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The role of erythropoietin levels and other hematological factors in the diagnosis of polycythemia vera in Iraqi patients

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Abstract:

BACKGROUND: According to the World Health Organization (WHO), erythropoietin (EPO) is only a minor criterion for the diagnosis of polycythemia vera (PV), but its diagnostic validity is controversial.

OBJECTIVES: The objective was to assess the diagnostic accuracy of EPO levels and the different combinations of the laboratory and clinical criteria, defined by the latest WHO report, as markers for the diagnosis of PV in Iraqi patients.

PATIENTS, MATERIALS AND METHODS: This cross-sectional study included 158 myeloproliferative neoplasm-suspected patients (48 PV, 47 essential thrombocythemia, 25 secondary thrombocytosis, and 35 nonclonal erythrocytosis). Patients were assessed for the presence of Janus Kinase 2 (JAK2) V617F mutation. Subsequently, JAK2V617F-negative patients were evaluated for the presence of JAK2 exon 12 mutations. Plasma EPO was measured in PV and nonclonal erythrocytosis patients.

RESULTS: Male was more prevalent among the nonclonal erythrocytosis patients. PV patients were older and had higher levels of all hematological variables examined in the study. Although all obtained EPO levels were normal, PV patients had significantly lower levels of EPO than nonclonal erythrocytosis. In addition, the hemoglobin and hematocrit had a better diagnostic accuracy than EPO levels in both male and female patients with PV. Furthermore, a better diagnostic accuracy was obtained when JAK2 mutation status was added to the evaluation of hemoglobin or hematocrit.

CONCLUSION: The low EPO level is not a good predictive marker for PV. Hemoglobin and hematocrit had equal predictive validity in the diagnosis of PV. It is convenient to evaluate JAK2 mutation as one of the major criteria in the diagnosis of PV.

Keywords:

Erythropoietin, Janus Kinase 2, myeloproliferative neoplasms, polycythemia vera

Introduction

Erythrocytosis denotes an increase in the red blood cell counts above the reference range for age and gender. It can be categorized as relative erythrocytosis due to diminished plasma volume or absolute erythrocytosis due to an actual increase in the red cell mass (RCM). Nonclonal erythrocytosis results from the proper physiological response to the increase in the erythropoietin (EPO) levels.[1] Nonclonal erythrocytosis has a wide range of differential diagnoses, including hypoxic lung disease, heart disease, smoking, and many others.[2] In contrast, primary erythrocytosis mostly occurs as a result of polycythemia vera (PV) which is a rare hematologic disorder. According to the 2016 World Health Organization (WHO) classification of hematological disorders, PV is one of the chronic myeloproliferative neoplasms (MPNs).[3] PV manifests itself as

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an abnormal increase in RCM and often as an increase in the total white blood count (WBC) counts and platelets level above the age- and sex-specific range.[3] PV patients usually present with ambiguous symptoms that may include fatigue, pruritus, early satiety due to splenomegaly, and increase the possibility of thrombotic events.[4] Extensive clinical, laboratory, and molecular evaluation is necessary for the differentiation between PV from nonclonal erythrocytosis.[3]

The most important complication and the principal cause of morbidity and mortality associated with PV are the thrombotic and hemorrhagic events. Besides, in rare cases, PV can transform into myelofibrosis or acute leukemia which will dramatically affect the patient's life span and quality of life.[5] Therefore, distinguishing PV from other MPN is essential. A gain of function mutations in the Janus Kinase 2 (JAK2) gene is the most common genetic abnormality detected among patients with PV.[6] About 95% of PV patients harbor mutation in JAK2V617F exon 14.[7] Alternatively, JAK2 exon 12 mutations are prevalent in the majority of JAK2V617F mutation-negative PV patients.[8] Genetic studies made the diagnosis of PV easier, but distinguishing subtypes of MPN still a challenge, especially distinguishing between PV, essential thrombocythemia (ET), and primary myelofibrosis (PMF) carrying the same point mutation in JAK2V617F.[3]

