done using the Sequencher Software 4.9 (Gene Codes, Ann Arbor, MI). For PCR product subcloning the TOPO Cloning Kit (Invitrogen / Life Technologies, Paisley, UK) was used according to manufacturers instructions. PCR products derived from single bacterial clones were sequenced as described above.

Statistical analysis

Differences in the distributions of chromosomal aberrations between different sample groups were tested statistically using the Kruskal-Wallis test (in the case of multiple comparisons) or the Mann-Whitney test (in the case of 2-sample comparisons). Differences in frequencies of individual chromosomal aberrations between chronic-phase MPN and sMF/AP or post-MPN AML, as well as between JAK2-V617F–positive and –negative samples were tested for significance using the Fisher exact test.

Real-time quantitative PCR (TaqMan)

This method allows a real time (RT) measure of the PCR amplification during the exponential phase of the reaction. It is based on the detection of fluorescence associated with amplification. This method is based on the use of two primers specific for the sequence of interest and of a fluorogenic probe that intercalate in the double helix of DNA. The TaqMan probe type is an oligonucleotide designed to be complementary to the target sequence; it containing at the 5’ a fluorescent marker with high energy that emits fluorescence, the Reporter, and at the 3' a fluorochrome with low energy that turns off the fluorescence of the reporter (quencher), both covalently linked. During the reaction, the cutting of the probe separates the reporter from the quencher fluorescent marker, with the result of an increase of the fluorescence emitted by the reporter to each cycle of the PCR process (the increase in fluorescence of the Reporter is directly proportional to the number of amplicons generated). The accumulation of PCR products is measured directly by monitoring the increase in fluorescence emitted by the reporter and is registered by the instrument ABI PRISM 7300 Sequence Detection System (Applied Biosystem). The buffer used contains a marker of passive reference (ROX) that is not participating in the PCR reaction. This serves as internal reference standard with which the signal of the reporter can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentrations or volumes of the samples. The normalization is accompanied by the division of the intensity of fluorescence emission of the reporter for the emission intensity of the passive marker, obtaining a defined ratio Rn (normalized reporter) for each reaction tube. The threshold

cycle or Ct is the cycle at which a statistically significant increase in Rn is first detected. The threshold is defined as the average standard deviation of Rn for the early cycles multiplied by a correction factor. The threshold cycle is the one corresponding to the moment in which the measuring system (Sequence Detecion System) begins to detect the increase in signal associated with an exponential growth of the PCR product. A treshold is chosen by the operator to intersect the curves of all samples in the exponential phase. The results of quantitative PCR are expressed as differences in the levels of expression between the gene of interest and the reference gene (RNAsiP, in our case) for each sample, then from the difference between the threshold cycle Ct of the gene of interest and that of the reference gene, obtainig the ΔCt. For each sample is then calculated the $\Delta\Delta$Ct by subtracting to the average ΔCt of samples under study the average ΔCt of the whole population of controls. The value of relative quantification (RQ), used to determine the relative concentration of the target, was expressed as $2^{-\Delta\Delta Ct}$.

TaqMan with SYBR-Green

Differently from the method described above, no specific probes are used, but a nonspecific intercalating fluorescent molecule (SYBR ® Green, Life Technologies) that binds to the PCR product. During the elongation an increase of fluorescence occurs, that corresponds to an increase in the number of copies of the amplicon.

Table 9. Primer sequences and PCR conditions for SYBR-Green analysis.

GENE	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	T° ann.	Amplificated fragment (bp)
RFX7	TGCTTATGGAGCAGCAAATG	GTGAGGATGAGGTTGGGGTT	56	207
NEDD4	GGAGTTGCCAGAGAATGGTT	TTGCCATGATAAACTGCCAT	60	153

EFTUD1	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	T° ann	Amplificated fragment (bp)
Esone 1	AAGCATGTCGCTATGCACTG	GCAAAACCAAAGCTTTCAGC	60	211
Esone 2	GGAAATAAGTAATCATGGTGCTCA	ATGTAGGGAAATGGCACTGG	60	248

GENOME ANALYSIS OF MYELOPROLIFERATIVE NEOPLASMS IN CHRONIC PHASE AND DURING DISEASE PROGRESSION

RESULTS

Whole-genome analysis of MPN patients

408 MPN samples has been investigated for chromosomal aberrations using high-resolution SNP microarrays that deliver 1.8 million copy number measurements and 0.9 million SNP genotypes per genome, in collaboration with the group of Prof. Kralovics, Center for Molecular Medicine (CeMM), Austrian Academy of Sciences, Vienna, Austria. The analysis for chromosomal aberrations revealed 153 samples (37.5%) with a normal karyotype, whereas 255 (62.5%) harbored at least 1 chromosomal aberration (**Figure 4**). All samples with UPD on chromosome 9p were positive for the JAK2-V617F mutation, whereas 6 of 7 samples with UPD on chromosome 1p were positive for the MPL-W515L mutation.

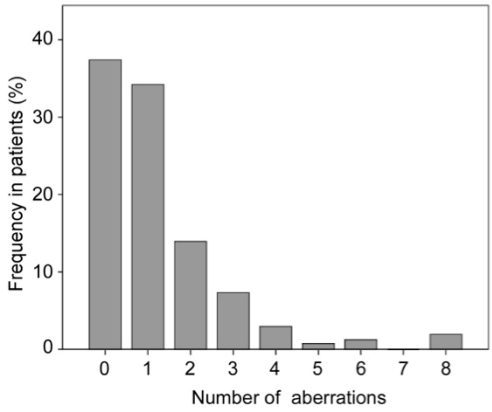


Figure 4. Distribution of chromosomal aberrations in the entire MPN cohort.(240)

Correlation of genomic aberrations with clinical parameters

To investigate whether chromosomal aberrations correlates with clinical features, we first focused on the patients in the chronic phase of the disease (n = 321). We detected a total of 50 gains, 76 deletions, and 147 UPDs, with sizes ranging from 0.1-146.3 Mb distributed across the genome (**Figure 5**). We compared the distribution of chromosomal aberrations between the 3 MPN disease entities (**Figure 6A**) and found that PV patients harbored significantly more chromosomal aberrations than ET ($P < .001$) or PMF ($P = .004$) patients. Because 92% of the PV patients were positive for the *JAK2* mutation and 68% of these patients harbored the associated 9pUPD, we examined whether the observed difference was mainly based on the high prevalence of 9pUPDs in PV patients. After data reanalysis excluding 9pUPDs, we could no longer observe a significant difference in chromosomal aberrations between the disease entities (**Figure 6A**). To investigate the relationship between *JAK2* mutational status and chromosomal aberrations, we compared the frequency of aberrations in patients with and without *JAK2* mutations (V617F and exon 12 mutations combined; **Figure 6B**). We observed a significant difference ($P < .001$), which was again lost after the exclusion of 9pUPDs from the analysis ($P = .392$). We also compared the distribution of individual recurrent chromosomal abnormalities (found in at least 3 patients) between *JAK2* mutation–positive and -negative patients. The only significant difference was the clustering of 9pUPD in *JAK2*-positive MPN patients (**Table 10**).

Table 10. Association of individual chromosomal aberrations with *JAK2* mutational status.

Aberration type		JAK2-mutant (n=297)	JAK2-wt (n=111)	P value	P value*
UPD	***9p***	***169 (56.90%)***	***0 (0.00%)***	***1.35×10^{-32}***	***3.38×10^{-31}***
UPD	1p	2 (0.67%)	6 (5.41%)	0.0062	0.1548
deletion	12p	2 (0.67%)	4 (3.60%)	0.0492	1
gain	9p	6 (2.02%)	0 (0.00%)	0.1959	1
trisomy	9	9 (3.03%)	1 (0.90%)	0.2983	1
UPD	7q	2 (0.67%)	2 (1.80%)	0.2990	1
deletion	3p	5 (1.68%)	0 (0.00%)	0.3293	1
deletion	4q	11 (3.70%)	2 (1.80%)	0.5278	1
deletion	11q	4 (1.35%)	0 (0.00%)	0.5783	1
deletion	5q	3 (1.01%)	2 (1.80%)	0.6164	1
deletion	6p	3 (1.01%)	2 (1.80%)	0.6164	1
UPD	22q	3 (1.01%)	2 (1.80%)	0.6164	1
deletion	12q	4 (1.35%)	2 (1.80%)	0.6653	1
UPD	14q	7 (2.36%)	1 (0.90%)	0.6888	1
gain	1q	11 (3.70%)	3 (2.70%)	0.7668	1
deletion	13q	11 (3.70%)	3 (2.70%)	0.7668	1
deletion	20q	11 (3.70%)	5 (4.50%)	0.7752	1
gain	3q	3 (1.01%)	1 (0.90%)	1	1
deletion	7q	7 (2.36%)	3 (2.70%)	1	1
deletion	7p	3 (1.01%)	1 (0.90%)	1	1
trisomy	8	6 (2.02%)	2 (1.80%)	1	1
UPD	11q	2 (0.67%)	1 (0.90%)	1	1
deletion	18p	4 (1.35%)	1 (0.90%)	1	1
UPD	19q	2 (0.67%)	1 (0.90%)	1	1
trisomy	21	2 (0.67%)	1 (0.90%)	1	1

*p values after Bonferroni correction for multiple testing; significant associations are highlighted. Abbreviations: UPD, uniparental disomy; wt, wild-type

This result indicates that patients positive for *JAK2* mutations do not carry more chromosomal aberrations compared with *JAK2*-negative patients. Furthermore, we did not detect an aberration specifically associated with JAK2-V617F–negative MPN. We further examined the correlation between the cytogenetic complexity of patients and disease duration and age. The median disease duration of the chronic-phase patients was 4 years (range 0-26), and the median age at sample was 65 years (range 22-92). As shown in **Figure 6C**, patients without chromosomal lesions and patients with increasing numbers of lesions did not significantly differ in their disease duration (P = .273). Conversely, we observed a significant difference related to the patient age at sampling. Patients with normal karyotypes were younger than those with complex karyotypes (P = .009; **Figure 6D**). Then, we compared the distributions of chromosomal aberrations in the 3 stages of disease progression (**Figure 6E**). Samples diagnosed with sMF/AP harbored significantly more chromosomal aberrations than samples in the chronic phase of the disease (P < .001) and significantly fewer aberrations compared with samples diagnosed with post-MPN AML (P < .001; **Figure 6E**).

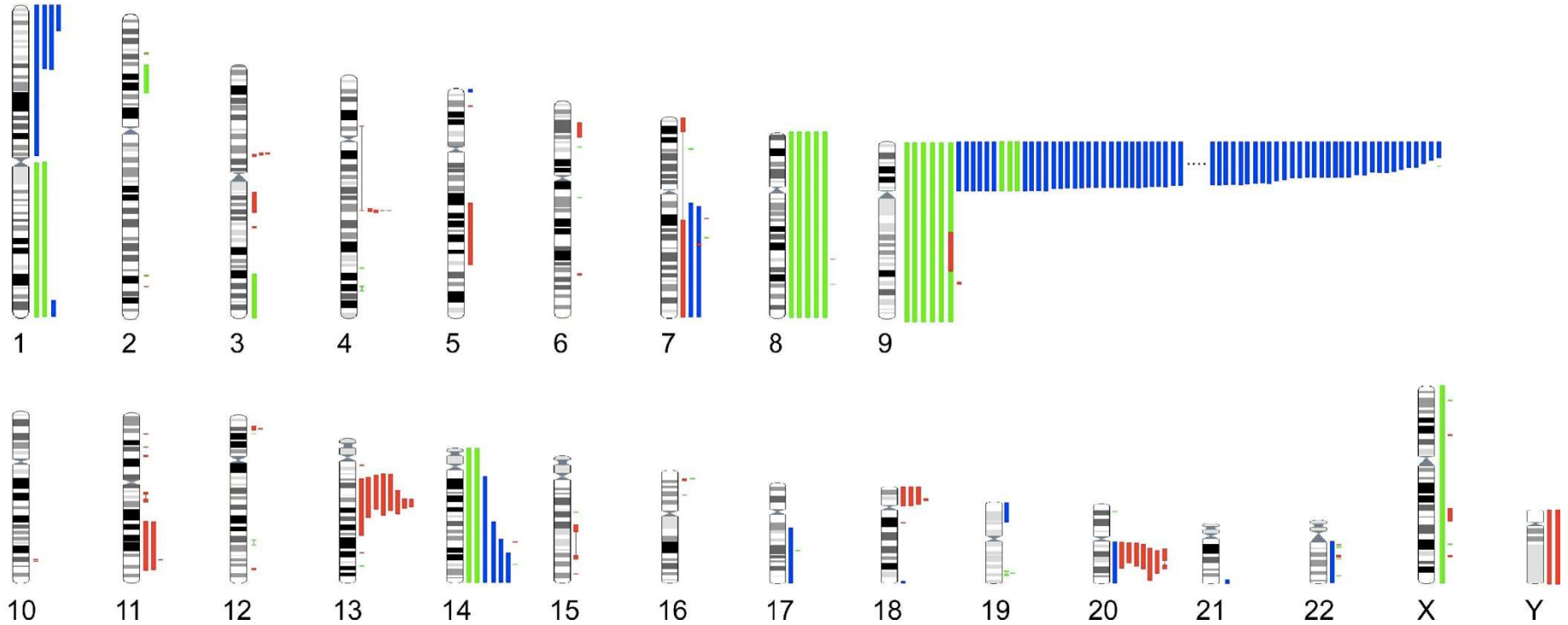


Figure 5. Karyoview of all chromosomal aberrations detected by Affymetrix SNP array analysis in 321 chronic phase MPN patients. Bars depict the physical position and the size of the aberration (green, gains; red, deletions; blue, uniparental disomies). Thin black lines that connect two bars indicate multiple aberrations in the same patient. Chromosomal ideograms based on www.ensembl.org release 56.

