Approach to Diagnosis of *BCR::ABL1*-Negative Myeloproliferative Neoplasms

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Introduction

Myeloproliferative neoplasms (MPNs) are defined by clonal proliferation of hematopoietic stem cells leading to uncontrolled proliferation of mature myeloid cells, which are hypersensitive to cytokine stimulation for proliferation, cell survival, and differentiation.[1] The incidence of MPNs is 2.3 to 2.8 per 100,000 persons/year and it is primarily a disease of elderly and slightly more common in males.[2] All the entities have distinguishing features based on affected cell lines and fibrosis in the bone marrow compartment.[3]

Chronicle

In 1951, Dameshek gave concept of MPNs and described MPNs as conditions with increased production of mature blood cells as a result of uncontrolled proliferation of hematopoietic precursors in the bone marrow. In 2008, the World Health Organization (WHO) classification coined the term "neoplasm" to highlight the clonal nature of these disorders.

In 2017, revised classification included following entities in MPNs:[1]

- Chronic myeloid leukemia (CML); *BCR::ABL1*+
- Polycythemia vera (PV)
- Essential thrombocythemia (ET)
- Primary myelofibrosis (PMF)
- Chronic neutrophilic leukemia (CNL)
- Chronic eosinophilic leukemia (CEL), not otherwise specified
- MPN, unclassifiable.

Recently, a remarkable advancement in the molecular understanding of MPNs led to the inclusion of juvenile myelomonocytic leukemia (JMML) in MPNs in 5th edition of the WHO classification; however, the International Consensus Classification for myeloid neoplasms and acute leukemia has not included this entity in the classification.[4,5]

The *BCR::ABL1*-negative MPNs are a family of hematologic neoplasms with overlapping clinicopathological features and are characterized by excess accumulation of one or more mature cell lineages of myeloid, erythroid, or megakaryocytic origin. The risk of occurrence of complications such as thromboembolic phenomena or the progression to acute myeloid leukemia (AML) differs between these types of MPNs and hence, correct diagnosis is imperative for effective management and long-term follow-up.[6]

Classical MPNs include three entities, namely, ET, PV, and PMF. Although the clinical features of these entities are different in their typical forms, the majority of the cases have overlapping clinicopathologic and morphologic features; hence, accurate diagnosis is often challenging.[7]

Historically, CML has been studied separately due to unique underlying molecular abnormality and specific targeted therapy (that is, tyrosine kinase inhibitors). CNL, CEL, and JMML are other MPNs, discussed separately due to different molecular pathogenesis and variable presentation.

Molecular Evolution

The *BCR::ABL1* fusion transcript in CML was identified in 1982, and since then advanced genomics has provided major insight into the understanding of several subtypes of MPNs via the discovery of principal pathogenetic causative mutations. Since then continuous advances have been made in the field of myeloid disorders.[8]

BCR::ABL1-negative MPNs are characterized by numerous somatic mutations which impact the patient outcome and disease progression. These mutations are *JAK2, CALR, or MPL*. Understanding of molecular pathogenesis of these disorders became easy after the identification of a single gain of function, point mutation in the Janus kinase2 *(JAK2)* gene in 2005.[7]

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At present, *JAK2*V617F mutation is the most common molecular feature in classical BCR::ABL1-negative MPNs, observed in 95% of cases with PV and 55 to 60% of cases of PMF and ET. Cases of PV which are negative for *JAK2*V617F often harbor *JAK2* exon12 mutation.

CALR mutations are not seen in cases of PV but are observed in up to one-fourth of cases of ET or PMF. Similarly, mutations in *MPL* are observed in about 10% of cases of ET or PMF. "Triple negative MPNs" are cases of ET and PMF in which all three mutations *(JAK2, MPL, and CALR)* are not detected and these constitute 15% of all cases.[3,8]

The latest WHO classification update is solely based on the incorporation of molecular markers as diagnostic criteria for the majority of hematological malignancies. This forms the basis of routine testing for these mutations in any suspected case of MPN, starting always with *BCR::ABL1* as its absence is a major criterion for classical MPNs.[3]