In 2016, the WHO delineated a number of clinical and biological criteria that can be used in the diagnosis of PV.[3] These diagnostic criteria are continuously evolving due to the progressions in the molecular testing.[3] JAK2 mutation status was first added to the major criteria for the diagnosis of PV by the WHO in 2007.[9]

Major changes were performed in the latest WHO report that includes upgrading bone marrow biopsy from minor to one of the major criteria due to its objectivity and reproducibility. In addition, the WHO lowered the hemoglobin levels into 16.5 g/dL, 16 g/dL in male and female, respectively, and hematocrit into 49.5%, 48.5% in males and females, respectively, because many evidences proved that the previous levels of hemoglobin and hematocrit were associated with underdiagnoses of PV. Furthermore, the WHO added the genetic testing to the MPN diagnostic algorithms.[3]

The usefulness of measuring EPO level is debatable, especially after the incorporation of JAK2 mutation in the major criteria for the diagnosis of PV. Therefore, below normal level, EPO was included as the only minor criterion in the latest WHO revision.[3] The sensitivity of below normal level of EPO in the diagnosis of PV is 64%, while the specificity is 92%.[10] Even though EPO levels have a good specificity, normal EPO might be encountered in PV patients due to many reasons, including high body mass index (BMI), smoking, and other comorbidities such as pulmonary disease and heart disease. Therefore, the role of EPO in the diagnosis of PV is debatable.[11]

Our aim in this study was to investigate the reliability of EPO as a diagnostic criterion for PV. Besides, we evaluated the sensitivity and the specificity of EPO together with other criteria defined by the WHO in the diagnosis of PV. Moreover, we tried to differentiate between PV and nonclonal erythrocytosis using clinical and laboratory information.

Patients, Materials, and Methods

This is a cross-sectional study that included all MPN-suspected patients who were attending the medical city, Baghdad Hospital, Department of Hematology, and the National Center for Hematology/Mustansiriyah University from December 2021 to May 2022. The diagnosis of PV was proved in 48 patients with erythrocytosis using the 2016 WHO criteria for the diagnosis of MPN.[3] The current research did not depend on the EPO level in the diagnosis of those patients. Thirty-five patients with erythrocytosis did not meet the WHO criteria and thus considered a nonclonal erythrocytosis. This study was approved by review ethical Committee of Mustansiriyah University. A written consent was obtained from all qualified patients. A venous blood was collected at the time of diagnosis in ethylenediaminetetraacetic acid tubes between 8:00 a.m. and 12:00 p.m. The relevant clinical and hematological variables were documented by reviewing the medical records.

QIAamp DNA Micro Kit (QIAGEN, Germany, cat. no 51304 and 51306) or ReliaPrep Blood gDNA Miniprep System (Promega, USA, cat. No. A5081) kits were used for DNA extraction. Quantification of DNA was done using spectrophotometer (Qubit 4 flowmeter from Thermo Fisher Scientific). Eligible patients were first evaluated for the presence of JAK2V617F mutation using either allele-specific polymerase chain reaction (PCR) as described in the previously published protocol[12] or JAK2 quantitative real-time PCR kit (single-nucleotide polymorphism [SNP], TURKY, Cat. No: 21QR-10-01) and according to the manufacturer's instructions. Subsequently, JAK2V617F-negative patients were evaluated for the presence of JAK2 exon 12 mutations using SNP biotechnology MPN screening kit (TURKEY, cat. No: 23R-20-10) according to the manufacturer's protocol.

Plasma EPO measurement was made using blood samples taken from PV and secondary erythrocytosis

patients. The measurement was based on the sandwich enzyme-linked immunosorbent assay (ELISA) using Human EPO ELISA Kit (Sunlong Biotech, China, Catalog Number: SL0679Hu). The ELISA microplate reader used for the analysis was Mindray, MR-96A. Sensitivity of the kit was about 0.7 pg/ml.