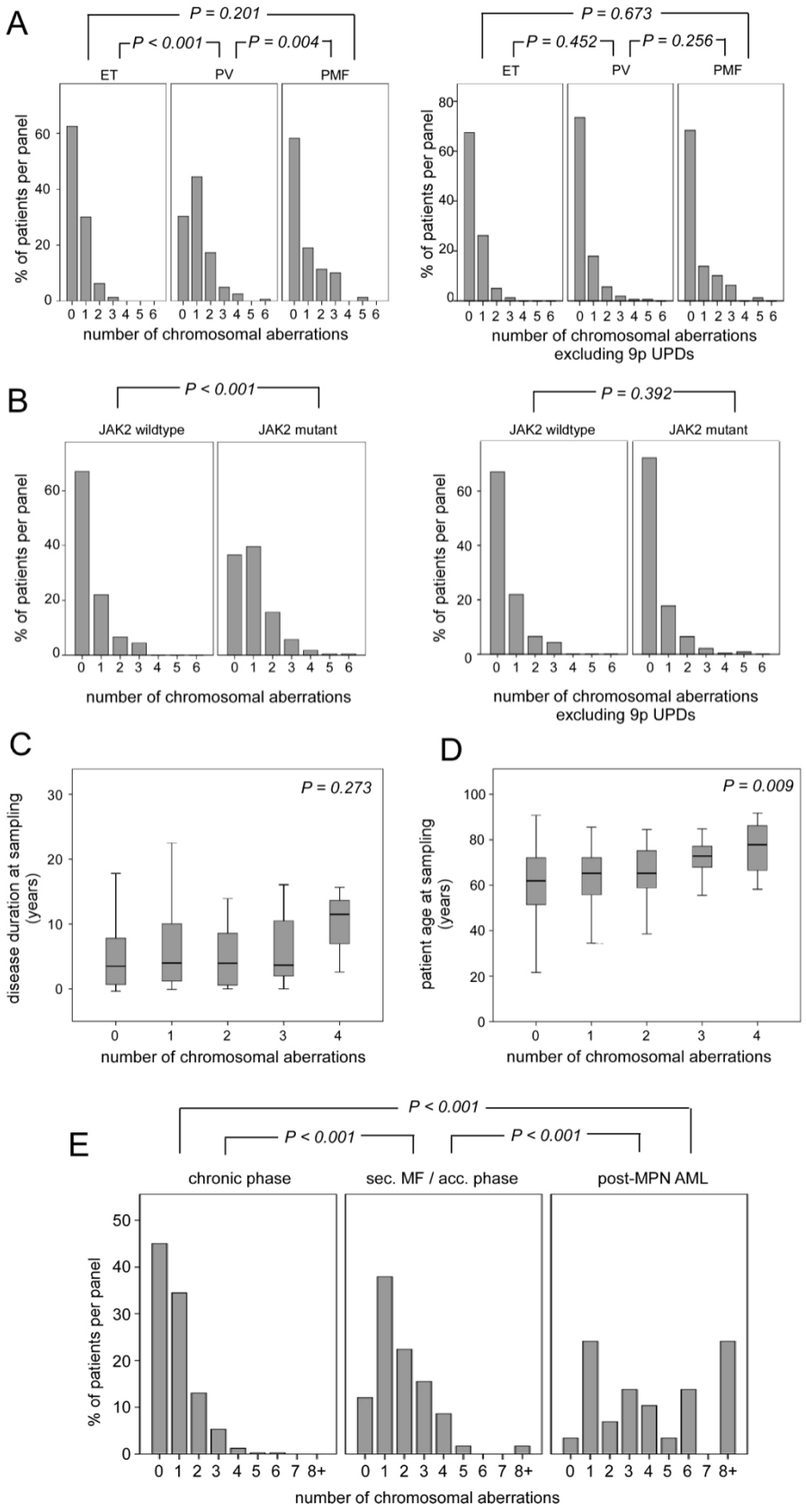


Figure 6. **Correlation of chromosomal aberrations with clinical parameters.** P values refer to differences between the indicated distribution patterns. Distribution of chromosomal aberrations in (**A**) the three MPN entities (PV, polycythemia vera; ET,

essential thrombocythemia; PMF, primary myelofibrosis) and (**B**) in patients positive or negative for *JAK2* mutations. Association of the number of chromosomal aberrations with (**C**) disease duration and (**D**) patient age (outliers are not shown). (**E**) Distribution of chromosomal aberrations comparing patients in chronic phase of the disease, patients with secondary myelofibrosis / accelerated phase and patients transformed to post-MPN AML.

Association of specific chromosomal aberrations with sMF/AP and post-MPN AML

To identify specific chromosomal lesions associated with disease progression, we compared the distribution of recurrent chromosomal abnormalities (found at least in 3 patients) between samples in chronic phase, sMF/AP, and post-MPN AML. Gains of chromosomes 1q and UPDs of 9p were significantly associated with sMF/AP compared with chronic phase (**Table 11**). Chromosome 1q and 3q amplifications; deletions of 7q, 5q, 6p, and 7p; and UPDs of 19q and 22q showed significant association with post-MPN AML compared with chronic phase (**Table 12**). We did not observe any associations of recurrent aberrations comparing sMF/AP with post-MPN AML. Common chromosomal aberrations in MPN, such as deletions of 4q (*TET2* gene deletions), 13q, and 20q, were evenly distributed among the groups. The chromosomal defects most significantly associated with sMF/AP and post-MPN AML transformation were gains of chromosome 1q (**Tables 11 and 12**). Recently it has been reported that the minimal amplified region on chromosome 1q harbors the *MDM4* gene, a potent inhibitor of p53. It was also showed that p53 mutations are associated with post-MPN AML(44). In the present study, we extended our analysis on this finding and sequenced the *TP53* gene in a total of 129 chronic-phase and the 29 post-MPN AML samples. We found two samples in the chronic phase (1.6%) and 6 samples with post-MPN AML (20%) carrying mutations in *TP53*. This result confirms the reported association of *TP53* mutations with post-MPN AML ($P<.001$). The chromosome 9p UPDs, which were also associated with sMF/AP, amplified JAK2-V617F mutations.

Table 11. Association of individual chromosomal aberrations with progression to secondary myelofibrosis/accelerated phase.

Aberration type		sMF/AP (n=58)	Chronic MPN (n=321)	P value	P value*
gain	***1q***	***7 (12.07%)***	***2 (0.62%)***	***3.98 x 10^{-5}***	***0.0010***
UPD	***9p***	***38 (65.52%)***	***120 (37.38%)***	***8.15 x 10^{-5}***	***0.0020***
deletion	12q	3 (5.17%)	1 (0.31%)	0.0122	0.3053
UPD	14q	4 (6.90%)	4 (1.25%)	0.0216	0.5393
UPD	11q	2 (3.45%)	0 (0.00%)	0.0231	0.5770
deletion	20q	5 (8.62%)	7 (2.18%)	0.0239	0.5963
deletion	7q	3 (5.17%)	2 (0.62%)	0.0272	0.6798
deletion	4q	4 (6.90%)	5 (1.56%)	0.0344	0.8605
deletion	12p	2 (3.45%)	2 (0.62%)	0.1125	1
deletion	7p	1 (1.72%)	0 (0.00%)	0.1530	1
trisomy	21	1 (1.72%)	0 (0.00%)	0.1530	1
UPD	1p	2 (3.45%)	4 (1.25%)	0.2299	1
UPD	22q	1 (1.72%)	1 (0.31%)	0.2830	1
trisomy	9	2 (3.45%)	5 (1.56%)	0.2916	1
UPD	7q	1 (1.72%)	2 (0.62%)	0.3933	1
deletion	13q	3 (5.17%)	9 (2.80%)	0.4051	1
deletion	3p	1 (1.72%)	3 (0.93%)	0.4869	1
gain	9p	1 (1.72%)	4 (1.25%)	0.5662	1
gain	3q	0 (0.00%)	1 (0.31%)	1	1
deletion	5q	0 (0.00%)	1 (0.31%)	1	1
deletion	6p	0 (0.00%)	1 (0.31%)	1	1
trisomy	8	1 (1.72%)	5 (1.56%)	1	1
deletion	11q	0 (0.00%)	3 (0.93%)	1	1
deletion	18p	0 (0.00%)	4 (1.25%)	1	1
UPD	19q	0 (0.00%)	0 (0.00%)	1	1

*p values after Bonferroni correction for multiple testing; significant associations are highlighted.

Table 12. Association of individual chromosomal aberrations with progression to post-MPN AML.

Aberration type		Post-MPN AML (n=29)	Chronic MPN (n=321)	P value	P value*
gain	***1q***	***5 (17.24%)***	***2 (0.62%)***	***5.20×10^{-5}***	***0.0013***
deletion	***7q***	***5 (17.24%)***	***2 (0.62%)***	***5.20×10^{-5}***	***0.0013***
deletion	***5q***	***4 (13.79%)***	***1 (0.31%)***	***1.82×10^{-4}***	***0.0046***
deletion	***6p***	***4 (13.79%)***	***1 (0.31%)***	***1.82×10^{-4}***	***0.0046***
deletion	***7p***	***3 (10.34%)***	***0 (0.00%)***	***5.16×10^{-4}***	***0.0129***
UPD	***19q***	***3 (10.34%)***	***0 (0.00%)***	***5.16×10^{-4}***	***0.0129***
gain	***3q***	***3 (10.34%)***	***1 (0.31%)***	***0.0019***	***0.0487***
UPD	***22q***	***3 (10.34%)***	***1 (0.31%)***	***0.0019***	***0.0487***
deletion	4q	4 (13.79%)	5 (1.56%)	0.0036	0.0904
trisomy	21	2 (6.90%)	0 (0.00%)	0.0066	0.1662
deletion	20q	4 (13.79%)	7 (2.18%)	0.0084	0.2102
deletion	12q	2 (6.90%)	1 (0.31%)	0.0189	0.4728
trisomy	9	3 (10.34%)	5 (1.56%)	0.0217	0.5413
deletion	12p	2 (6.90%)	2 (0.62%)	0.0359	0.8970
UPD	1p	2 (6.90%)	4 (1.25%)	0.0808	1
UPD	11q	1 (3.45%)	0 (0.00%)	0.0829	1
trisomy	8	2 (6.90%)	5 (1.56%)	0.1073	1
deletion	13q	2 (6.90%)	9 (2.80%)	0.2289	1
UPD	7q	1 (3.45%)	2 (0.62%)	0.2291	1
deletion	3p	1 (3.45%)	3 (0.93%)	0.2936	1
deletion	11q	1 (3.45%)	3 (0.93%)	0.2936	1
gain	9p	1 (3.45%)	4 (1.25%)	0.3528	1
deletion	18p	1 (3.45%)	4 (1.25%)	0.3528	1
UPD	9p	11 (37.93%)	120 (37.38%)	1	1
UPD	14q	0 (0.00%)	4 (1.25%)	1	1

*p values after Bonferroni correction for multiple testing; significant associations are highlighted.

The role of other de novo AML-specific mutations in post-MPNAML

To gain deeper insight into other pathways involved in the transformation to AML, we screened all post-MPN AML patients for point mutations common in de novo AML affecting the genes *RUNX1, FLT3, NPM1, IDH1*, and *IDH2*. Sample 393 carried 2 nonsense mutations in *RUNX1* and also tested positive for somatic trisomy 21. Sample 396 had a hemizygous single-gene deletion of *RUNX1*. Sample 373 carried a L29S missense mutation and sample 381 harbored 1 missense mutation and a 5-bp insertion. Three patients harbored mutations in *FLT3* and 1 patient carried a *NPM1* insertion. IDH1- R132G, IDH2-R140W, and IDH2-R140Q mutations were detected in samples 407, 304, and 381, respectively.

CDRs that mapped to single genes

Common deleted regions (CDRs) were mapped as the minimum overlap of all deletions detected for a particular chromosome in our cohort. We found 6 such regions that mapped to single-target genes. The CDR of chromosome 4q harbors only the known target gene *TET2* (**Figure 7A**), and the CDR of chromosome 7p contained only the *IKZF1* gene (**Figure 7B**). Mutations of both genes have been reported previously(141, 142, 230). The deleted regions of chromosome 7q mapped to *CUX1* (**Figure 7B**). Chromosome 7p and 7q deletions were among the lesions most significantly associated with post-MPN AML. Two patients tested positive for an acquired 7qUPD in the same chromosomal region. Sequence analysis of *CUX1* in these 2 patients did not reveal any mutations. When we sequenced the enhancer of zeste homolog 2 gene (*EZH2*), a described target of 7qUPDs(245, 246) we identified mutations in both patients. A third patient harboring a 7qUPD had wild-type *EZH2* but harbored a homozygous single-gene deletion of *CUX1* within the UPD region. A close neighboring gene of *CUX1* is *SH2B2*, a homolog of the SH2B adaptor protein 3 gene (*SH2B3 LNK*). Aberrations in *SH2B3* have been associated with myeloid malignancies(99, 150). Because chromosome 7q aberrations were associated with post-MPN AML, we sequenced *SH2B2* in the 29 leukemic patients of our cohort, and identified a single somatic missense mutation (G113C) in 1 patient. The CDR on

chromosome 3p (71.2-71.3 Mb) mapped to the forkhead box P1 (*FOXP1*) gene (**Figure 7C**). We found 6 patients with overlapping deletions on chromosome 12p. The smallest of these deletions mapped to the ets variant 6 gene (*ETV6*; **Figure 7D**). Three patients harbored chromosome 21 trisomies. In another patient with post-MPN AML, a deletion containing only the *RUNX1* gene on chromosome 21q was detected (**Figure 7E**).