Newer techniques in use like next-generation sequencing (NGS) have shown some additional somatic mutations which are observed in patients aforementioned three mutations (*JAK2, MPL, or CALR)* and these include RNA splicing factors, transcriptional regulators, and epigenetic modifiers. These mutations are not directly related to the pathogenesis of MPNs but they do influence the clinical course.[8]

Characteristic mutations of JMML are somatic mutations in *PTPN11, KRAS, NRAS, or RRAS*. Germline mutations in *NF1* or clinical diagnosis of neurofibromatosis type 1, loss of heterozygosity of *CBL*, and germline *CBL* mutation are other pointers towards JMML.[9]

Diagnosis of CEL needs exclusion of certain mutations, including BCR::ABL1, *JAK2, PDGFRA, PDGFRB, FLT3, or FGFR1*. However, clonal cytogenetic abnormality and/or other somatic mutations can be observed.[9]

The genetic signature of CNL is the mutations in the colony-stimulating factor 3 receptor *(CSF3R).* However, additional mutations such as *SETBP1, ASXL1, SRSF2*, and signaling mutations may be found in many cases.[9]

2022 Revision of the WHO Classification of Classical MPNs

Anomalies in blood cell counts, bone-marrow morphology of hematopoietic components in the background of the above mutations form the basis of the WHO diagnostic criteria for classical MPNs (►**Table 1**).[9]

Significance of the Need to Differentiate All MPNs from One Another

The risk of complications such as thromboembolic phenomena and/or the progression to AML differs among different MPNs and hence, distinction among these is crucial. Patients of PV and ET may experience venous and/or arterial thrombosis due to increased viscosity; however, the majority of them have an indolent course. On the other hand, PMF patients have bone marrow fibrosis, vascular complications, and constitutional symptoms due to excessive inflammatory cytokines release.[10]

Approach to a Case of BCR::ABL1-Negative MPNs

Step 1. When to Suspect a Case as MPN?

Although the median age at diagnosis is in sixth decade for various MPNs with males more commonly affected; however, this differs from disease to disease, and patients may have a variable presentation. They may be asymptomatic and detected on routine screening at OPD visit for some other illness or trivial symptoms. Less commonly, patients may have severe constitutional symptoms, ET may present with changes in vision and headaches as well as splenomegaly, thrombosis, and bleeding episodes. The most common symptoms in PV are headache, pruritus, blood clots as well as other symptoms due to high hemoglobin (Hb); however, slightly increased Hb levels can be observed in *JAK2V617F*-mutant PMF as well as ET.

Patients of PMF present with the maximum burden of symptoms and the worst prognosis among all MPNs and may present with abdominal discomfort and early satiety, fatigue, night sweats weight loss, and fever.[1,11]

Cases of JMML are exclusively seen in childhood with 75% of cases occurring in the aged less than 3 years with the variable presentation and are associated with disorders resembling Noonan syndrome and in cases of neurofibromatosis type 1. Constitutional symptoms or signs and symptoms secondary o infections are the presenting features in the majority but most of them are not picked up clinically.[11]

Unusual presentations for MPNs include thrombosis at any unusual site like abdominal vein thrombosis, cavernous sinus venous thrombosis, and splenic vein thrombosis. A significant proportion of patients with *JAK2* mutation presenting with thrombosis with normal blood counts at presentation may convert to full-blown MPN during follow-up. Rumi and Cazzola observed similar frequencies of occurrence of thrombosis in cases with *JAK2* mutation diagnosed as ET or PV, and it was four times that of cases of ET with *CALR* mutation. The incidence of *JAK2* exon12 and *MPL* mutations is not well studied.[12]

Step 2. Role of Basic Laboratory Workup

After a patient presents with the above signs and symptoms, usually a complete hemogram is the first requested investigation and this coupled with peripheral blood smear examination may reveal high hemoglobin, leucocytosis, and/or thrombocytosis.