Statistical analysis

Minitab 19 software was used to analyze data. Relevant variables distribution was checked by the Anderson–Darling test. We summarized the numerical variables as median and range, while the categorical variables were presented as count and relative frequency (%). Unpaired two-sample *t*-test or the nonparametric Mann–Whitney test was used to assess the differences in the distribution of quantitative variables. On the other hand, qualitative variables were compared using Fisher's exact test. The sensitivity and specificity of plasma EPO, hemoglobin, and hematocrit levels and the presence of the mutation were calculated to evaluate their predictive accuracy in the diagnosis of PV. For the EPO, the cutoff point used was the lower limit of the normal EPO values. While for the hemoglobin and hematocrit, the thresholds determined by the WHO were adapted (hemoglobin and hematocrit were >16.5 g/dL and 49% in males, respectively, and >16 g/dL and 48% in females, respectively).[3] The correlation between variables was done using nonparametric pair-wise spearman's correlation. EPO, gender, and smoking status were used to fit univariate logistic regression models. $P < 0.05$ was considered statistically significant.

Results

Of the 158 study cohort, 48 (62.5% of males) were diagnosed as PV in accordance with the 2016 WHO criteria, and 35 (94.3% of males) patients were diagnosed as nonclonal erythrocytosis. Although both PV and nonclonal erythrocytosis are more common among male patients, nonclonal erythrocytosis had a higher percentage of males than PV ($P < 0.002$) [Table 1]. Patients with PV were significantly older than the nonclonal erythrocytosis. Furthermore, nonclonal erythrocytosis was more prevalent among the current smokers ($P < 0.02$), while PV was more prevalent among the nonsmokers ($P < 0.002$). No difference in BMI was noticed between the two groups. 97.9% of PV patients were found to have mutation in JAKV617F, and 2.1% had mutation in JAK2 exon 12. Conversely, no mutation was detected in the nonclonal erythrocytosis group [Table 1].

Table 2 shows the hematologic and clinical characteristics of the study cohort. PV patients had a significantly higher level of hemoglobin and hematocrit than nonclonal erythrocytosis. The total WBC, absolute neutrophils, and basophils counts were higher in PV than nonclonal erythrocytosis. No difference was observed in the level of monocytes between the two groups. The median platelets count was lower in nonclonal erythrocytosis than PV. Importantly, patients with confirmed diagnosis of PV and nonclonal erythrocytosis had the serum EPO levels within the normal reference range, except two patients with nonclonal erythrocytosis who had serum EPO levels above the normal reference range (one is a current smoker). Despite the fact that almost all patients in our study cohort had normal EPO levels, PV patients had significantly lower EPO levels than nonclonal erythrocytosis ($P < 0.001$).

It is worth noting that 35.4% of PV patients presented with splenomegaly at diagnosis; meanwhile, in the nonclonal erythrocytosis patients, only 5.7% of them had splenomegaly at diagnosis; the difference was statistically significant ($P < 0.002$). No differences were noted in the frequency of hepatomegaly and hepatosplenomegaly between the two groups.

Likewise, the frequency of thrombotic events in PV and nonclonal erythrocytosis was computed [Figure 1].

Table 1: The baseline demographic characteristics of patients with polycythemia vera and secondary erythrocytosis

	PV (*n*=48)	NO PV (*n*=35)	*P*
Age mean (years)	64 (24-83)	32 (17-70)	<0.0001
Gender, *n* (%)			
Male	30 (62.5)	33 (94.3)	<0.002
Female	18 (37.5)	2 (5.7)	<0.002
BMI (kg/m^2)	27.55 (17.26-32.65)	27.99 (19.03-41.91)	NS
Smoking status, *n* (%)			
Never	38 (79.2)	19 (45.3)	<0.002
Current	4 (8.3)	10 (28.6)	0.02
Former	6 (12.5)	6 (17.1)	NS
Mutation status, *n* (%)			
JAK2V617F	47 (97.9)	0	<0.0001
JAK2 exon 12	1 (2. 1)	0	NS

Data were expressed either as median and range or as relative frequencies. BMI=Body mass index, PV=Polycythemia vera, NS=Not significant