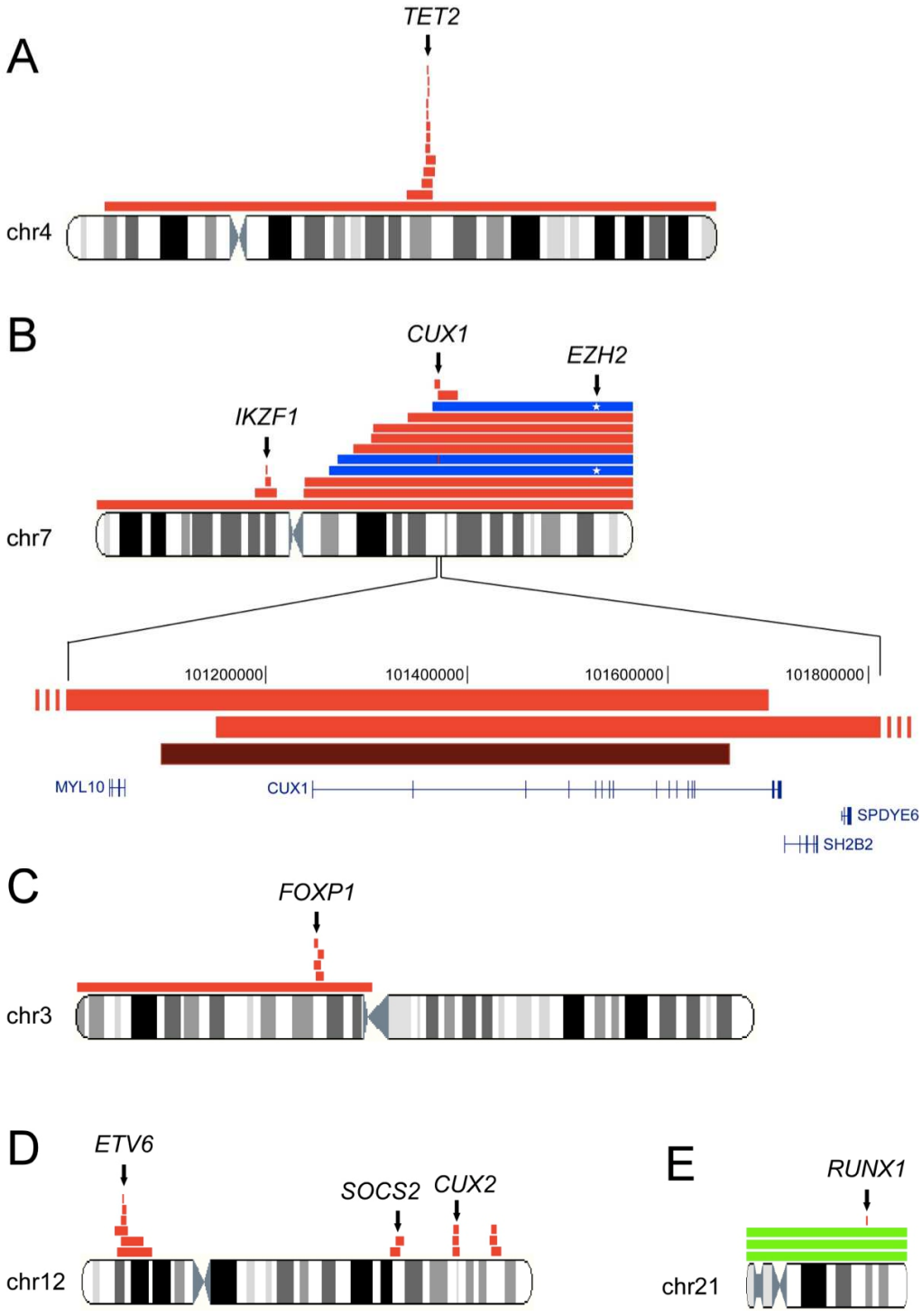


Figure 7. **Commonly deleted chromosomal regions in MPN that map to single target genes.** Red horizontal bars indicate deletions, dark red – homozygous deletion, blue bars - UPDs. (A) Recurrent deletions on chromosome 4q target *TET2* gene. (B) Complexity of chromosome 7 aberrations in MPN. The common deleted region on 7p (*IKZF1)* and 7q (*CUX1)* are shown. Two events of 7qUPD associated with an *EZH2* mutation (*) are shown in blue. Another 7qUPD is negative for *EZH2* mutations but has homozygous *CUX1* deletion. Two overlapping deletions in the *CUX1* gene locus and the homozygous deletion of *CUX1* are shown at higher magnification. (C) Five overlapping deletion events on chromosome 3p define the target gene as *FOXP1*. (D) Recurrent deletions on chromosome 12. The target of 12p deletions is *ETV6*, while one of the commonly deleted regions on 12q contains *CUX2*. (E) Aberrations on chromosome 21q affecting *RUNX1*. Single gene deletion of *RUNX1*, trisomy 21 with 2 nonsense mutations in *RUNX1*. chr, chromosome; Mbp, mega base pairs; UPD, acquired uniparental disomy; MPN, myeloproliferative neoplasm.

CHROMOSOME 11 ABERRATIONS

PATIENTS SAMPLES

In order to systematically analyze chromosome 11 aberrations in myeloid malignancies, the number of blood samples was increased by the group of Dr. Kralovics, Center for Molecular Medicine (CeMM), Austrian Academy of Sciences, Vienna, Austria, for a total of 813 peripheral blood samples, including also patients from the Masaryk University Brno, Czech Republic, the University of Belgrade, Serbia, and the Innsbruck University Hospital (manuscript submitted). A total of 813 samples from 773 patients was analyzed and genotyped at high-resolution with Affymetrix Human 6.0 SNP microarrays. For 40 patients we had two samples from different disease stages available (e.g. chronic phase MPN and post-MPN AML).

This cohort included 180 *de novo* acute myeloid leukemia (AML), 62 chronic myeloid leukemia (CML), 101 myelodysplastic syndrome (MDS) (61 chronic phase, 40 post-MDS AML) and 470 myeloproliferative neoplasm (MPN) (426 chronic phase, 44 post-MPN AML) samples.

RESULTS

Chromosome 11 aberrations in myeloid malignancies

Chromosome 11 aberrations were detected in 52 of 813 (6.4%) samples (**Figure 8**). The 52 samples were from 50 patients, for 2 patients we had 2 samples from different disease stages. The samples harbored between 1 to 3 genetic changes on chromosome 11, except for sample 42 which had a complex chromosome 11. Excluding sample 42, we detected a total of 30 deletions, 11 gains and 17 UPDs. (**Figure 9**). In MPN, aberrations of chromosome 11 significantly associate with post-MPN AML compared to chronic phase MPN ($P<0.001$, Fisher's exact test, **Figure 8**). These findings indicate that genes located on chromosome 11 potentially contribute to disease progression if genetically altered. Associations of chromosome 11q LOH with disease progression or bad prognosis have been described before in B cell chronic lymphatic leukemia (B-CLL) (247)or neuroblastoma (248). Chromosome 11q23 abnormalities were associated with a poor outcome in infant acute lymphoblastic leukemia (ALL) (249).

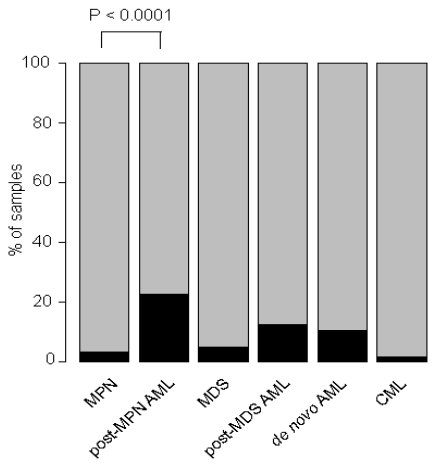
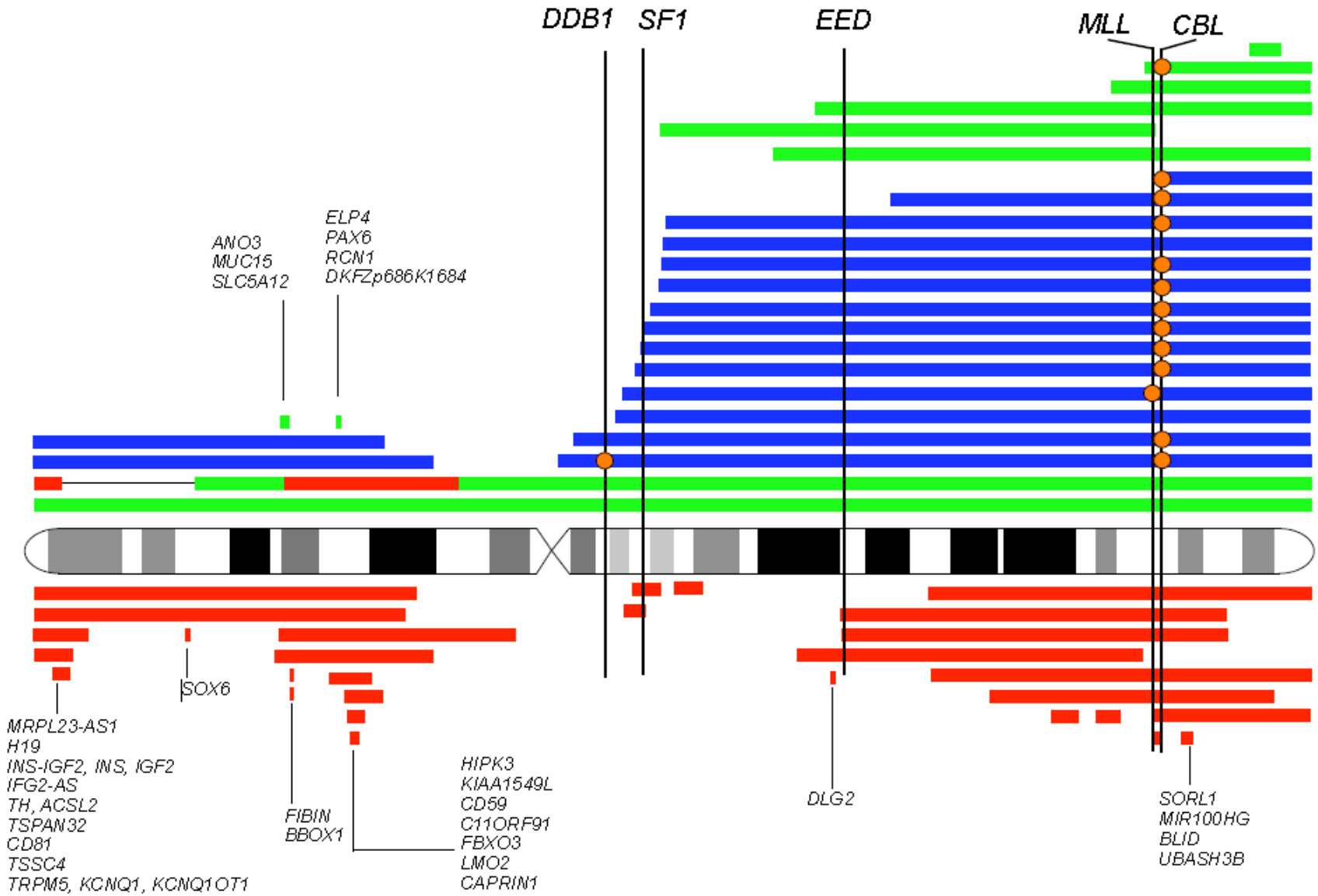


Figure 8. Fraction of samples that harbor chromosome 11 aberrations (black bars) for each disease entity in percent. The P-value indicates an association of chromosome 11 aberrations with disease progression in MPN.

Figure 9. Summary of chromosome 11 aberrations.

Large chromosomal aberrations are indicated with colored bars around the ideogram of chromosome 11. Green – gains; red – deletions; blue – uniparental disomies. The position of the bars relative to the chromosome ideogram indicates the position and size of the aberration. For the two patients of which two samples were analyzed recurrent aberrations are depicted only once. The positions of *CBL, MLL, EED, SF1* and *DDB1* are indicated by vertical lines. Mutations in these genes are depicted by orange circles along these lines. Common deleted regions are indicated at the bottom of Figure 9 listing the genes they contain.


DDB1
SF1
EED
MLL
CBL
ANO3
MUC15
SLC5A12
ELP4
PAX6
RCN1
DKFZp686K1684
SOX6
MRPL23-AS1
H19
INS-IGF2, INS, IGF2
IFG2-AS
TH, ACSL2
TSPAN32
CD81
TSSC4
TRPM5, KCNQ1, KCNQ1OT1
FIBIN
BBOX1
HIPK3
KIAA1549L
CD59
C11ORF91
FBXO3
LMO2
CAPRIN1
DLG2
SORL1
MIR100HG
BLID
UBASH3B

CBL is a frequent target of chromosome 11q aberrations

We found that UPDs of chromosome 11q are the most recurrent defects in the dataset. A number of studies have shown that, 11q UPDs were associated with mutations of the *CBL* gene (143, 144, 250). Almost all mutations of *CBL* described to date have been confined to exons 8 and 9 or their exon-intron junctions (143, 144, 250). Therefore, we sequenced these two exons of *CBL* in all samples that harbored chromosomal aberrations overlapping the *CBL* locus. Of the 14 patients that had 11q UPDs we detected SNVs in 9 patients. One patient harbored a 6 bp tandem duplication (**Figure 10A**). Out of 6 patients that had 11q gains overlapping *CBL*, one had a somatic mutation in *CBL* (C384Y). PCR subcloning revealed that the gain amplifies the mutant allele. No mutations were detected in the 7 patients with deletions overlapping *CBL*. For the patients where we had control tissue available, the somatic origin of the variants detected in *CBL* was confirmed. In order to identify mutations in other exons of *CBL* or in other genes that potentially associate with 11q aberrations we performed whole exome sequencing on samples from 3 patients with 11q uniparental disomy and two samples with 11q gains which did not have mutations in exons 8 and 9 of *CBL*. For two of the samples with UPDs we had matched control samples available, for which we also performed whole exome sequencing. Therefore we were able to directly search for somatic mutations in these two samples solely based on whole exome data. Of the five whole exome sequenced samples, only one sample showed a somatic mutation in *CBL* at the 3' splice site of exon 7.

CBL mutations associate with leukemic transformation of MPN

In order to test if *CBL* mutations in particular associated with disease progression, we sequenced *CBL* exons 8 and 9 in a total of 52 post-MPN AML samples and 288 chronic phase MPN samples. *CBL* mutations were present in 1% of chronic phase and 10% of post-MPN leukemic patients, respectively. Thus, *CBL* mutations are significantly associated with post-MPN leukemia (P=0.0032; Fisher's exact test). A particularly informative patient had two mutations of *CBL* affecting exon 8 (W408C) and the 5' splice site of exon 9 (**Figure 10B**). Both mutations were somatic and PCR subcloning and sequencing revealed that these two mutations were on independent DNA strands. As all the bacterial clones analyzed contained only one of the mutations and no clones with wild type *CBL* were

detected, we concluded that the patient harbored a compound heterozygous progenitor clone with distinct mutations of both *CBL* alleles (**Figure 10B**).

Mechanisms that increase mutant CBL dosage

Our data on *CBL* suggest that there are several different genetic mechanisms by which the malignant clone can increase the mutant *CBL* allele dosage (**Figure 10C**). The first mechanism is via mitotic recombination resulting in UPD. The second mechanism is amplification of the mutant allele by duplication. Another possibility is the inactivation of wild type alleles by two independent point mutations (compound heterozygosity). Interestingly, it seems possible that loss of a single *CBL* allele (haploinsufficiency) might be oncogenic as 7 patients in our cohort carried hemizygous *CBL* deletions (**Figure 9**). In support for this hypothesis, heterozygous *Cbl* deficiency in mice showed accelerated blast crisis compared to *Cbl* wild type animals in a BCR-ABL transgenic murine model (144). In addition, hemizygous deletions of *CBL* have been recently shown by others in MDS and related disorders (145).

