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THE IMPORTANCE OF JAK2V617F AND BCR-ABL TESTING IN THE DIAGNOSIS OF CHRONIC MYELOPROLIFERATIVE NEOPLASMS: A SINGLE CENTRE EXPERIENCE

A. ILEA[1] A.M. VLĂDĂREANU[2, 3]

Abstract: *This article reports the molecular diagnosis findings regarding chronic myeloproliferative neoplasms made in a Molecular Biology Laboratory over a seven years' experience (between 2009 and 2015). Ritus Biotec ltd is an ELN (European Leukemia Net) certified diagnostic laboratory for quantification of BCR-ABL. 1373 patients were subjected to Jak2 V617F testing in this time frame. The patients were presumed with BCR-ABL negative chronic myeloproliferative neoplasms. 674 patients (50,29%) were found to be Jak2 V617F positive. 1141 BCR-ABL tests were also performed for suspected CML patients, 482 (42%) of which were positive. Both diagnosis tests (for BCL-ABL transcript and Jak2 V617F point mutation) were performed on a number of only 614 patients with various chronic myeloproliferative disorder subtypes. These results are consistent with other similar works from the scientific literature and demonstrate the importance of molecular testing for an accurate diagnosis and a correct assessment of the clonal character of proliferations.*

Key words: *BCR-ABL Transcript, Jak2 V617F Mutation.,* *Chronic Myeloid Leukaemia, Myeloproliferative Neoplasms.*

1. Introduction

Chronic myeloproliferative neoplasms (MPN) are clonal proliferations of hematopoietic stem cells that involve one, two or all of the cellular lines of myeloid precursors, characterized by uncontrolled development and mature myeloid cells accumulation.

This disorder group was first described and defined in 1951, by Dameshek. He named five entities included in the myeloproliferative disorders group: chronic myeoid leukemia(CML), polycytemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), and erythroleukemia. Dameshek put forward

[1] Ritus-Biotec Molecular Biology Laboratory-Codlea, Brasov.
[2] Emergency University Hospital, Bucharest.
[3] Carol Davila University of Medicine and Pharmacy, Bucharest
[*] Correspondent author: ancamariailea@yahoo.com

the idea that these disorders share a hyper stimulation of myelopoiesis as a result of a stimulus unknown at the time (a "hitherto undiscovered stimulus") and that each of these diseases are phases of a single myeloproliferation. See [4]

It was later discovered that there is a "stimulus" pathogenically connected to a disease, the Philadelphia chromosome, which was only present in CML patients (1960-Nowell). Later discoveries cleared up some pathogen issues, which, in turn, determined an important change in diagnosis criteria and classifications. See: [13]

In 2008, WHO released a new classification of haematological diseases based on molecular testing used in diagnosis. The concept of neoplastic was also put forward for the first time to underline the clonal aspect of these proliferations (a character that was first proven in 1967). See: [13]

This classification was based on several markers of which very important are the BCR-ABL fusion oncogene and JAK2V617F point mutation.

Myeloproliferative neoplasms are currently divided into two groups: BCR-ABL positive myeloproliferations (CML) and BCR-ABL negative myeloproliferations (with 7 subtypes: Polycythaemia Vera (PV), Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF), Chronic neutrophil leukaemia(CNL), Eosinophilic leukaemia and hypereosinophilic syndrome (CEL and HES) Systemic mastocytosis(SM)and unclassifiable chronic myeloproliferative neoplasms(MPNu). See: [2], [14], [17]

The most common BCR-ABL negative MPN entities are PV, TE, and PMF.

BCR-ABL negative MPNs have a clonal origin and show some common features: hyper cellular bone marrow, overproduction with no relation to growth factors' action and accumulation of mature blood elements at the periphery, splenomegaly, asthenia, bleeding or thrombotic events, a possibility of leukemic transformation.

It was found that Jak2 V617F point mutation is present in 90% of PV cases and 60% of PMF and ET cases, which makes it an important diagnosis criterion. See: [2]

Latest data from the literature shows the existence of complex pathogenic mechanisms that still raises questions. There were identified other molecular markers involved: 3%–5% of ET and 5%–8% of PMF patients have point mutations at codon 515 of the gene encoding the thrombopoietin receptor *MPL* (W>L, K or A). In 2%–4% of JakV617F negative PV are detected abnormalities located in exon 12 of Jak2. About 60%–80% of *JAK2* and *MPL* negative patients with ET and PMF have mutations in the exon 9 of *CALR* (mutations discovered in 2013).See [13] Thus currently only 10-15% of patients with ET and PMF remain molecularly uncharacterized (are defined as "triple negative" for the mutations described above). See: [10], [16]

Another reason why molecular markers have become essential in the diagnosis of chronic myeloproliferative neoplasms is the emergence of revolutionary drugs such as low molecular weight drugs like tyrosine kinase inhibitors. Currently, there are tyrosine kinase inhibitors that target BCR-ABL and are successfully used in CGL treatment (imatinib, also known as "the magic bullet"), as well as Jak2 inhibitors used in PMF therapy and in PV cases that are refractory to or intolerant of Hydrea.