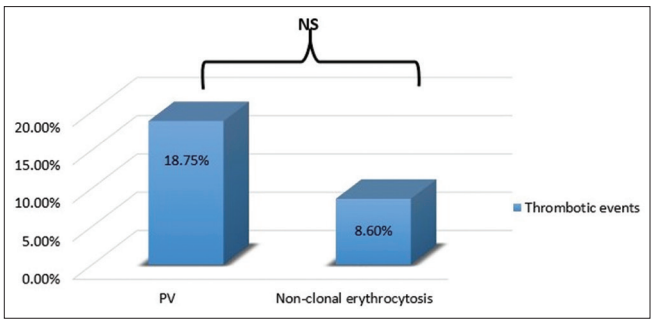

Figure 1: Thrombotic events in PV and nonclonal erythrocytosis. PV: Polycythemia vera

Table 2: The baseline hematological and clinical characteristics of patients with polycythemia vera and secondary erythrocytosis

	PV *n* (48)	No PV *n* (35)	*P*
Hemoglobin (g/dL)	17.3 (16.2-23)	16.8 (16-22)	<0.02
Hematocrit (%)	54.65 (45.8-72)	49.48 (48.4-65.3)	<0.001
Leukocyte (×10³/μL)	13.63 (7.55-47.1)	7.82 (4-17.19)	<0.001
Absolute neutrophils (×10³/μL)	10.38 (3.69-32.9)	4.56 (1.55-12.56)	<0.001
Eosinophils (×10³/μL)	0.287 (0.01-1.09)	0.183 (0.02-0.543)	<0.008
Basophils (×10³/μL)	0.142 (0.02-0.76)	0.06 (0.00-0.7)	<0.001
Monocytes (×10³/μL)	0.64 (0.01-8.42)	0.58 (0.29-1.2)	NS
Platelets (×10⁹/L)	562.5 (168-1500)	243 (116-476)	*P* <0.001
Erythropoietin (pg/mL)	82.123 (77.54-88.75)	85.9 (77.9-974.4)	<0.001
Hepatomegaly (%)	12.5	2.9	NS
Splenomegaly (%)	35.4	5.7	<0.002
Hepatosplenomegaly (%)	12.5	0	NS

Data were expressed as median and range. PV=Polycythemia vera, NS=Not significant

In our cohort, no statistical significance was noted in the frequency of thrombotic events in PV patients and nonclonal erythrocytosis, although more PV patients presented with thrombotic events (18.75%) than nonclonal erythrocytosis patients (8.6%). It is worth noting that all PV patients presented with thrombotic events at diagnosis carry mutation in JAK2V617F.

Next, we examined the correlation of EPO with the other relevant variables in patients with PV as shown in Table 3. EPO was positively correlated with the BMI ($P < 0.001$). In contrast, no correlations were observed between EPO and age or between EPO and the different hematological variables such as hemoglobin, hematocrit, leukocytes, absolute neutrophils, basophils, monocytes, eosinophils, and platelets counts.

In addition, to demonstrate whether smoking and gender can have an influence over EPO levels, univariate logistic regression models were fitted as shown in Figure 2. Tobacco smoking and gender failed to significantly predict the serum EPO levels.

Next, we evaluated the diagnostic accuracy of hemoglobin, hematocrit, and plasma EPO level as indicators for the diagnosis of PV. Hemoglobin and hematocrit were measured in 158 patients (PV 48, ET 47, nonclonal erythrocytosis 35, and secondary thrombocytosis 25), while EPO levels were measured in PV and nonclonal erythrocytosis patients only. Our results showed that the hemoglobin and hematocrit had a better diagnostic accuracy than EPO levels in both male and female patients with PV; this is because EPO measurement has a sensitivity of zero in our cohort. Meanwhile, EPO measurement was highly specific to PV (100% specificity in male and female) [Table 4].