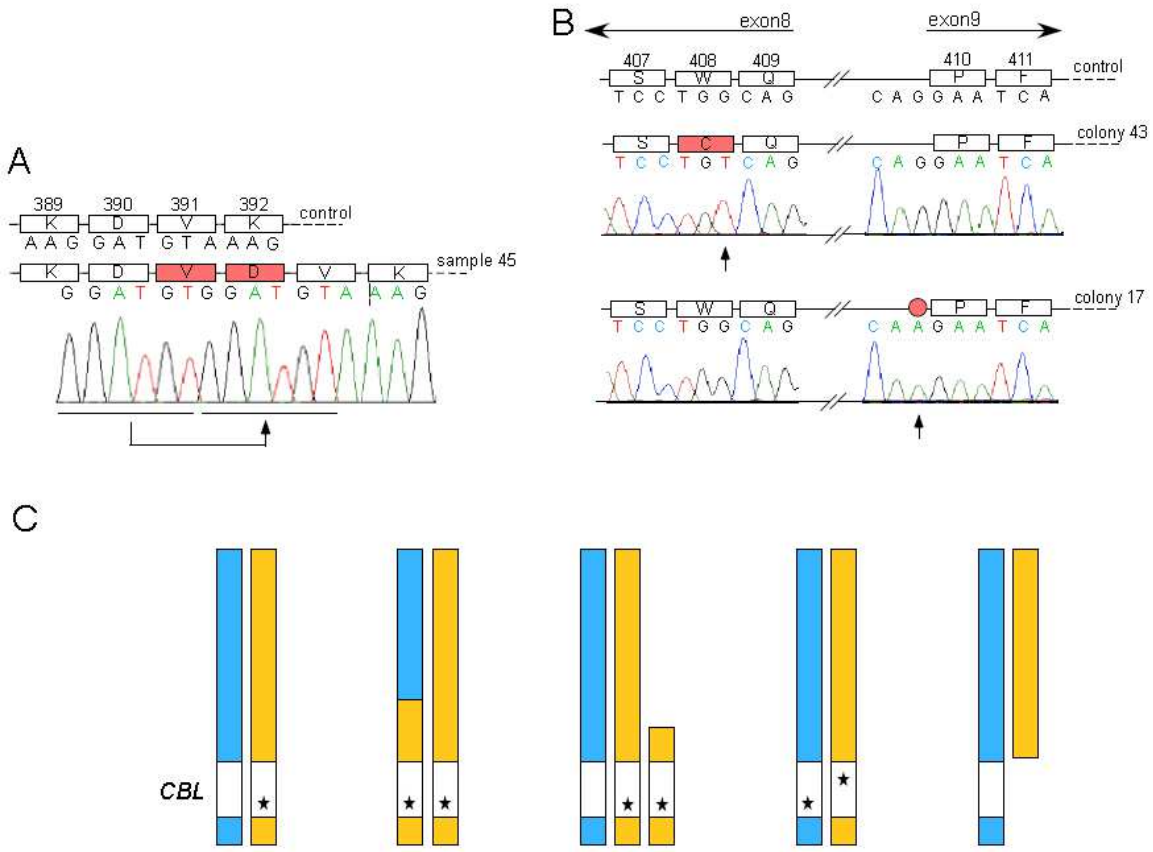


Figure 10. Mutational patterns in CBL. **A:** A 6 bp tandem duplication in CBL leads to the insertion of the amino acids valine (V) and aspartic acid (D) after position 390. **B:** One sample identified in a cohort screen for mutations in *CBL* exons 8 and 9 carried two mutations, one in exon 8 (W408C) and a second one in intron 8 at the splice acceptor site (G to A). PCR subcloning and analysis of colony DNA revealed that the two mutations are on different alleles. Depicted are two representative colonies. Colony 43 has the mutation in exon 8 but not in intron 8 whereas colony 17 shows the opposite case. **A+B:** Depicted are the genomic (letters) as well as the respective amino acid (box chains) sequences. Numbers indicate amino acid positions in the Cbl protein. Amino acids, which are substituted due to mutations are in red boxes. The splice site alteration is a red circle. Black arrows indicate the positions of the mutations below the Sanger sequencing traces. **C:** Overview of *CBL* mutagenesis in MPN. Different genetic mechanisms are involved in increasing mutant gene dosage of *CBL*. Each panel shows schematically the two parental copies of chromosomes 11 (blue and yellow) and the *CBL* gene (white rectangles). Mutations are indicated with asterisks. From left to right: heterozygous mutation in *CBL*; uniparental disomy introduces homozygous *CBL* mutations; gain of a part of chromosome 11q leads to a duplication of the *CBL* mutation while one wild type allele is still present; compound heterozygosity established by two different mutations on the different alleles of the *CBL* gene in one cell. In addition, the loss of a part of chromosome 11q deleting one *CBL* allele and leaving the other allele unaffected (wild type *CBL*) is likely to introduce phenotypes due to haploinsufficiency.

A mutation of DDB1 associated with 11q UPD

Recently, mutations in the splicing factor 1 gene *SF1* and a member of the polycomb complex 2 (*EED)* were found in myeloid malignancies (147, 251). Both genes are located on chromosome 11q (**Figure 9**), however we did not find any mutations in these two genes by either whole exome or Sanger sequencing of *EED* and the C-terminal proline-rich region of *SF1* that was found to be the mutational hotspot of the gene(252). All samples

that had aberrations spanning the two loci were analyzed (**Figure 9**).We performed whole exome sequencing data of five samples and attempted to identify genes other than *CBL* that might be associated with aberrations of chromosome 11q. In two of the patients, we were able to perform a paired analysis as whole exome sequenced T lymphocyte DNA was available as germline control. In one case we did not find any somatic mutations with an allelic frequency > 50% what is expected for all variants within the fully clonal 11qUPD region of this patient. Another case harbored in addition to the somatic mutation in *CBL* described above another somatic mutation in *DDB1* (**Figure 11**). The *CBL* and *DDB1* mutations in this sample were validated by Sanger sequencing and shown to be homozygous and fully clonal (**Figure 11**). The *Polyphen2* tool used to predict functional effects of human non-synonymous single nucleotide variants estimated the variant in *DDB1* to be "probably damaging" with the highest probability score of 1. *DDB1* was originally identified in patients suffering from *Xeroderma pigmentosum*, with inherited deficiency in nucleotide excision repair (NER). The gene was cloned together with its binding partner *DDB2* with which it forms the DDB protein complex (253). Later, *DDB1* was found to form an E3 ubiquitin ligase complex together with *CUL4A*, *ROC1* and a variable fourth protein that determines the target specificity of the E3 ligase. More than 30 different proteins have been identified as binding partners (254). The ubiquitination activity of DDB1-CUL4A-ROC1 complexes has been shown to play important roles in NER (reviewed by (255)) but also in regulating the expression of the tumor suppressor *CDKN2A* (256). *CDKN2A* gene expression is associated with histone 3 – lysine 4 (H3K4) trimethylation mediated by the MLL-RBBP5-WDR5 complex. RBBP5 and WDR5 are two of the binding partners of the DDB1-CUL4A-ROC1 complex. The authors could show that *DDB1* expression is required together with *MLL* for proper *CDKN2A* transcriptional activation (256). Inactivating mutations of *DDB1* are therefore likely to contribute to cancer not only by impairing NER, but also by preventing the transcription of tumor suppressor genes. It remains to be seen if the described example of a concerted action of *DDB1* and *MLL* is unique or if there is a systematic relationship between these two genes that might play a role in hematologic malignancies.

In the remaining three samples that were whole exome sequenced we identified a number of SNVs and small indels that we could validate by Sanger sequencing. Only one

gene appeared recurrent in this dataset, *HEPHL1*. Two patients harbored both an SNV in the *HEPHL1* gene, which function of *HEPHL1* is not known.

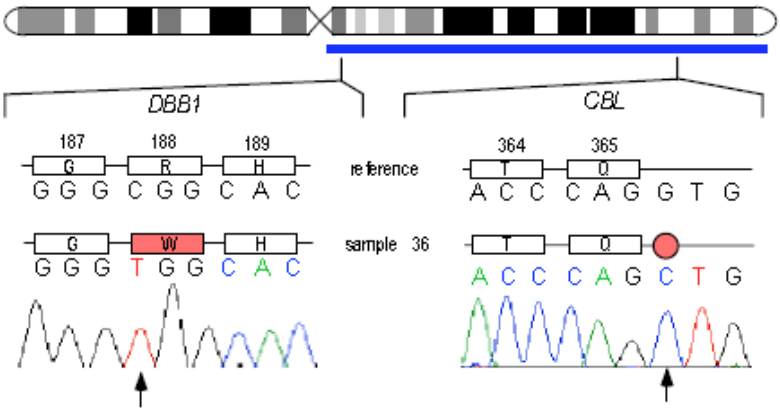


Figure 11. Sample harboring an 11q UPD as indicated by the blue bar below the chromosome 11 ideogram. We found two somatic mutations in *DDB1* and *CBL*. As can be seen in the Sanger sequencing traces, both mutations are homozygous due to amplification by the UPD. The red circle indicates a splice site mutation. Reference and mutant sequences are shown. The arrows indicate the site of mutations below the Sanger sequencing traces.

A tandem duplication within exon 3 of MLL associated with 11q UPD

In order to find small scale genetic alterations that are either too small to be detected by Affymetrix microarrays or too large to be detected by the GATK pipeline, we analyzed exome coverage data obtained after alignment of the NGS reads to the human reference genome. We compared the coverage data of each of the five exome datasets to a set of control samples to identify regions of focal deletions or gains on chromosome 11q. In one sample we were able to detect a focal amplification in exon 3 of *MLL* (**Figure 12**). Independent analysis by Sanger sequencing revealed a 513 bp tandem duplication in *MLL* exon 3. This duplication translates to an in-frame duplication of 171 amino acids from position 528 to 698 of the MLL protein (uniprot ID Q03164-1) (**Figure 12**). We do not have control tissue of this patient available to test for the somatic or germline origin of this

duplication. However, this duplication was absent in 196 controls ruling out the possibility of a common germline polymorphism. *MLL* is a well-studied target gene for translocations in various hematological malignancies (257). Tandem duplications in *MLL* have been described but usually affect the region from exon 3 to exon 9, 10 or 11 (258). Small tandem duplications like the 513 bp duplication within exon 3 described here have not been reported so far.

By comparing all chromosome 11q deletions detected by Affymetrix microarrays we identified two common deleted regions (CDRs) that contained only few genes as indicated in **Figure 9**.

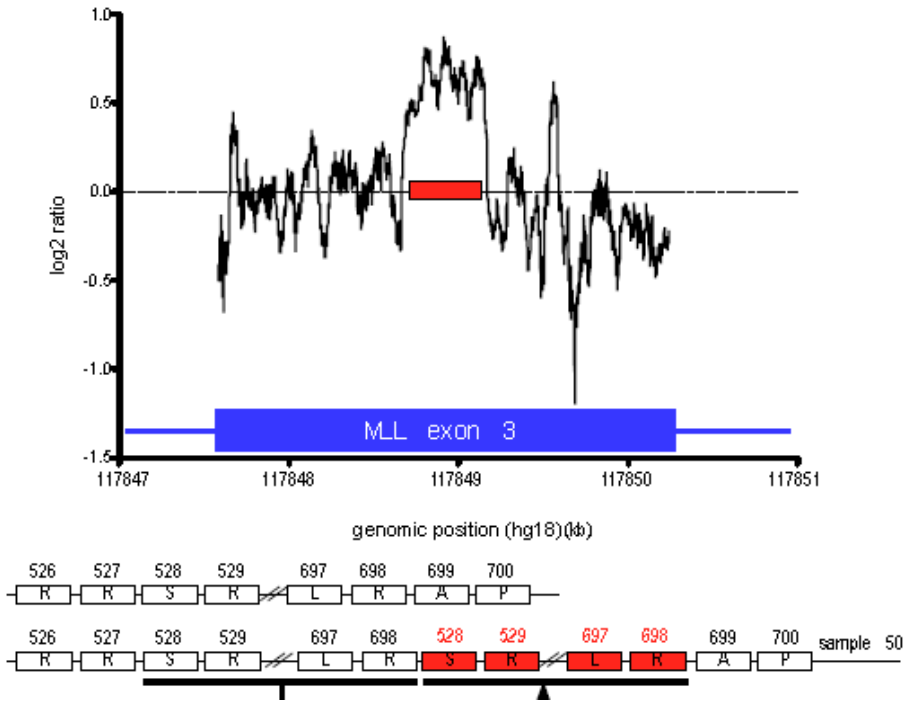


Figure 12. In one sample a tandem duplication in *MLL* exon 3 was detected. The top graph shows whole exome coverage data across *MLL* exon 3. The data is plotted as the log2 ratio of the normalized coverage in the patient sample divided by the median normalized coverage of 8 independent control samples at each genomic position (X-axis). The position of the duplication is indicated by the red bar. Sanger sequencing confirmed an in-frame tandem duplication of 171 amino acids as shown at the bottom.

CDRs of chromosome 11p associate with de novo AML or target LMO2

On chromosome 11p we identified 4 CDRs (**Figure 9**). The most telomeric CDR contained 14 genes. Interestingly, we found a significant association of aberrations spanning this CDR with de novo AML compared to secondary AML (P = 0.013). It is likely that one or more of the genes in this region play a particular role in *de novo* AML pathogenesis. The most centromeric CDR on chromsome 11p contains the *LMO2* gene. In one sample we detected a deletion spanning the *LMO2* locus and an SNV in *LMO2* in the remaining allele (c.G388A; p.G130S, Uniprot ID P25791-3) which was hemizygous in Sanger sequencing traces (**Figure 13**). The *Polyphen2* tool estimated the variant to be "probably damaging" with the hightest probability score of 1. Due to lack of control tissue in this patient we could not analyze the somatic or germline origin of this SNV. Based on the available data we conclude that there is a full loss of *LMO2* activity in this patient. We tested all other patients with aberrations overlapping the *LMO2* locus, but were not able to find any mutations in the coding region or at splice sites of *LMO2*. The deletions were detected across several different pathologies. *LMO2* is frequently involved in translocations in T-cell leukemia (259). It is expressed in different fetal tissues (260) and the full knockout in the mouse was shown to be embryonic lethal (261). Warren et al. showed that *LMO2* is essential for erythroid development in the mouse. The group confirmed that this defect is intrinsic to the hematopoietic system and specific for the erythroid lineage by in vitro differentiation assays (261). Interestingly the patient in our study showed anemia with hemoglobin level of 97 g/L at the time of sampling.