2. Materials and Methods

The two sets of molecular testing results (for BCR-ABL transcript and Jak2 V617F point mutation) performed for patients with a presumptive diagnosis of chronic myeloproliferative neoplasm from 2009 to

2015 were subject to analysis.

1141 BCR-ABL tests were performed for presumed onset BCR-ABL positive MPN patients and 1373 Jak2 V617F tests were performed for presumed BCR-ABL negative MPN patients.

Our study focused on patients that underwent both sets of tests, for both BCR-ABL transcript and Jak2 V617F point mutation. The 614 patients are between 19 and 90 years old (median age: 61.5years), they are both male and female (48% male; 52%female) and came from different areas of Romania. All patients had presumptive diagnosis of MNP in compliance with recommendations of clinical practice guidelines (European Society of Medical Oncology Guidelines, ESMO, 2015 Vannucchi et al.). See: [16]

This work is part of a doctoral research study approved by the Ethics Committee of the Carol Davila University of Medicine and Pharmacy, Bucharest.

Diagnosis criteria are describe in Table 1

Regarding CML -with an incidence of 1-2 /100,000 inhabitants/year , median age 60 years- the diagnosis is based on clinical data according ESMO guidelines (nonspecific symptoms : weight loss , fatigue, splenomegaly ascertained in about 50 % of cases), on laboratory data (characteristic feature is leukocytosis with basophilia and the presence of immature granulocytes) and requires mandatory confirmation by cytogenetic (showing t(9; 22)(q3.4;q1.1),) and molecular (RT -PCR) to detect transcript BCR- ABL. (Baccarani et.al , 2012; ESMO. See: [1]

Diagnostic criteria and MPNs incidence in Europe (ESMO, 2015) Table 1

MPN bcr-abl negative Incidence: n/100000 inhabitants/year Median age at diagnosis ranges	**Major criteria**	**Minor criteria**	**Diagnosis requirements**
PV 0.4–2.8 65–74 years	1. Hemoglobin > 18.5 g/dl (men) > 16.5 g/dl (women) or any other evidence of increased red cell volume 2. Presence of JAK2V617F or JAK2 exon 12 mutation	1. BM trilineage myelo-proliferation 2. Sub-normal EPO level 3. Endogenous erythroid colony growth	Both major criteria and one minor criterion or the first major criterion and two minor criteria
ET 0.38 – 1.37 64–73 years	1. Platelet count ≥ 450× 10^9/l 2. Megakaryocyte proliferation with large and mature morphology 3. Not meeting WHO criteria for CML, PV, PMF, MDS or other myeloid neoplasm 4. Presence of *JAK2*V617F or other clonal marker (*MPL)*or no evidence of reactive thrombocytosis		all four major criteria
MFP 0.1 – 1 69–76 years	1. Megakaryocyte and atypical proliferation accompanied by either reticulin and/or collagen fibrosis, or [c]	1. Leukoerythroblastosis 2. Increased serum LDH level 3. Anemia 4. Palpable splenomegaly	all three major criteria and two minor criteria

MPN bcr-abl negative Incidence: n/100000 inhabitants/year Median age at diagnosis ranges	**Major criteria**	**Minor criteria**	**Diagnosis requirements**
	2. Not meeting WHO criteria for CML, PV, MDS or other myeloid neoplasm 3. Presence of *JAK2*V617F or other clonal marker or no evidence of reactive BM fibrosis		

The nucleic acids (genomic DNA and total RNA) were obtained by standard purification procedures of integral peripheral blood using Qiagen IVD Kits.

The concentrations of nucleic acids were measured using NanoDrop spectrophotometer (NanoDrop ACTgene, USA). V617F mutations were detected using ARMS assay (Amplification Refractory Mutation Screening) described by Jones et al. in Blood, 2005 Sept 15, 106 (6). See: [7]

The ARMS assay uses two pairs of primers (tetra-primers) that amplify in the same reaction a control fragment as well as fragments corresponding to V617F mutation and wild-type sequence.

The resulting amplicons were: a 463bp control fragment and fragments corresponding to V617F mutation (279bp) and to wild-type sequence (229bp).