Neutrophilic leucocytosis (with left shift) and basophilia point toward a diagnosis of CML. In the absence of basophilia, reactive causes of neutrophilia such as infective or inflammatory disorders should be ruled out and a basic laboratory workup should include C-reactive protein, procalcitonin, and blood culture. Here, a high leukocyte alkaline phosphatase score helps rule out reactive causes of neutrophilia.

Thrombocytosis may be observed in all MPNs. At the same time, thrombocytosis is more commonly due to secondary reactive causes; hence, all such causes such as iron deficiency

Table 1 The WHO diagnostic criteria for BCR::ABL1-negative myeloproliferative neoplasms (MPNs)

Polycythemia vera (PV)	Essential thrombocythemia (ET)	Primary myelofibrosis (PMF) —early/prefibrotic stage (pre-PMF)	PMF, overt fibrotic stage:
Major criteria: 1. Elevated hemoglobin concentration: 16.5 g/dL in men and 16.0 g/dL in women; or elevated hematocrit of 49% in men and 48% in women, or increased red blood cell (RBC) mass of 25% above mean normal predicted value. Increased RBC mass, however, has been removed as a component of major criteria in WHO 5th ed. [WHO 5 ed] 2. Presence of JAK2 V617F or JAK2 exon 12 mutation 3. Bone marrow (BM) biopsy showing age-adjusted hypercellularity with trilineage proliferation (panmyelosis), including prominent erythroid, granulocytic, and increase in pleomorphic, mature megakaryocytes without atypia	**Major criteria** 1. Platelet count $>450\times109$ /L 2. BM biopsy showing proliferation mainly of the megakaryocytic lineage, with increased numbers of enlarged, mature megakaryocytes with hyperlobulated staghorn-like nuclei, infrequently dense clusters; no significant increase or left shift in neutrophil granulopoiesis or erythropoiesis; no relevant BM fibrosis 3. Diagnostic criteria for BCR::ABL1-positive CML, PV, PMF, or other myeloid neoplasms are not met 4. JAK2, CALR, or MPL mutation	**Major criteria** 1. BM biopsy showing megakaryocytic proliferation and atypia,* BM fibrosis grade, increased age-adjusted BM cellularity, granulocytic proliferation, and (often) decreased erythropoiesis 2. JAK2, CALR, or MPL mutation or presence of another clonal marker or absence of reactive BM reticulin fibrosis 3. Diagnostic criteria for BCR::ABL1-positive CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms are not met	**Major criteria** 1. BM biopsy showing megakaryocytic proliferation and atypia,* accompanied by reticulin and/or collagen fibrosis grades 2 or 3 2. JAK2, CALR, or MPL mutation or presence of another clonal marker or absence of reactive myelofibrosis. 3. Diagnostic criteria for ET, PV, BCR::ABL1-positive CML, myelodysplastic syndrome, or other myeloid neoplasms are not met
Minor criterion: Subnormal serum erythropoietin level	Minor criteria Presence of a clonal marker or absence of evidence of reactive thrombocytosis	Minor criteria: Anemia not attributed to a comorbid condition leukocytosis $>11\times109$ /L, palpable splenomegaly, lactate dehydrogenase level above the above the reference range	**Minor criteria** Anemia not attributed to a comorbid condition leukocytosis $ 11 3 109 /L palpable splenomegaly lactate dehydrogenase level above the above the reference range leukoerythroblastosis
Diagnosis requires either all 3 major criteria or the first 2 major criteria plus the minor criterion **Note:** *BM biopsy may not be required in patients with sustained absolute erythrocytosis (hemoglobin concentrations of 18.5 g/dL in men or 16.5 g/dL in women and hematocrit values of 55.5% in men or 49.5% in women) and the presence of a JAK2 V617F or JAK2 exon 12 mutation*	Diagnosis requires either all major criteria or the first 3 major criteria plus the minor criteria	The diagnosis of pre-PMF or overt PMF requires all 3 major criteria and at least 1 minor criterion confirmed in 2 consecutive determinations	

anemia, infections, inflammatory disorders, and malignancy must be ruled out at first.[1]