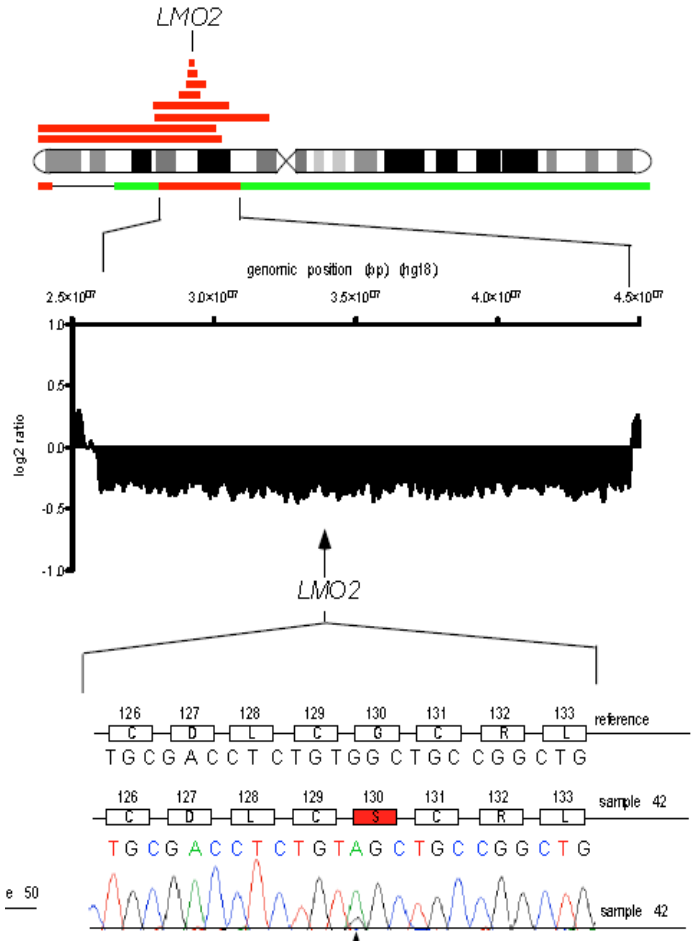


Figure 13. A common deleted region on chromosome 11p targets LMO2. All deletions in the analyzed cohort that span the LMO2 locus are depicted next to the chromosome 11 ideogram. Red bars indicate deletions, green bars indicate gains. In one sample which harbored a deletion spanning the LMO2 locus we detected a point mutation in LMO2 on the other allele. The middle section shows a signal intensity plot measuring copy number from Affymetrix microarrays. The plot depicts signal intensity (log_2 scale) differences between the patient and a healthy control pool for each probe (as implemented in the Affymetrix Genotyping Console software). The point mutation in LMO2 as identified by Sanger sequencing is depicted at the bottom of panel.

FOCUS ON A SINGLE PATIENT WITH POLYCYTHEMIA VERA AND COMPLEX TRANSLOCATION

CLINICAL FEATURES

The patient, a man of 61 years, at the onset of the disease presented hemoglobin 23.2 g dL with an hematocrit value of 71.5%, white blood cells and platelets within normal range. He was diagnosed with Polycythemia Vera on the basis of hemoglobin level, of the bone marrow biopsy showing hyperplasia of erythropoietic and megakaryocytic lineages, and of the presence of endogenous erythroid colonies (EEC). Neither the examination of the karyotype nor the JAK2V617F mutation had been performed, since the latter was not yet known at the time of diagnosis. Clinically, the patient had a splenomegaly of 5 cm below the left costal margin. For the following ten years, the patient was followed at another center and treated with hydroxyurea and phlebotomies. The only clinically significant event was a deep vein thrombosis of the lower limbs three years after diagnosis. We visited again the patient ten years after diagnosis, when he reported worsening of general conditions, fatigue and night sweats. The spleen appeared increased in size up to 14 cm below the costal margin. The CBC count showed the appearance of leukocytosis despite treatment with hydroxyurea (WBC 17.4 $x10^9$/L), hemoglobin 15.4 g/dL and hematocrit 52.9%, platelet count 212 x 10^9/L. The JAK2V617F mutation was positive with an allele burden of 85% and the circulating CD34+ cell count was $14x10^6$/L. A bone marrow re-evaluation showed the presence of hyperplasia of myeloid and erythropoietic lineages and grade 1 fibrosis according to the European classification(60). LDH was increased (595 U/L), while the peripheral blood smear showed no leukoerythroblastic picture but only few dacrocytes. On these basis, the patient presented the features of a polycythemia vera with trend in evolution to secondary myelofibrosis, however without fulfilling the criteria for this diagnosis(59). The conventional karyotype performed from bone marrow blood showed the presence of a translocation t(15;6). A bone marrow biopsy and cytogenetic examination repeated after one year showed an overt evolution in post polycythemia vera myelofibrosis due to worsening of bone marrow fibrosis from grade 1 to grade 2, while the cytogenetic alteration was still present. . The patient did not present any mutation in EZH2, ASXL1 and IDH1/2 genes.

Molecular characterization of the not reciprocal, mono allelic translocation t(15;6)

The cytogenetic examination showed a deletion in the long arm of chromosome 15 in the region q21.3 in all the mataphases analyzed. The same test repeated on peripheral blood lymphocytes was instead consistent with a normal male karyotype (46, XY), allowing to conclude that the translocation is somatic. A first FISH analysis showed that the region 15q22, that includes the PML gene, was translocated and inserted in the short arm of chromosome 6.

To define the exact breakpoints, we used a Genome-Wide Human SNP Array 6.0 (Affymetrix), while to determine the orientation of the translocated fragment we performed a FISH analysis with BAC clones. Moreover, we analyzed granulocytes also with a next generation technology, the Illumina mate pair sequencing, thanks to the collaboration with prof. Tefferi, Mayo Clinic, Rochester, MN, USA. From that integrated approach we were able to conclude that the region of chromosome 15 included between 62,316,971 bp and 77,265,237 bp, bands 15q25.1 - 15q22.2, resulted translocated into the region 6p21.2 - 6p22, maintaining the centromere-telomere orientation. The same region in chromosome 6, between 18,163,058 bp and 29,891,198 bp, was deleted. Moreover, the translocated region was flanked by a deleted region (54,121,513 bp-62,316,971 bp)

Two breakpoins take origin from this translocation:

-in the first, the chromosome 6 at the basis 18,163,058, that lies in the intronic region between exon 4 and exon 5 of the KDM1B gene (forward strand), was placed side by side to the chromosome 15 at the basis 62,316, 971, in the intron between exons 6 and 7 of VPS13C gene (reverse strand) (GRCh37/hg19). No fusion genes origins at this level.

-in the second one, the basis 29,891,198 of chromosome 6 was juxtaposed to the 77,265,237 base of chromosome 15. On both chromosomes, the base pair position lies in intergenic regions (NCBI36/hg18).

SNP analysis showed that on the derivative chromosome 15, the deletion and translocation made close the FAM148A gene and a genomic region without genes included between NEDD4 (not deleted) and RFX7 gene (deleted). **Figure 14.**

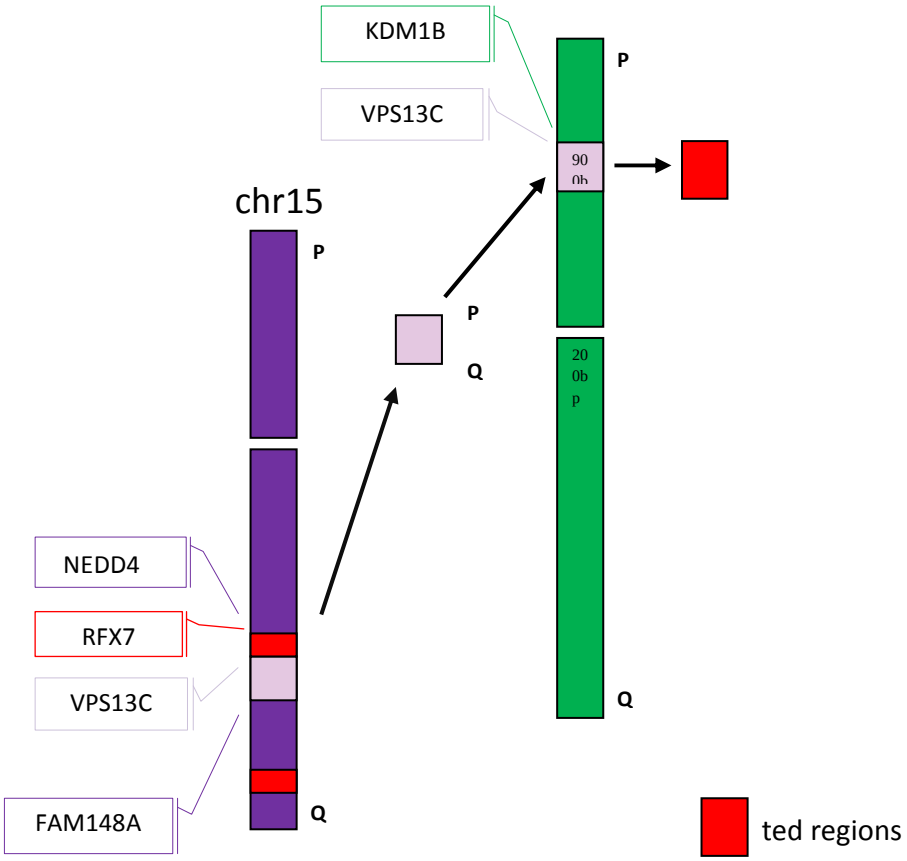


Figure 14. Diagram of translocation t(15;6).

As a consequence of the translocation, the function of different genes can be altered through various mechanisms, such as haploinsufficiency, loss of functional domains, changes in the regulation of expression due to fusion with promoter sequences of other genes or to a to position effect. To investigate this aspect in our case we studied the gene expression and/or we looked for new mutations in genes with potentially interesting function for the pathogenesis of Polycythemia Vera .

KDM1B

The KDM1B gene, also known as AOF1 or LSD2, maps in the 6p22.3 region and is composed by 22 exons. It is the second histone demethylase in the family of flavin-dependent amine oxidases with specificity for H3K4me1 and H3K4me2 and reveals a

demethylase-independent repression function. It is composed by a zinc-finger domain for the DNA binding and a highly conserved SWIRM domain that is required for its enzymatic activity. KDM1B is found to localize to chromosomes during the mitotic phase of the cell cycle, and represses transcription when tethered to DNA. Demethylation of H3K4 is critical for establishing the DNA methylation imprints during oogenesis(262). LSD2/AOF1/KDM1b catalyzes demethylation of mono- and di-methylated H3K4 and plays an important role in transcriptional regulation and genomic imprinting(263).

Because in our case the KDM1B gene loses both Zn finger e SWIRM domains, we sequenced the exons that encode for the functional domains to look for mutations that can have a functional role through haploinsufficiency, but we failed to discover any mutation. Moreover, we evaluated KDM1B expression in granulocyte cells of our patient, samples from other patients with MPNs and healthy controls. As showed in **figure 15**, the translocation did no't affect the KDM1B expression.

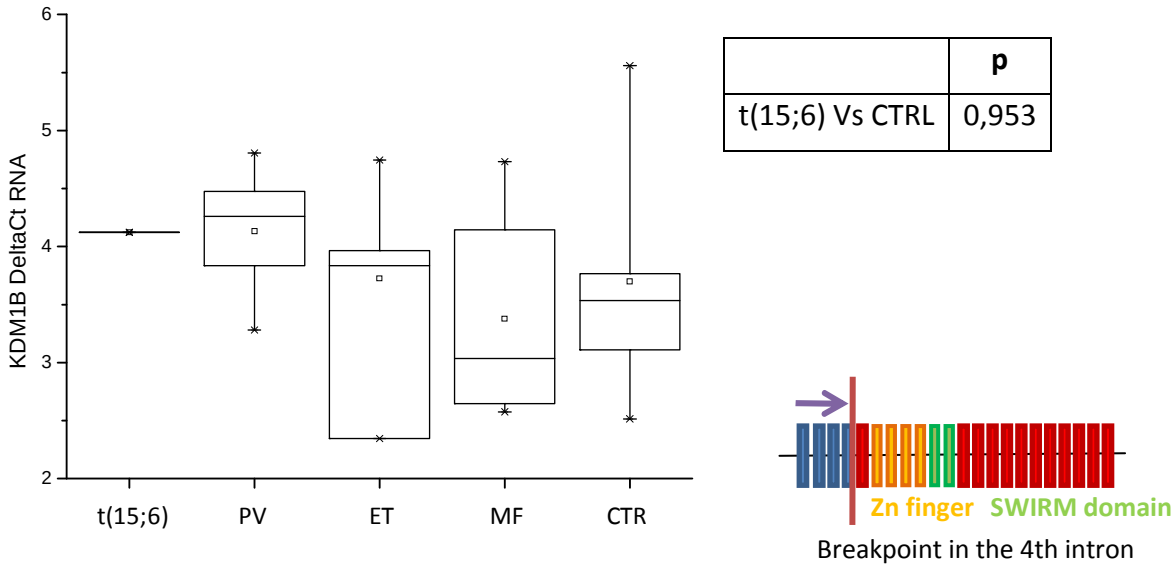


Figure 15. Gene expression of KDM1B and structure of the protein with its functional domains. The red line indicate the breakpoint, the arrow the direction of transcription. The bars represent the exons; in blue are reported exons maintained after translocation.

VPS13C

The VPS13C maps in the 15q22.2 region and its structure includes 85 exons. It encodes a member of the vacuolar protein sorting-associated 13 gene family, but its function is not well characterized to date. Moreover, after translocation it maintains all exons that encode for the functional domains, so we reserved to evaluate expression and mutations eventually in the next future.