The precision of hemoglobin levels as a diagnostic marker in patients with PV was higher when combined with JAK2V617 mutation; sensitivity increased by

Table 3: The correlation of erythropoietin with the demographic and laboratory indices of patients with polycythemia vera

	r	*P*
Age (years)	0.198	0.181
BMI (kg/m²)	0.843	*P*<0.001
Hemoglobin (g/dL)	-0.207	0.158
Hematocrit (%)	0.002	0.99
Leukocyte (×10³/L)	0.226	0.093
Absolute neutrophils (×10³/L)	0.187	0.207
Eosinophils (×10³/L)	0.207	0.121
Basophils (×10³/L)	-0.070	0.676
Monocytes (×10³/L)	0.250	0.130
Platelets (×10⁹/L)	0.033	0.822

BMI=Body mass index, PV=Polycythemia vera, EPO=Erythropoietin

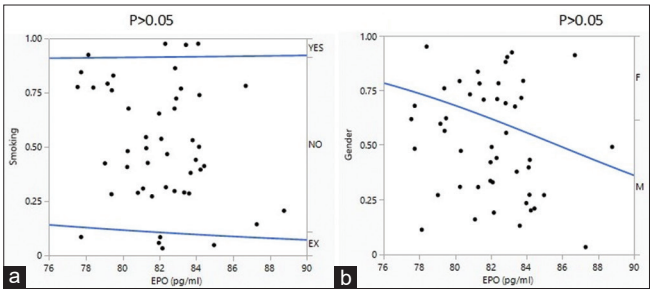

Figure 2: Logistic fit of EPO. (a) Logistic fit of EPO levels by smoking. (b) Logistic fit of EPO by gender. EPO: Erythropoietin

28.6%, and 22.6% in male and female, respectively, and specificity increased by 38.3%, and 2.4% in male and female, respectively.

Furthermore, the sensitivity and specificity of hematocrit levels were measured in all study groups using threshold defined by the WHO. Measuring the hematocrit and hemoglobin showed a similar diagnostic validity. Likewise, the evaluation of hematocrit or hemoglobin and JAK2V617F as markers for PV gave better diagnostic values than hematocrit or hemoglobin alone [Table 4].

As shown in Table 5, we assessed the precision of different combinations of PV diagnostic criteria. A sensitivity of 34.4% and specificity of 80.4% were obtained using hemoglobin or hematocrit levels above thresholds defined by the WHO. Adding JAK2V617F mutation to the hemoglobin or hematocrit slightly improved the diagnostic accuracy of PV. On the other hand, lower sensitivity and higher specificity were obtained by adding the WBC count above the level designated by the WHO to the hemoglobin or hematocrit. Again, adding JAK2V617F mutation to this combination had changed the results slightly. Adding the platelets count to the previous combination had an increased specificity to 100% and lowered the sensitivity to 22.5% [Table 5].

When we used the hemoglobin or hematocrit, the WBC and platelet counts in JAK2V617F-positive MPN patients, the sensitivity and specificity did not change dramatically as displayed in Table 5.

Discussion

Erythrocytosis is relatively common in clinical practice, and in most cases, it is secondary to other conditions. The differentiation between PV and nonclonal erythrocytosis is critical, since early diagnosis and treatment of PV patients may reduce the risk thrombosis and many other vasomotor complications. Even though the exact estimation of the prevalence of nonclonal erythrocytosis is difficult, it is higher than the prevalence of PV. 6%–8% of patients with abstractive lung disease[13] and 2%–8% of patients with obstructive sleep apnea present with nonclonal erythrocytosis,[14] while the prevalence of PV is about 22/100,000.[15] In our study, the prevalence of nonclonal erythrocytosis (42.2%) was <PV (57.8%). This is because we included only patients who met the WHO criteria for the diagnosis of PV and had their complete medical information available. In addition, the limited number of genetic test we were able to run on patients with erythrocytosis was also another factor that