In cases of erythrocytosis, there is a need to rule out reactive causes like smoking, diseases associated with hypoxia, drugs, tumors, or dehydration. A detailed clinical history should be coupled with arterial blood gas testing, chest X-ray, ultrasonography of the abdomen, history of any fluid loss, serum lactate dehydrogenase, and serum erythropoietin (EPO) levels followed by molecular testing for *JAK2* mutation.[1]

Patients of PMF may present various combinations of single lineage or multilineage cytopenia or, the most common feature as anemia with leucoerythroblastic blood picture in the elderly along with splenomegaly, and hence, the approach to diagnosis of PMF typically starts with the evaluation of anemia and splenomegaly.[1]

Persistent eosinophilia is a pointer towards CEL; hence, a workup should start with ruling out reactive causes of eosinophilia, like parasitic infections, chronic inflammatory diseases, autoimmune disorders, or tumors; hence, clinical details and peripheral smear examination should be complemented by stool examination and autoimmune workup followed by BCR::ABL1 testing and tissue biopsy to look for end-organ damage in cases of hypereosinophilic syndromes.

Step 3. Role of Molecular Testing

The beginning of molecular workup with *BCR::ABL1* is a prerequisite for *BCR::ABL1*-negative MPNs.

The molecular approach may be sequential beginning with ruling out all the reactive causes and keeping patients on regular follow-up and blood counts, followed by molecular testing in resource-limited settings.

In patients with high suspicion of MPN and advanced centers, a primary reflex panel for MPN (including *BCR::ABL1* followed by *JAK2, CALR, and MPL*) should be done. Suspected cases of PV may be directly advised for serum EPO and *JAK2* mutation (both *JAK2V617F* and exon 12 mutation).

MPN Panel for Molecular Testing

Molecular testing for MPN comprises a primary MPN reflex panel and an advanced MPN panel.

Primary MPN reflex panel comprises screening by *BCR::ABL1* then followed by *JAK2* (V617F & exon 12), *CALR*, and *MPL* mutation.[13]

Advanced panels should be advised if all the above primary panel mutations are negative to rule out the possibility of CEL, CNL, and JMML (in the case of childhood MPN). Advanced panel testing includes *CSF3RT618I*, a very specific mutation positive for CNL. Most of cases of JMML harbor mutations (germline or somatic) in genes *NF1, CBL, NRAS, KRAS, RRAS, FLT3*, and *PTPN1*.[14] Diagnosis of CEL requires exclusions of FIP1L1-PDGFRA along with the exclusion of rearrangements of *PDGFRA, PDGFRB, FGFR1 and PCM1-JAK2*.[15]

There are various methods to detect mutations, the ones most widely practiced are highly sensitive quantitative reverse-transcription polymerase chain reaction (RT-PCR), droplet digital PCR, or NGS assays with very high sensitivity.[4]

Step 4: Role of Bone Marrow Examination

The next step after a clinical suspicion supported by basic laboratory investigations and molecular testing is marrow examination.

Bone marrow examination is not only a major criterion for diagnosis of *BCR::ABL1*-negative MPNs, but also helps assess the percentage of blasts which should be less than 20% in MPNs. Clinical suspicion supported by a complete hemogram and molecular testing provides us a tentative diagnosis which is confirmed by bone marrow examination.[9]

Cases of PV reveal a hypercellular marrow with panmyelosis which is the proliferation of all lineages with pleomorphic, mature megakaryocytes. In suspected cases for PV with *JAK2* mutation and subnormal EPO levels, bone-marrow examination may be avoided if Hb or hematocrit values are more than 18.5g/dL or 55.5% in males respectively, or more than 16.5g/dL or more than 49.5% in females respectively.[9]

In suspected cases of ET and PMF, marrow examination is mandatory in all irrespective of other parameters. In PMF, megakaryocytic morphology (megakaryocytic proliferation and atypia, abnormal para-trabecular localization with cloud-like nuclei) and fibrosis as assessed by reticulin stain help in diagnosis. In the prefibrotic phase, reticulin fibrosis is usually not more than grade 1; however, bone marrow cellularity is increased and there is granulocytic predominance, and (often) reduced erythropoiesis whereas reticulin and/or collagen fibrosis grades 2 or 3 is observed in the overt fibrotic stage.[9]

In ET, bone marrow is usually normocellular with megakaryocytic prominence. Megakaryocytes reveal giant, mature forms having hyperlobulated nuclei. Further, there is usually no significant prominence of granulopoiesis or erythropoiesis, and a few cases may have mild reticulin fibrosis (not more than grade 1a).[9]

In CNL, hypercellular bone marrow reveals granulocytic predominance with a normal maturation pattern; however, myeloblasts are less than 5% of the nucleated cells.