RFX7

Located in the 15q21.3 band, is deleted in our patient. It is a member of the regulatory factor X (RFX) family of transcription factors expressed widely and heavily expressed in many different tissue types including kidney (tumor tissues), thymus, brain, and placenta(264). It was reported as part of a set of genes identified as outcome predictors of metastatic outcome in breast cancer(265). We did not find any statistically significant difference in its expression by comparison of our patient with healthy controls. (**Figure 16**)

NEDD4

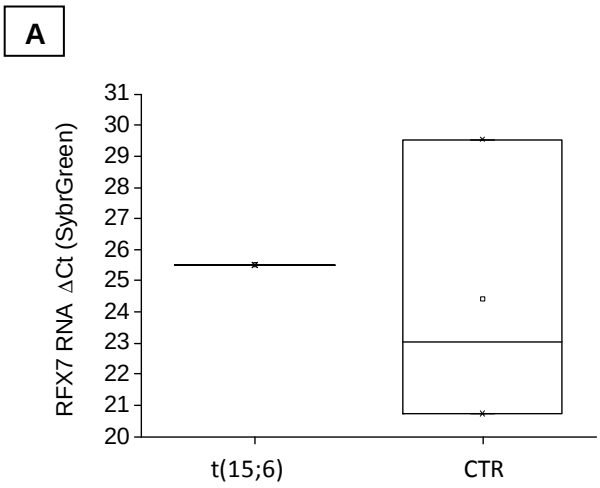
Located in the band 15q21.3, is not deleted nor interrupted but close to the breakpoint, so a position effect was conceivable. NEDD4-1 as an ubiquitin ligase for the tumor suppressor PTEN, that can polyubiquitinate PTEN and thus targets PTEN for proteasomal degradation(266). Moreover, NEDD4 could also positively regulate PTEN through monoubiquitination of PTEN in human and mouse cells. Monoubiquitinated PTEN was stabilized by its accumulation in cell nuclei, and it retained its ability to antagonize AKT and cause apoptosis(267). NEDD4 is also overexpressed in colorectal cancers, where it promotes growth of colon cancer cells independently of PTEN and PI3K/AKT signaling(268), and NEDD4-1 overexpression also augmented the tumorigenicity of lung cancer cells(269). NEDD4 had the same expression level in the patient in exam and in healthy subject. (**Figure 16**)

FOXB1

Located in the deleted fragment of chromosome 15, it encodes for a member of the Human Forkhead-box (FOX) family, that consists of at least 43 members. Even if FOXB1 alterations have not been reported in hematological neoplasms, we investigated this gene due to the importance of various members of FOX family in human diseases. FOXA1 gene is amplified and over-expressed in esophageal and lung cancer. FOXM1 gene is up-regulated in pancreatic cancer and basal cell carcinoma due to the transcriptional regulation by Sonic Hedgehog (SHH) pathway. FOXO1 gene is fused to PAX3 or PAX7 genes in rhabdomyosarcoma. FOXO3 and FOXO4 genes are fused to MLL gene in hematological malignancies. Deregulation of FOX family genes leads to congenital disorders, diabetes mellitus, or carcinogenesis(270).

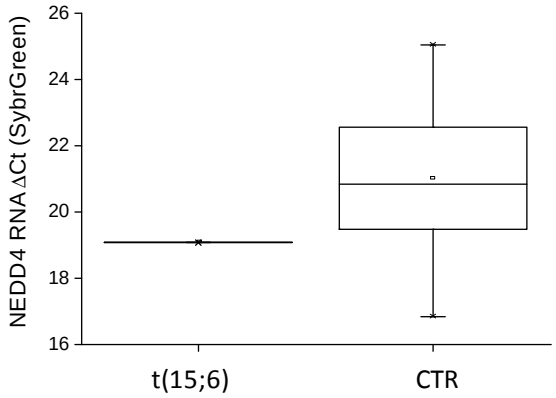
From our expression analysis, the FOXB1 levels appear to be reduced in the patient with the translocation, in unselected Polycythemia Vera and Essential Thrombocythemia patients when compared to healthy controls. However, further analysis is needed to confirm the data. (**Figure 16**).

We also sequenced FOXB1 gene in our patient, without finding any mutation.



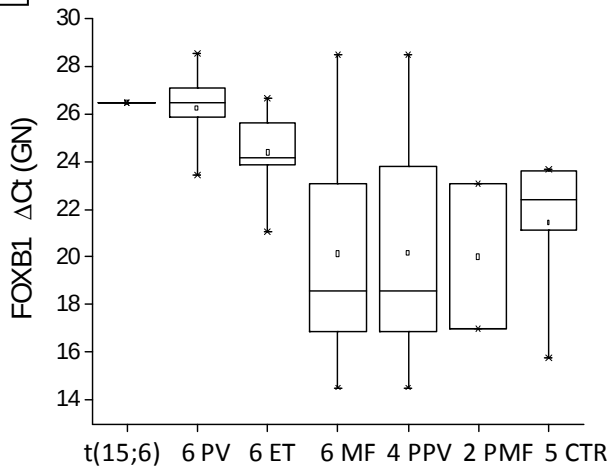
	p
t(15;6) Vs CTRL	0.857

B



	p
t(15;6) vs CTRL	0.032

C



	p
T(6;15) Vs CTRL	0.134
T(6;15) Vs PV	0.923
PV Vs CTRL	0.0067
ET Vs CTRL	0.04
MF Vs CTRL	0.518
PPV Vs CTRL	0.57
PMF Vs CTRL	0.56
PV Vs PPV	0.03

D

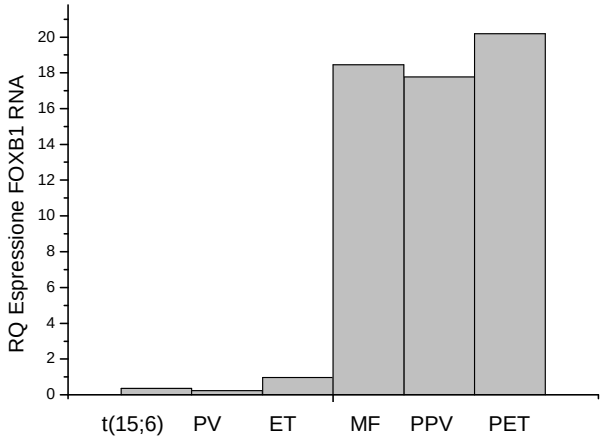


Figure 16. Comparison of expression levels of RFX7 (A), NEDD4 (B) (Sybr-Green) and FOXB1 gene (TaqMan gene expression assay) expressed as ΔCt (C) and RQ (D) in the patient with t(15;6), other MPN patients and healthy controls. Analysis performed in duplicate.

Molecular characterization of the not reciprocal, mono allelic translocation t(15;18)

The analysis of the sample of our patient with the Illumina mate pair sequencing technology allowed to identify a second translocation involving still chromosome 15 and the chromosome 18. Through the SNP array analysis was already identified a region of mono allelic deletion on chromosome 18q11.21-22, but using this new generation technology has been possible to determine that a fragment of 10KB of Chr15 between bands 15q25.1 and 15q25.1 inserted into 2.75MB deletion of Chr18. In particular the region of chromosome 15 between 79,486,994 and 79,476,511 bp inverted and went in the short arm of chromosome 18 (reversing the order centromere-telomere), in correspondence with the deleted region of between 9,586,502 and 12,160,974 bp.

Moreover, at the level of chromosome 15, the data obtained from the analysis of SNP array demonstrate that the translocated part is flanked by a deleted region, located after 79,512,256 bp, that lead to partial loss of the gene EFTUD1 (chr 15q25.2) **(Figure 17).**

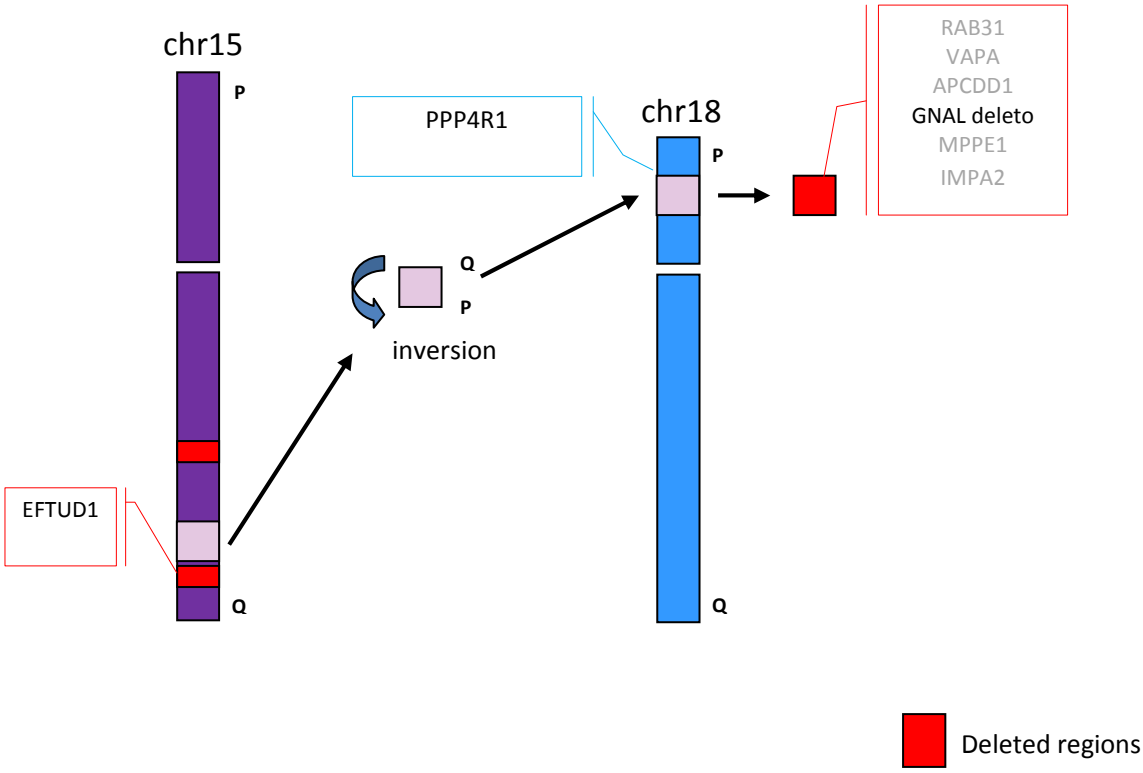


Figure 17. Diagram of translocation t(15;18).

In the derivative chromosome 18, the first breakpoint is formed by an intergenic region of chr 15q (79,486,994) that became in close proximity to the intron between exons 12 and 13 of gene PPP4R1 of chr 18p (9,586,502), without giving rise to fusion genes. Instead, the second breakpoint involves an intergenic region in both chromosomes. At the level of chromosome 15, the loss of the deleted and translocated fragments leads to the deletion of the gene EFTUD1. The deleted region of chromosome 18p contains several genes.

PPP4R1

Encode for the regulatory subunit 1 of the protein phosphatase 4. PPP4R1 interacts with PPP4C which is implicated in the regulation of histone acetylation, DNA damage checkpoint signaling, NFκB activation, and microtubule organization at centrosomes. The downregulated expressions of *ZFP161, PPP4R1* and *YES1 has been* correlated with the luminal B subtype of breast cancer, suggesting their potential involvement in the genesis of a particularly aggressive form with 18p loss(271). In the case on exam, PPP4R1, through

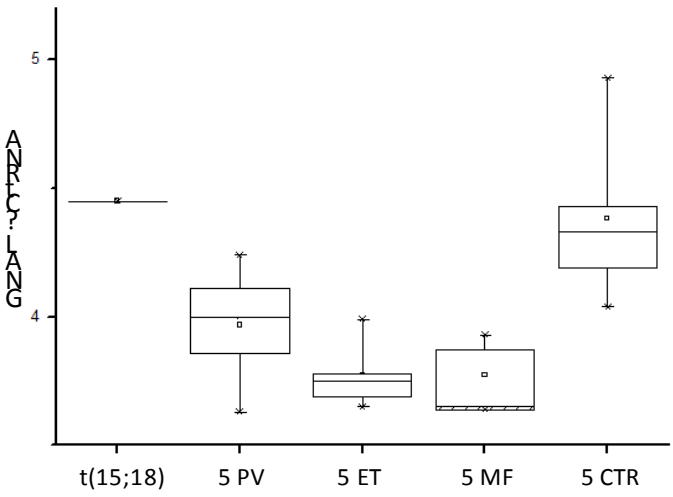
interrupted by the translocation, maintains the functional domain, so we did not pursued in further analysis.

GNAL

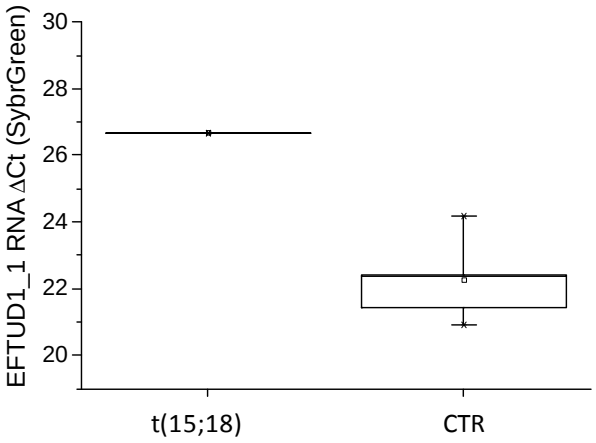
Following the identification of genes including in the deleted regions of chromosomes 15 and 18, we focused on the evaluation of gene GNAL (chr 18p11.21), that consists of 16 exons and encodes a G protein-alpha. Previous reports have identified downregulation of GNAL in leukaemic cells(272) Moreover, GNAL is downregulated significantly by V617F in HeLa cells, while its knockdown in the HepG2 cancer cell line significantly increases cellular proliferation. In both systems, disease-related JAK mutations result in differential regulation of this Gα subunit to promote cellular proliferation and tumorigenesis, supporting the possibility that this locus has an important effector function downstream from JAK/STAT signaling (273). In our case we first assessed the expression pattern of this gene, using TaqMan Gene Expression Assay Applied Biosystems. From the results we observed a downregulation compared to other cases of MPNs but with comparable expression levels as healthy controls **(Figure 18).** We also sequenced the entire gene but without finding any mutation.

EFTUD1

EFTUD1 (elongation factor Tu GTP binding domain containing 1) is an elongation factor not well characterized yet. Sequencing of the coding exons didn't show any mutation. Gene expression analysis revealed a down regulation in the patient with translocation compared to healthy subject.



		P value
GM Vs	PV	0,13458
GM Vs	TE	0,00938
GM Vs	MF	0,02696
GM Vs	CTR	0,86749
PV Vs	TE	0,14270
PV Vs	MF	0,19390
PV Vs	CTR	0,05408
TE Vs	MF	0,99589
TE Vs	CTR	0,00551
MF Vs	CTR	0,01267



	p
t(15;18) vs CTRL	0.032

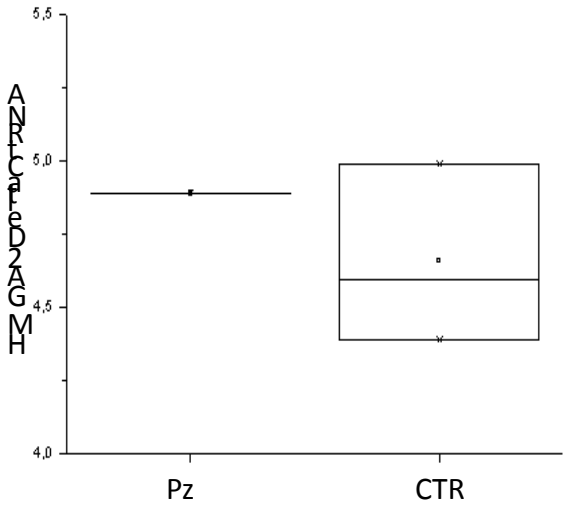
Figure 18. Gene expression analysis on EFTUD1 and GNAL genes.