The amplicons were resolved on 2% agarose gels (through electrophoresis) and visualized after staining with ethidium bromide and using UV transluminal. Every experiment used specific controls: positive (with mutant allele) and negative (with wild-type allele). (Figure 1)

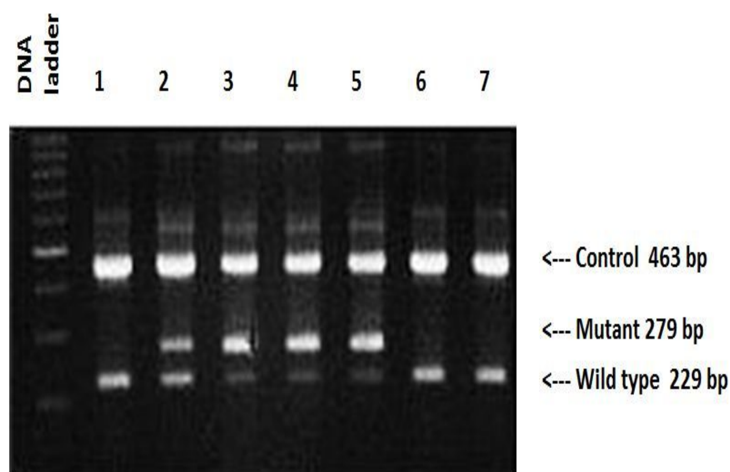

Fig. 1. *JAK2V617F detection by ARMS*
PCR: Representative results: Tracks 1,6,7 are negative, 2 is positive heterozygous and 3,4,5 positive homozygous.

We also used a semi-quantitative assessment of the presence of the Jak2 V617F mutation by amplifying a series of homozygous mutant DNA dilutions in wild type DNA.(Figure 2).

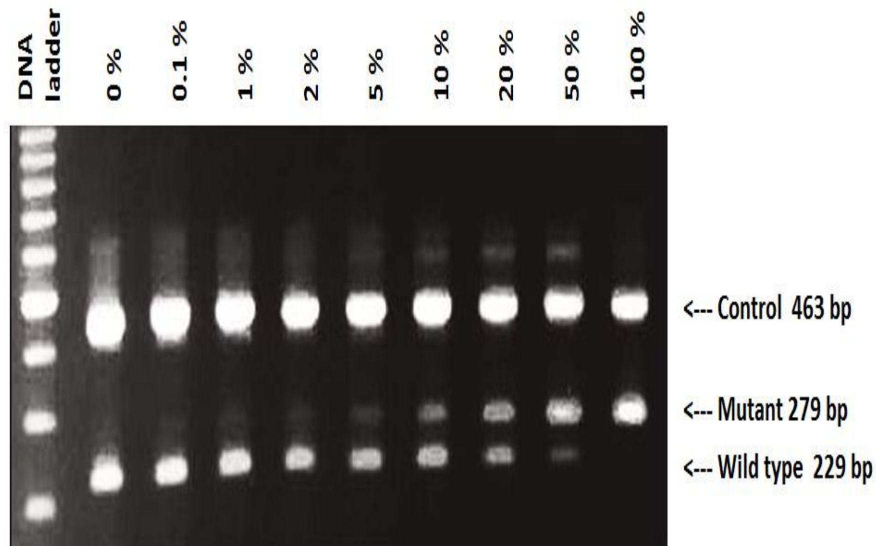

Fig. 2. *Sensitivity of ARMS PCR Amplified dilutions of DNA from a homozygous sample with normal DNA*

We can thereby asses the allele load, which allows us to distinguish homozygous genotypes from heterozygous genotypes and establish the accuracy of this method – this method is able to detect the JAK2 V617F mutation in 1-2% dilutions.

Detection and quantification of BCR-ABL transcript was performed by Standard Real-time PCR protocol (Europe against Cancer, Gabert et al in Leukemia, 2003) on the Applied Biosystems® StepOnePlus™. See: [5]

The Ritus Biotec laboratory is European Leukemia Net certified for BCR-ABL quantification.

The methods employed in the laboratory are periodically validated and evaluated by participating in external quality control schemes (UK NEQAS, United Kingdom.)

PCR and Real Time PCR tests were performed at onset and, in some particular situations, also during the clinical course of the disease.