Step 5: Integration of Clinical and Laboratory Features

The integrated approach (clinical details, hemogram, basic laboratory test, bone marrow examination, and molecular studies) helps reach an accurate diagnosis in the most of cases.

Driver mutations associated with MPN; *JAK2*V617F, *JAK2* exon12, *MPL*, and *CALR* need to be identified accurately that is important not only to diagnose PV, ET, or PMF and but also to differentiate wild-type or triple-negative cases.[4]

Differential Diagnosis

1. **ET versus pre-PMF with thrombocytosis:** It is particularly important to differentiate ET and prefibrotic PMF frequently presenting with thrombocytosis (false ET) as there is comparatively less tendency to fibrotic progression in cases of (true) ET. Further, there is also reduction in life expectancy in case of early PMF compared to true ET. The distinction between these two is primarily based on morphology as shown in ►**Table 2**.[16]

2. **PV with monocytosis versus CMML:** Monocytosis can be observed in cases of PV (~20%) and PMF (~15%) portends a poor outcome. Further, monocytosis, if present at the time of presentation, can mimic chronic myelomonocytic leukemia (CMML). Monocytosis, however, is not pathognomic for CMML and can be due to reactive and clonal processes (including MPNs) such as PV and PMF. The distinction in these cases relies on bone marrow morphology and molecular studies. Further, studies are exploring utility of flow cytometry in distinction of CMML from other causes of monocytosis.[17]
3. **Nonclonal marrow fibrosis with PMF:** Bone marrow fibrosis is a histologic finding and can be observed in various diseases, including malignancies, endocrine disorders, autoimmune diseases, and infections. Autoimmune myelofibrosis (AIMF), an uncommon entity, may be primary or can be secondary to a defined autoimmune disorder. Distinguishing between PMF and non-neoplastic AIMF is very important as the prognosis and management are different. This distinction, however, can be complicated by overlapping findings in the two disease entities. The morphologic criteria that favor AIMF rather than PMF, as proposed by Vergara-Lluri and colleagues, are mentioned in ►**Table 2**.[18]

The Course of the Disease

MPNs are heterogenous neoplasms with a wide spectrum of stepwise or clonal progression into various stages. These may undergo bone marrow failure due to myelofibrosis or ineffective hematopoiesis or may accelerate and transform into blast phase leading to acute leukemia.[19]

Life span of patients with MPN is often reduced compared to the general population and the survival rate observed in PMF is generally the lowest compared with cases of PV, and in cases of PV compared with those of ET.[1]

The disease course and survival of MPNs are variable and range from a few months (with rapid leukemic progression) to several decades. Hence, uncertainties in prognosis and the similarities in the clinical and morphological phenotypes at diagnosis necessitate inclusion of molecular parameters in classification.[1]

Correlation Among Genotype, Phenotype, and Clinical Outcome

Patients of PV carry an increased tendency of thrombosis and may progress to myelofibrosis or to a blast crisis leading to AML.