HMGA2 analysis

HMGA2 (high mobility group AT-hook 2), which map in the 12q14.3 region, encodes for a type of nonhistone chromosomal protein. This family is often referred to as architectural proteins, participate in a wide variety of cellular processes including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the induction of neoplastic transformation and promotion of metastatic progression of cancer cells. The members are characterized by the presence of 3 copies of a conserved DNA-binding peptide motif (AT-hook) that preferentially binds with the minor groove of many AT-rich promoter and enhancer DNA regulatory elements (274, 275).

HMGA2 play also a crucial role in self-renewal and control of differentiation of a variety of stem cells, including cancer stem cells. In particular, overexpression on HMGA2 leads to a growth advantage in benign tumors and cancers. In most cases these tumors harbor a rearrangement of chromosome 12q13-15, causing a truncation or deletion of HMGA2 3'UTR. This alteration has been described in patients with MPN, MDS and MPN/MDS(276), and HMGA2 overexpression was reported as higher in patients with JAK2V617F positive PMF than in PMF patients without the mutation(277). To our knowledge, two case of polycythemia vera with HMGA2 involvement are described; in one a t(12;21)(q14;q22) involving the HMGA2 gene was present, while the other showed a t(3;12)(q26;q14) with breakpoint mapped within the 3'UTR of the gene. In both cases increased expression level of HMGA2 was present, suggesting that overexpression may contribute to the pathogenesis of the disease, regardless of the partner chromosome involved in the translocation. Moreover, in the case of involvement of 3'UTR region, the upregulation of HMGA2 was likely due to a position effect (278, 279).

We then examined whether the gene could be overexpressed in our case, assuming that its overexpression could be associated with genomic instability such as that postulated in an individual with cytogenetic abnormalities. The evaluation of the expression by Real Time PCR showed no difference compared to healthy controls and other subjects with polycythemia vera. **(Figure 19)**



	p
t(15;6)vs CTRL	0.58

Figure 19. Analysis of gene HMGA2 in granulocyte cells. The analysis was performed in duplicate for each sample.

Analysis of the fusion gene SETD1B/GTF2H3 obtained as a result of del(12q)

Analysis with mate pair sequencing, carried out by the research group of Prof. Tefferi of the Mayo Clinic in Rochester, USA, showed, in addition to the two translocations described above, a deletion on the long arm of Chr 12, which leads to loss of the chromosomal portion 12q24.31b-q24.31d; this deletion was confirmed by the Genome-Wide Human SNP Array 6.0 analysis.

The identified breakpoint localizes to the bases 122,260,315 in exon 11 of the gene SETD1B and 124,136,890 in exon 7 of the gene GTF2H3. From this deletion a fusion construct SETD1B/GTF2H3 originates that was confirmed using three different pairs of primers to amplify the same region (**Figura 20**):

SETD1B_EX11B_F+GTF2H3_EX7R(242bp);

SETD1B_EX9_F+GTF2H3_EX11_R(924bp);

SETD1B_EX7_F+GTF2H3_EX13_R (1351bp).

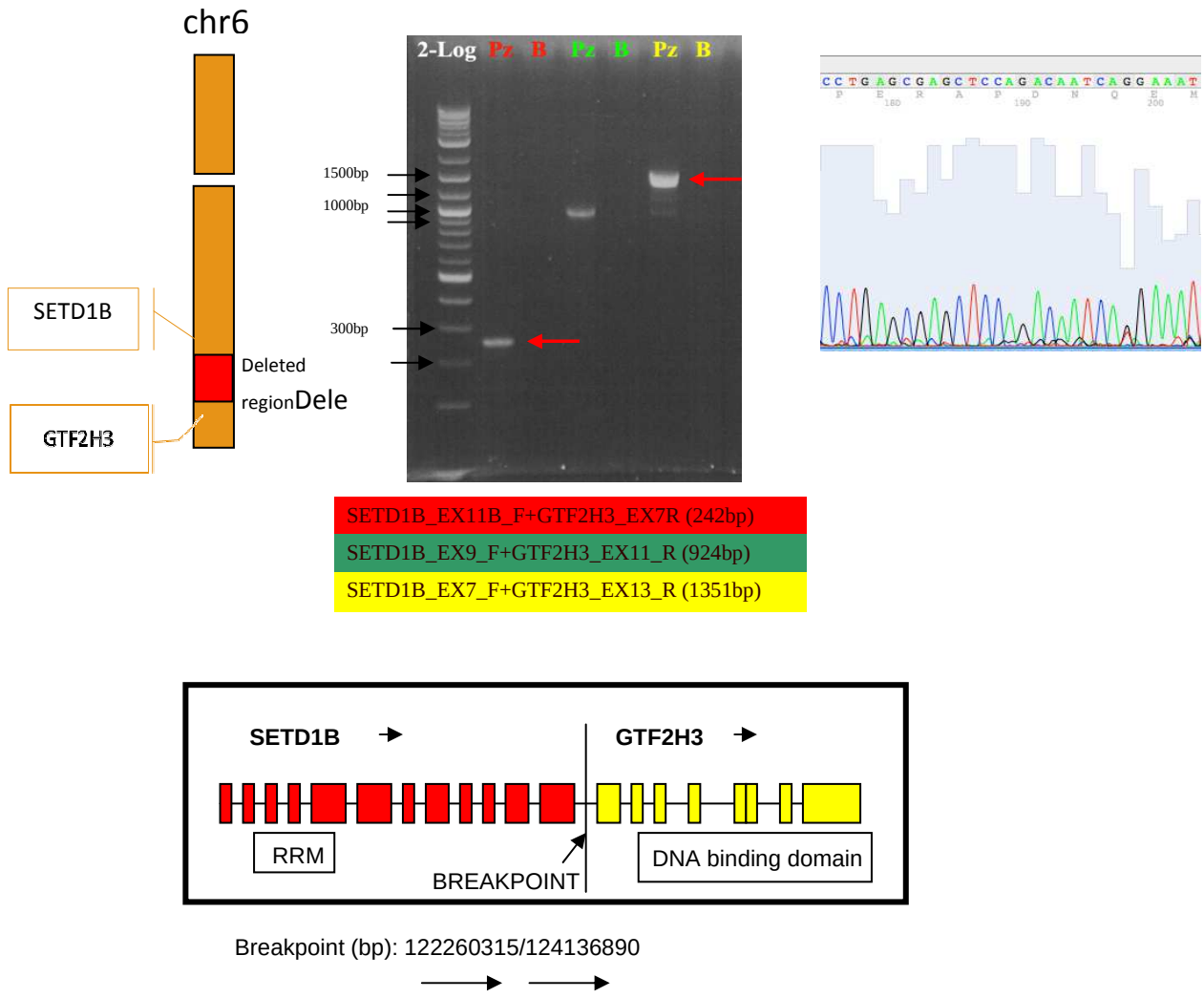


Figura 20. Identification of the fusion gene SETD1B/GTF2H3.

SETD1B

The gene SETD1B is part of the family of Set1-like enzymes, including Setd1a, Setd1b, mixed-lineage leukemia (MLL) 1, MLL2, and MLL3/4. Many of these enzymes have been implicated as critical epigenetic regulators of development. For example, rearrangements of the MLL1 gene are associated with aggressive acute leukemia in both children and adults; the MLL2 gene is amplified in some solid tumors; and the MLL3 gene is deleted in cases of myeloid leukemia. SET1B is a component of a complex that contains 5 noncatalytic components, including CXXC1, RBBP5, ASH2, WDR5, and WDR82; this

complex produces trimethylated histone H3 at Lys4, with a consequent role in epigenetic control of chromatin structure and gene expression. SETD1B contains conserved SET domain, which normally interacts with the other components of the complex, in the C terminus and an RNA recognition domain in the N terminus. (280, 281) In the subject under study, SETD1B loses the SET domain, while maintains the domain RRM, necessary to bind RNA .

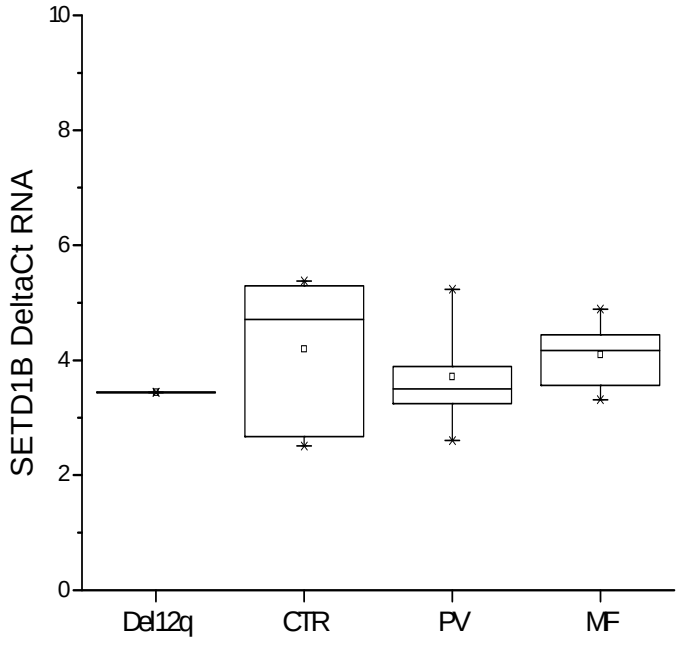
GTF2H3 (general transcription factor IIH, polypeptide 3)

This is the 34-kD subunit transcription factor IIH, associated with the RNA polymerase II transcription complex, which is involved in transcription and transcription-mediated DNA repair.(282)

As a first step we looked for the same fusion construct SETD1B/GTF2H3 by RT-PCR in other patients with MPN, in order to assess if it could be a cryptic deletion recurrent in these pathologies, not detectable with conventional cytogenetic techniques. We then selected among our patient database 20 PV, 20 ET, 15 PMF, PPV 5 and 4 PET subjects to evaluate the presence of the fusion gene by using all three PCR reaction that amplified fragment respectively of 1351 bp, 924 bp and 272 bp. In total we analysed 64 cases of MPNs, and we found the fusion transcript only in our subject under study.

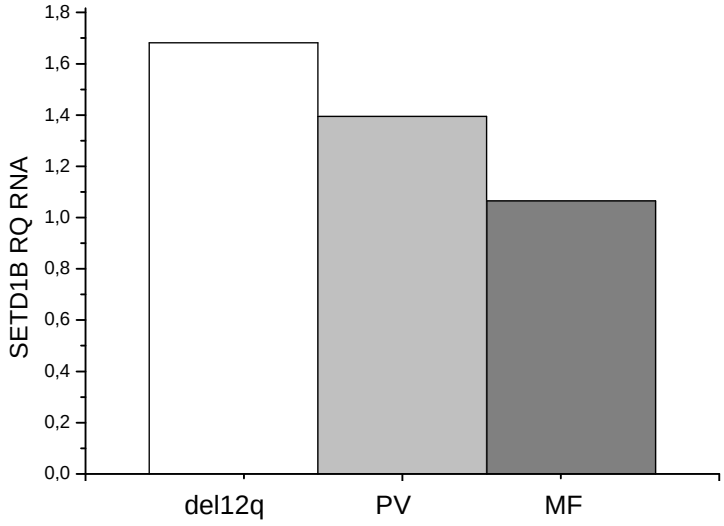
We performed direct sequencing of exons coding for the functional domains of both genes (GTF2H3 and SETD1B). In SETD1B gene mutations were not found, while in the gene GTF2H3 was found a point mutation 26015A> G in exon 11, which, however, does not lead to an amino acid substitution: 246P> P. Subsequently we evaluated the expression pattern of the retained portions of both genes using the Applied Biosystems Assay, comparing our subject with 8 PV, 7 PMF and 7 healthy controls.

From the results obtained in real-time PCR, SETD1B gene is not differentially expressed, while GTF2H3 is very significantly overexpressed compared to controls, PV and MF **(Figure 21 and 22)** .



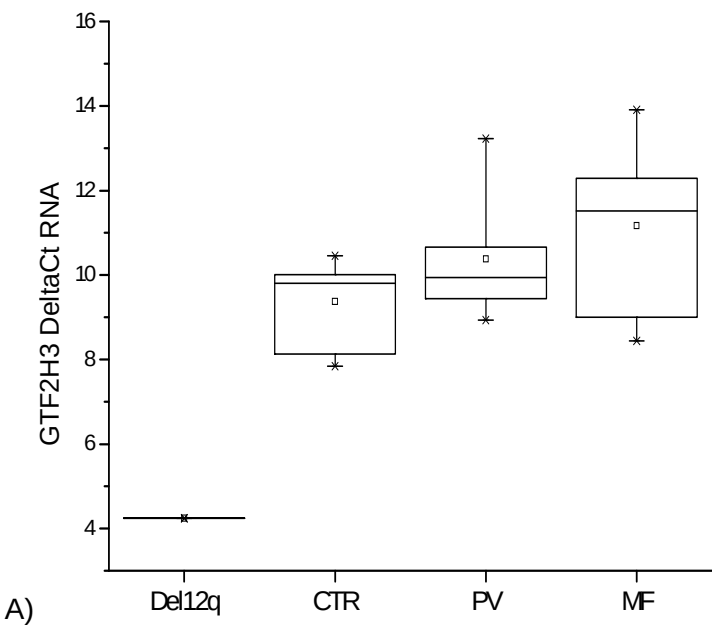
	P value
Del12q Vs CTR	0,59395
Del12q Vs PV	0,74467
Del12q Vs MF	0,29084
CTR Vs PV	0,38234
CTR Vs MF	0,85776
PV Vs MF	0,28871

A)



B)

Figura 21. Analysis of gene SETD1B expression in granulocytes. The analysis was performed in duplicate for each sample. The image A) shows the results as ΔCt, while Figure B) as relative quantification (RQ).