3. Results and Discussions

1141 BCR-ABL tests were performed for presumed CML patients, 482 (42%) of which were positive. 1373 Jak2 V617F tests were performed for presumed BCR-ABL negative MPN patients. 688 (50.10%) (Below table)

Table 2

Distribution of JAK2V617F screened patients according to diseases and gender

Diagnosis: No. of patients	**Jak2V617F positive** No. of patients (%) F%	**Jak2V617F negative** No. of patients (%) F%
PV (424 patients) From which Polyglobulia (81 patients)	194 (45.7%) F:50% 8 (9.8%) F:50%	230 (54.6%) F:15 % 73 (90.12%) F:10%
TE (462 patients)	236 (51%) F:64%	226 (49%) F:68%
PMF (213 patients)	114 (54%) F:50%	99 (46%) F:45%
MPNu (274 patients)	144 (52.55%) F:43.75%	130 (47.44%) F:50%

Legend: F% – percentage of females;
PV – Polycythemia Vera;
ET – essential thrombocythemia;
PMF – primary myelofibrosis;
MPNu – myeloproliferative neoplasms unspecified subtype.

In PV suspicions (424 patients), the JAK2 V617F mutation was only present in 45.7% of the cases, a smaller percentage than the one found in literature (97% in Baxter et al., 74% in Levine et al.,65% in Kralovics et al). See: [3], [8, 9]. The variations can be explained by the accuracy of the methods involved: the method of PCR allele-specific used by Baxter has a high sensitivity (2%) compared to DNA sequencing and microsatellites mapping. The methods used by Kralovics et al (microsatellite mapping and DNA sequencing methods) can reach a sensitivity of 20%. Our method has a high sensitivity (1-2%).

A potential cause for this difference is the inclusion of several cases of reactive polyglobulia, especially since, in Romania, there is a high percentage of secondary polyglobulia causes by smoking induced hypoxia (27% of Romania's population are smokers, according to a study conducted by IRESS).

The careful research of reference diagnoses found that 81 patients had a diagnosis of "polyglobulia". Of these polyglobulia cases, 90.12% didn't present the mutation and 90% of patients were males (90%). JAK2 V617F testing proves its importance in differentiating the reactive polyglobulia from clonal polyglobulia.

For ET and PMF, results are comparable to the ones found in literature. In TE cases, JAK2 V617F was present in 236 (51%) patients, mainly females (63%) (in literature: 57% in Baxter et al, 32% in Levine et al. and 23% in Kralovics et al). In PMF cases, 114 patients out of 213 (54%) tested positive for the mutation, equal distributed based on gender (in the literature: 50% in Baxter et al, 35% in Levine et al., 57% in the Kralovics et al.). See: [3, 8, and 9]

Of the 614 tested for both tests, 549 patients had a clinical suspicion of BCR-ABL negative MNP and 65 patients had a clinical suspicion of BCR-ABL positive MPN (CML-like) (table below)

Result distribution in MPN groups Table 3

Diagnosis No of patients	**Jak2V617F positive**	**Bcr-abl positive**	**Double positive**	**Double negative**
NPM BCR-ABL neg. 549	297 (54.09%)	16 (2.91%)	7 (1.14%)	229 (41.71%)
NPM (MLC like) 65	23 (35.38%)	9 (13.84%)	*	33 (50.79%)

The 7 patients that were double positive were carefully monitored and presented some phases of CML phenotype.

Of the 65 patients included in the MPN CGL like group, the disease was only confirmed in 9 patients (13.84%).

In 33 patients, accounting for 50.79% of the group, both tests came back negative, and in 23 patients (35.28%), the results for Jak2 V617F were positive. Most of the CML-like cases with Jak2 V617F proved to be prefibrotic PMF cases in various stages of the disease's evolution.

A particularly interesting case was found in the 7 patients that presented both molecular markers. It is a rare, but not impossible occurrence (the percentage we obtained, 1.14% (7/614), is comparable to the ones found in literature, from 0.37% (5/1320 in Cappetta et al, 2013, to 2.25% (7/314 in Pieri et al, 2011). See: [6], [12], [15]

These particular cases were closely monitored by repeated testing. According to classic diagnosis criteria, the patients fell into different MPN entities: two PV cases, two ET cases, two PMF cases and one unclassified NMC. Close monitoring and laboratory data showed a change in MPN phenotype. More specifically, in certain phases of the disease's evolution, all patients presented a typical CML phenotype, which was confirmed once the BCR-ABL major marker was discovered. BCR-ABL translocation confers a growth advantage compared to Jak2 V617 and, as such, it proliferates in granulocytes, resulting in a CML phenotype. JAK2V617F expression induced phenotype occurs only when the transcript BCR-ABL is absent / reduced due to tyrosine kinase inhibitors therapy.

The distribution of JAK2V617F mutation among the BCR-ABL negative MPN subtypes was as follows: the mutation was noticed in 66% of PV cases, in 56% of ET cases and in 54% of PMF cases.