There is an increased risk of thrombosis as well as bleeding in cases of ET and these cases may take much more aggressive course compared to PV. In ET, cases with *JAK2*V617F mutation carry a much higher risk of thrombosis and progression, while *CALR*-mutant ET involves a comparatively lower risk. On the other side, triple-negative ET is a slowly progressive disorder with a low incidence of vascular events and less risk of progression.[1]

PMF has the highest burden of symptoms and the poorest prognosis among all MPNs, with a much higher risk of progression. *CALR*-mutant PMF has shown a better survival compared with other genotypes. Triple-negative PMF is again an aggressive MPN characterized by a much higher risk of transformation into acute leukemia.[1]

Risk Stratification of PMF

Cases of MPNs carry increased risk of vascular complications that include thrombosis as well as bleeding, the former being

Table 2 Features differentiating MPNs with other entities[16–18]

Morphological feature	MPN	Differential diagnosis
• Cellularity(age-adjusted) • Myeloid-to-erythroid ratio • Dense megakaryocyte clusters • Megakaryocyte size • Megakaryocyte nuclear lobulation • Reticulin fibrosis, grade1 b	**ET** Normal Normal Rare Large/giant Hyper lobulation Grade 1b very frequent	**Pre-PMF with** Increased Increased Frequent Variable Bulbous/hypolobulated More frequent
• Features of dysplasia • Monocytosis • Mutation profile	**PV withmonocytosis** Absent Not a strict criterion JAK2	**CMML** More often seen Persisting >3 months (TET2, SRSF2, ASXL1, and SETBP1)
	PMF leukoerythroblastic reaction Eosinophilia or basophilia present Grade 2-3 fibrosis Granulocytic hyperplasia Absence of lymphoid aggregates Dysplastic features may be seen	**Nonclonal myelofibrosis (AIMF)** Rarity or absence Absence Mild degree of BMF (usually MF1). Absence of osteosclerosis and bone changes Presence of hypercellular marrow characterized by erythroid and megakaryocytic hyperplasia Presence of lymphoid aggregates Absence of dysplastic features in any of the lineages, especially the megakaryocytes

Abbreviations: AIMF, autoimmune myelofibrosis; BMF, bone marrow fibrosis; CMML, chronic myelomonocytic leukemia; ET, essential thrombocythemia; MPN, myeloproliferative neoplasms; PMF, primary myelofibrosis; PV, polycythemia vera.

more common. In addition, the proportion of patients with ET and PV may evolve in to secondary myelofibrosis, while all MPN cases may potentially advance to a blast phase into AML. Therefore, risk stratification is an important end part of a diagnostic algorithm.[1]

The initial International Prognostic Scoring System (IPSS) for PMF was later modified to a dynamic prognostic model (DIPSS). Presently, refined Dynamic International Prognostic Scoring System (DIPSS Plus) is in use widely and it includes eight predictors of inferior survival that include age, Hb level, total leucocyte count, platelet count, percentage of circulating blasts, presence of constitutional symptoms, need for red cell transfusion, and unfavorable karyotype.[20]

Allele Burden of JAK2 for Risk Assessment of Thrombosis

Age more than 60 years and past history of thrombosis are two major risk factors for thrombosis. However, recent literature suggests that in cases of PV and ET, the chances of vascular events proportionately increase with the amount of mutated *JAK2*V617F allele burden.[21]

In addition, *JAK*2V617F allele burden (ratio of mutant allele to total allele) has been correlated with certain clinical features, such as a higher incidence of pruritus and splenomegaly and risk of thrombosis in patients with PV and ET; however, there is no conclusive association, particularly in cases of PMF.[22]

Studies have demonstrated a higher *JAK2* allele burden in PV than in ET. A recent study showed that a *JAK2* allele burden more than 50% favors a diagnosis of prefibrotic PMF over ET. Further, in study by Park et al, median allele burden of *JAK2* V617F was significantly lower for ET than for PV or PMF. However, utility of this allele burden to distinguish subtypes of Ph-negative MPN has not been evaluated systematically.[23]

Conclusion

BCR::ABL1-negative MPNs are a diverse group of neoplasm. Advances in genomics have led to newer improved diagnostic approaches. Updated molecular data are presently utilized for developing new predictive and prognostic models, and for monitoring of disease responses to newer targeted drugs. There is a need for tailored treatment for different disease entities as well as the availability of *JAK* inhibitor; hence, adequate workup and accurate diagnosis are important that require integration of clinical and laboratory data.

Authors' Contributions
M.S. was involved in writing–original draft and data curation. A.H.L.P. contributed to conceptualization, resources, and writing of the original draft.

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None declared.

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