	P value
Del12q Vs CTR	0,00287
Del12q Vs PV	0,00328
Del12q Vs MF	0,01382
CTR Vs PV	0,12479
CTR Vs MF	0,04578
PV Vs MF	0,36057

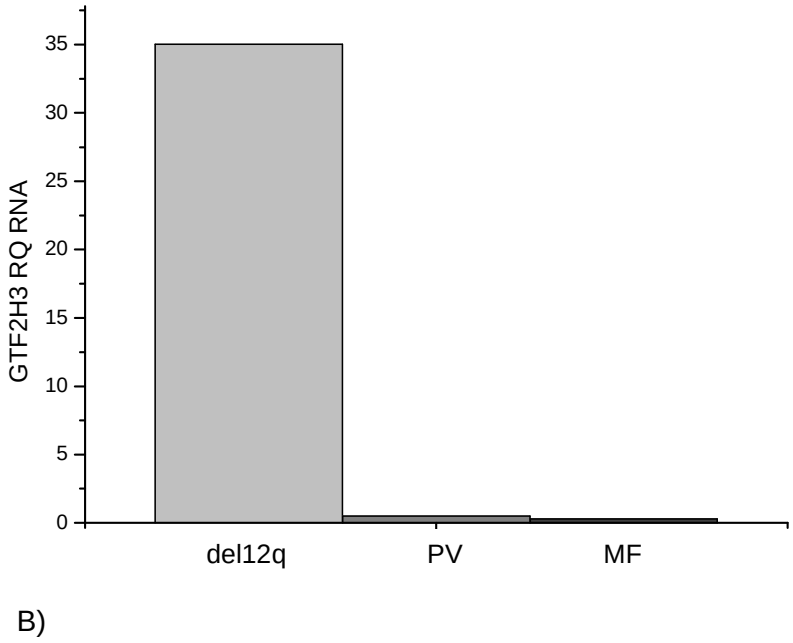


Figura 22. Analysis of gene GTF2H3 expression in granulocytes. The analysis was performed in duplicate for each sample. The image A) shows the results as ΔCt, while Figure B) as relative quantification (RQ).

Future perspectives include to assess if from the SETD1B/GTF2H3 chimeric transcript arises a fully functional fusion protein, and in case to perform functional tests in vitro after cloning.

Since SETD1B is homologous to MLL1, gene implicated in a series of genetic rearrangements in acute lymphoid leukemia and myeloid leukemia, we planned to evaluate through FISH analysis if SETD1B rearranged also with genes other than GTF2H3. For this analysis we collaborated with the group of Dr. Storlazzi the Department of Genetics and Microbiology of the University of Bari that analyzed metaphases and nuclei of mononuclear cells of 13 patients with PPV-MF. They found that in 2 patients a rearrangement was indeed present, even if in a small percentage of the analyzed nuclei (3/412). Further studies are ongoing to confirm the data obtained and to extend the series.

We also looked if in our series of patients analyzed with SNP array method, if this region of 12q (regardless of the fusion gene) was altered in a larger series of patients with MPNs. We found 14 additional patients showing deletions of 12q that have a common deleted region compared to our subject under examination. Our future plans include the analysis of minimal common deleted region, which locates between 122,277,433 and 122,907,179 bp, and includes the following genes:

-HPD: involved in the degradation pathway of tyrosine.
-PSMD9: encodes a subunit to non-ATPase regulatory subunit of the 19S proteasome.
-WDR66: belongs to a family of proteins is implicated in the regulation of the MPV, and polymorphisms have been associated with changes in the MPV.
-BCL7A: antiapoptotic BCL family of genes, involved in the rearrangement with IgH and MYC in Burkitt's lymphoma.
-MLXIP: interacts with MLX (transcription factor with a role in the proliferation and differentiation) forming heterodimers that bind E-boxes activating the transcription.
-LRRC43: unknown function.
-IL-31: active STAT1, STAT3, STAT5, but not STAT6. implicated in the pathogenesis of allergies, upregulates VEGF and activates the p38 MAPK.
-B3GNT4: Involved in the synthesis of oligosaccharides.
-DIABLO: activates apoptosis by inhibiting caspase inhibitor proteins.

-VPS33A: involved in the distribution of proteins in vesicles, mainly endosomes and lysosomes.

-CLIP1: binding protein localized at the level of cytoplasmic microtubules. Expressed in cells of Reed Stenberg in vivo and in monocytes in vitro.

DISCUSSION

The Ph-negative chronic myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) became one of the most studied topics in the field of oncohaematology in recent years due to the identification of some recurrent molecular alterations, such as mutations of JAK2 and MPL, that had a relevant impact on the understanding of the pathogenesis of typical MPN and on the identification of molecular target for new drugs. The JAK2 V617F mutation, which induces the constitutive kinase activity of the protein ("gain-of-function") and the activation of various intracellular downstream signaling pathways, like STAT5, STAT3 and MAP kinase, is present in the almost all cases of PV (95-99%) and in 50-60% of cases of ET and PMF. MPL mutations, which result in the substitution of tryptophan 515 with leucine (W515L) or lysine (W515K), have been identified in 5-10% of patients with PMF and 8% with TE, while they are absent in PV. However, neither these genetic abnormalities nor new mutations identified in other genes like TET2, ASXL1, EZH2, IDH1 and IDH2 are sufficient to explain different clinical and biological features of these disorders.

Other than single gene mutations, studies of MPN using classical metaphase cytogenetics reported around 30% of patients with aberrant karyotype (131, 132, 134). These abnormalities could point to locus containing gene mutations, such as is the case of trisomy of chr 9 where JAK2 resides, could suggest the existence of genes that influence the phenotype of the disease, or might hide new mutations with a potential pathogenetic role. Therefore, in collaboration with some other groups involved in research on MPN, including Prof. Kralovics in Vienna, Dr.. Storlazzi in Bari, Prof. Martinelli in Bologna and Prof. Tefferi in Rochester, we performed the analysis of a large cohort of classical MPN patients at high resolution using Affymetrix 6.0 SNP array, that represents a valuable

complement to metaphase cytogenetics(130, 283). We found chromosomal changes in more than 50% of the patients, a higher percentage compared to conventional cytogenetic analysis due to the fact that SNP arrays allow the detection of small size aberrations as well as UPDs, both undetectable by metaphase cytogenetics. It is known that mitotic recombination resulting in UPD often amplifies oncogenes or tumor suppressor gene mutations. In MPN, chromosome 9pUPD amplifies the JAK2-V617F mutation(137), 1pUPD amplifies *MPL* mutations(21, 139, 140) and chromosome 11qUPD has been shown to amplify mutations of *CBL*(143). We detected chromosome 9pUPDs in 169 patients, all of which tested positive for *JAK2* mutations. Of the seven 1pUPD cases only six associated with mutations in the *MPL* gene. The remaining case raises the possibility that chromosome 1p harbors an unknown gene mutation relevant for MPN pathogenesis.

In contrast to previous reports showing that PMF patients have higher rate of chromosomal abnormalities, we did not observe association of the number of chromosomal aberrations with any of the three MPN disease entities (130). A recent publication suggested that the JAK2-V617F mutation induces genomic instability and might account for chromosomal lesions observed in MPN(284), but in our cohort JAK2-V617F positive patients did not show higher frequency of chromosomal aberrations. Disease duration did not associate with the frequency of chromosomal aberrations in the studied patients. However, the age of the patients at the time of sample was positively correlated with the number of defects. In line with this finding, there have been reports showing that ageing stem cells acquire chromosomal defects(285) and also a study in PV showed a similar correlation(134).

MPN patients diagnosed with secondary myelofibrosis or accelerated phase (sMF/AP) had significantly more chromosomal aberrations than patients in chronic phase of the disease, and patients with post-MPN AML harbored more aberrations than both chronic phase and sMF/AP patients, as previously reported(132). Chromosome 9p UPDs is more frequent in sMF/AP in comparison to chronic phase, suggesting that a high burden of JAK2-V617F predisposes for disease progression. JAK2-V617F homozygosity had already been associated with a higher risk for secondary myelofibrosis in PV and ET(40).

Chromosome 1q amplifications associated both with sMF/AP and post-MPN AML compared to chronic phase but no significant difference was observed between sMF/AP and post-MPN AML. All observed chromosome 1q gains amplify the *MDM4* gene, which is a potent inhibitor of p53(286, 287). *TP53* itself has been shown to be involved in the leukemic transformation process in MPN(288, 289), what we confirmed in this study.
We also showed that lesions of chromosomes 7, 5, 6, 19, 22 and 3 significantly cluster in post-MPN AML compared to chronic phase. It remains to be seen if the transformation-associated aberrations identified here will have prognostic value.

Six common deleted regions in our cohort mapped to single target genes. We identified a new putative tumor suppressor gene on chromosome 7 that encodes the cut-like homeobox transcription factor *CUX1*. *CUX1* is a DNA-binding protein playing a role in gene transcription and cell cycle regulation and it is involved in hematopoiesis(290, 291), however its precise role in MPN pathogenesis remains to be addressed in functional studies. Recently, Thoennissen and colleagues reported an MPN patient harboring a 0.88 Mb deletion on chromosome 7q including only the two genes CUX1 and SH2B2(292). Our data indicate that CUX1 is a major target of chromosome 7q deletions, and that deletions of SH2B2 might play a role in a smaller subset of patients. We also mapped two known tumor suppressor genes on chromosome 4 (*TET2*) and on chromosome 7p (*IKZF1*) reported earlier(141, 230). The CDR of chromosome 3p mapped to the forkhead box P1 transcription factor (*FOXP1*). *FOXP1* is a member of the large family of forkhead transcription factors, which are involved in various cellular processes. Differential expression of *FOXP1* has been observed in several types of tumors including both over-expression and loss of expression (293). Loss of heterozygosity of the region on chromosome 3p14 that harbors *FOXP1* is common in cancer(294). *FOXP1* has been shown to play important roles in B-cell development(295). Its role in myeloid malignancies is so far poorly understood. *ETV6* is a transcription factor of the ets family and the only gene in the CDR of chromosome 12p. It was originally identified in a translocation between chromosomes 5 and 12 in a patient with CMML(296). Since then it has been shown to be involved in more than 40 translocations in several different hematological malignancies(297). Different fusion genes of *ETV6* with other genes including *ABL1*, *JAK2*, or *RUNX1* have been described(298). Besides translocations, also deletions of *ETV6* are reported, often affecting the remaining allele not involved in the translocation (299).

Common deleted regions have been mapped to a small region including *ETV6* and *CDKN1B*(298). To our knowledge this is the first report of a single gene deletion of *ETV6* in MPN.

RUNX1 or *AML1*, has been previously implicated in leukemic transformation of myeloproliferative neoplasms(173, 288). Both mutations and translocations of *RUNX1/AML1* are frequently encountered in various leukemias. *RUNX1*, runt-related transcription factor 1, is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters and is thought to be involved in the development of normal hematopoiesis (300). Inactivating mutations in *RUNX1* result in differentiation arrest. Our observation of both point mutations and chromosomal aberrations at *RUNX1* locus in post-MPN AML samples confirms its role in leukemic transformation.

With *FOXP1*, *IKZF1*, *CUX1*, *ETV6*, and *RUNX1* five out of the six target genes of common deleted regions are transcription factors. This finding indicates that transcription factor networks may play crucial roles in MPN pathogenesis.

It seems that the pathogenesis of *de novo* AML and post-MPN AML involves different genetic mechanisms, since we found mutations of *RUNX1*, *FLT3*, *IDH1* and *IDH2* at lower frequencies than reported in *de novo* AML. Interestingly, 6 out of 29 post-MPN AML patients carried no chromosomal aberrations except 9pUPDs and were negative for all leukemia-associated mutations tested. This indicates that there are yet unknown pathways of leukemic transformation in MPN.

Thanks to the collaboration of the group of Prof. Kralovics with other centers, it was possible to extend the number of cases to perform a systematic analysis of chromosome 11 aberrations. Chromosomal translocations involving chromosome 11 have been studied intensively in the past but could not be assessed in our study in dependance of the technologies used. These novel technologies however allowed the detection of different genetic events at a scale and resolution that had not been possible until recently, thus facilitating the discovery of novel candidate genes that might play a role in the initiation or progression of hematological malignancies. We showed that not all 11q UPDs could be

associated with mutations in the *CBL* coding region as others have shown as well (143, 144, 250). It is possible that 11q UPDs amplify alterations of regulatory regions of *CBL* thereby targeting *CBL* without affecting its coding region. With mutations in *DDB1* and *MLL* we identified candidate genes that could be alternative targets of chromosome 11q UPD. The large number of deletions on chromosome 11 indicates the presence of several important tumor suppressor genes. We were able to find recurrent deletions assembling as CDRs, which contain only a few genes. It is a matter of further investigations to identify the target genes of these CDRs.

As a last part of my reserach, I focused on the characterization of a molecular alteration initially found with conventional cytogenetics (t15;6) in a patient with PV evolving to PPV-MF. This study confirms that next-generation sequencing methods are able to identify additional rearrangements not revealed by conventional cytogenetic, suggesting the existence of cryptic cytogenetic abnormalities. In the case under study, these alterations are represented by translocation t(15;18) and del(12q). For the translocation t(15;6), we identified and defined the breakpoints and performed the analysis of the expression of genes potentially involved in translocations and deleted, such as RFX7, NEDD4, FOXB1 and KDM1B . The genes RFX7, NEDD4 and KDM1B, did not seem to be affected by the presence of the translocation, while the deleted gene FOXB1 resulted dowregulated in subject under examination, in PV and ET patients compared to healthy controls. The direct sequencing of that gene in the granulocyte of the patient did not discover any mutations in the coding portion.

For the translocation t (15; 18) we were able to identify and characterize the breakpoints; the only gene with a theoretically interesting function for MPN was the deleted GNAL, for which we observed a downregulation compared to other cases of MPNs but comparable expression levels as healthy controls. The data obtained from the SNP analysis demonstrated that the region of the translocated chromosome 15 is flanked by a deleted region that would lead to partial loss of the gene EFTUD1 which, to our evaluation, is found to be down-regulated in the subject under examination compared to healthy controls. Further studies are needed to determine if the expression levels and the rupture of the gene has some functional consequence.

Finally, in assessing the deleted portion of chromosome 12q, we found the juxtaposition of two genes, SETD1B and GTF2H3 that gives rise to a fusion gene. By evaluating a large series of patients with MPNs, about 60, the fusion transcript was found only in our subject under consideration. For this reason we wanted to evaluate if this region of 12q (independently of the fusion gene) was altered in other patients with MPNs. We found other 14 patients with deletions of 12q that have a common deleted region, and it will be interesting to investigate in the future about some genes included in this region, such as DIABLO and BCL7A. Furthermore, the downregulation of GTF2H3 that we found in our subject compared to healthy controls, PV and MF, can confirm the fact that the presence of the fusion gene influences the normal function of this transcription factor.

In conclusion, the aim of this study was to identify chromosomal aberrations and potential new genes specifically involved in the molecular pathogenesis and in disease progression of MPN. Furthermore, such genes may represent novel diagnostic markers useful for diagnosis and new molecular targets in the therapeutic approach to chronic myeloproliferative neoplasms.

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