The data we obtained was consistent with other findings (65% to 97% for PV patients, 23% to 57% for ET cases, and 35% to 57% for PMF cases). See: [3], [8], [9]

The table below shows results distribution in BCR-ABL negative MPN subtypes.

Distribution for bcr-abl negative MPNs Table 4

Diagnosis	Jak2 V617F positive	BCR-ABL positive	Double positive	Double negative
PV	22 (66%)	*	2 (6%)	9 (28%)
ET	35 (56%)	*	2 (3%)	6 (41%)
PMF	96 (54%)	2 (1%) *	2 (1%)	0 (44%)
MPN us	44 (52.6%)	14 (5%) *	1 (0.4%)	14 (42%)

*double positive patients

In the case of PV patients, the percentage (66%) was higher compared to the one obtained by only testing the 424 patients for Jak2 (45.7%). For the 33 patients that were doubly tested, the diagnosis criteria for PV were stricter by comparison to the first group of 424 patients, for which Jak2 testing served as a screening meant to differentiate between clonal polyglobulia and reactive polyglobulia.

We can notice the presence of the BCR-ABL transcript in 14 patients with unknown subtype of MPN and in two patients with suspected PMF. In this case, these patients fall into the BCR-ABL positive MPN group and can benefit from targeted treatment. If they would have only been subjected to Jak2V617F testing, this categorization would have been wrong.

Regarding the Jak2 V617 allele load, we noticed that a homozygous type was found in 77% of the JAK2 V617F positive PV cases and only in 20% of the JAK2 V617F positive ET cases (Figure 3).

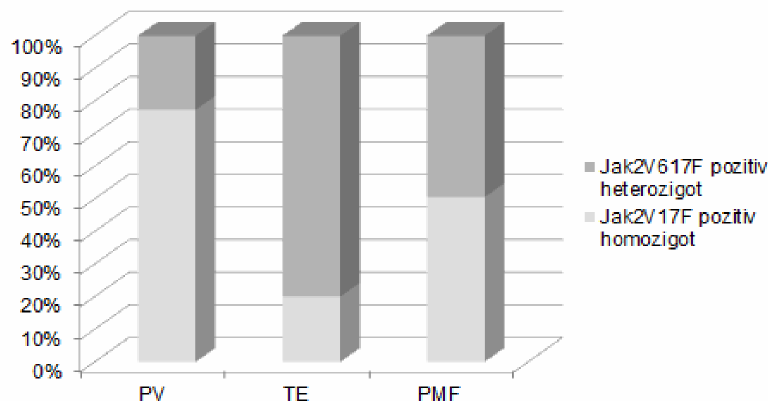

Fig. 3. *Distribution of JAK2V617F mutant allelic burden among diseases (homozygous vs. heterozygous)*

These distributions match the ones described in literature. This combination reinforces the hypothesis that the difference between the various phenotypes of MPN resides in the level of the allele load: a low Jak2 V617F mutation level would induce an ET phenotype dominated by thrombocytosis, and the increased mutation levels would lead to a PV phenotype. Experiments performed on transgenic mice

and studies made on erythroid colonies supported this hypothesis since 2009. See: [11]

The data reported in this study clearly proves the importance of detecting each of the molecular markers. The Jak2 V617F mutation is present in half of the patients with suspected BCR-ABL negative MPN, and the BCR-ABL transcript is present in 42% of the patients with suspected CML.

Our study also reveals the limits of a diagnosis based mainly on clinical and laboratory criteria. Of the 65 CML-like cases, we were able to confirm the disease in only 9 (13.8%). Also, of the patients with suspected BCR-ABL negative MPN, 16 cases (2.91%) were positive for BCR-ABL.

Particular cases with a concurrent existence of both markers contradict the idea that these mutations are mutually exclusive and raise new questions on the pathogenesis of MPN.

Therefore, for a correct diagnosis of chronic myeloproliferative neoplasms, the screening for detecting both JAK2V617F mutation and BCR-ABL transcript becomes mandatory.

Moreover, the new tyrosin-kinase inhibitors therapy for MPN treatments, are also/especially requiring a correct molecular diagnosis because these markers represent therapeutic targets as well.

4. Conclusion

An accurate diagnosis of MPN subtype (CML, PV, ET, and PMF) requires more tests of molecular biology: Jak2 exon 12 mutations, MPL, newly discovered CALR mutations. Furthermore, bone marrow biopsy is required for a firm diagnostic.

Oncogene Bcr-abl and point mutation Jak2V617 detection maintain their importance as screening tests, and should be performed to diagnose any suspected MPN. We would like to emphasize that such tests are performed by noninvasive methods, and do not require highly advanced and/ or too expensive technology.

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