Table 4: The diagnostic precision of hemoglobin, hematocrit, and erythropoietin thresholds limit as indicators of polycythemia vera

	n	False-positive cases (*n*)	False-negative cases (*n*)	Sensitivity (%)	Specificity (%)
The entire cohort	155				
Male	98				
Hemoglobin >16.5 g/dL	98	26	5	37.3%	61.7%
Hematocrit >49%	98	20	2	36.8%	70.5%
EPO pg/mL	63	0	30	0%	100%
Female	57				
Hemoglobin >16 g/dL	57	1	0	30.5%	97.6%
Hematocrit >48%	57	1	0	32.1%	97.4%
EPO pg/mL	20	0	18	0%	100%
MPN patients with JAK2V617F	76				
Male	44				
Hemoglobin >16.5 g/dL	44	0	3	65.9%	100%
Hematocrit >49%	44	0	2	66.7%	100%
Female	32				
Hemoglobin >16 g/dL	32	0	0	53.1%	100%
Hematocrit >48%	32	0	0	53.1%	100%

The entire cohort included patients with PV, ET, secondary erythrocytosis, and secondary thrombocytosis except EPO measured only in PV and secondary erythrocytosis patients. JAK2V617F-positive MPN patients comprised both PV and ET. EPO=Erythropoietin, MPN=Myeloproliferative neoplasm, PV=Polycythemia vera, ET=Essential thrombocythemia

Table 5: The precision of different combinations of diagnostic criteria for the diagnosis of polycythemia vera

Entire cohort	False-positive cases (*n*)	False-negative cases (*n*)	Sensitivity (%)	Specificity (%)
Hemoglobin >16.5/16 g/dL or hematocrit >49%, 48%	21	3	34.4	80.4
Hemoglobin >16.5/16 g/dL or hematocrit >49%, 48%, + WBC above normal range	8	12	27	92
Hemoglobin >16.5/16 g/dL or hematocrit >49%, 48%, + WBC above normal range, +PLT>450 *10^9/L	0	17	22.5	100
MPN patients with JAK2V617F				
Hemoglobin >16.5/16 g/dL or hematocrit >49%, 48%	9	12	36.3	73.1
Hemoglobin >16.5/16 g/dL or hematocrit >49%, 48%, + WBC above normal range	29	2	26.1	91.7
Hemoglobin >16.5/16 g/dl or hematocrit >49%, 48%, + WBC above normal range, +PLT PLT>450 *10^9/L	0	17	21.8	99

The cohort included patients with PV, ET, secondary erythrocytosis, and secondary thrombocytosis. MPN=Myeloproliferative neoplasm, PV=Polycythemia vera, ET=Essential thrombocythemia, WBC=White blood cell, PLT=Platelet

contributes to low frequency of nonclonal erythrocytosis in our cohort. Patients with PV had more severe clinical course and significantly higher values of all laboratory variables analyzed in this study.

Moreover, in this study, we tried to evaluate the precision of the WHO diagnostic criteria as markers for PV in Iraqi patients with erythrocytosis. Currently, the WHO included the low EPO level as the only minor criterion for the diagnosis of PV. The inclusion of EPO was based on multiple literatures published previously on the discovery of JAK2 mutations.[16] The diagnostic value of the EPO level had become questionable after the discovery of JAK2 mutations.

In our study cohort, we found that measuring EPO level was not a useful tool in distinguishing PV from nonclonal erythrocytosis or ET in Iraqi patients. In addition, depending on the EPO levels can lead to misdiagnosis of PV, because all PV patients in our cohort had EPO levels within normal. These results are different from other studies which showed that measuring EPO is a good diagnostic marker for PV.[10] Lupak *et al*., 2020, showed that the low EPO levels had a moderate predictive accuracy, and it is of no value when JAK2V617F mutation is positive.[5] We suggest that one of the reasons behind our results is that smoking in the general places is not forbidden in our country, and therefore, a large number of patients in our cohort might be considered passive smokers. In addition, our results could be attributed to the method we used in measuring plasma EPO levels. Different studies used different methods; Ancochea *et al*., in 2014, illustrated that EPO is a good predictive marker for PV only when it is measured through CEIA method.[11] Besides, the EPO levels in our cohort were not correlated with hemoglobin or hematocrit, which indicates that there are other factors that might affect them such as hypoxia and smoking. On the other hand, EPO correlated positively with the BMI.[5] These findings make EPO an unreliable diagnostic marker for PV.

In the current study, we revealed that the diagnostic accuracy of the criteria defined by the WHO was insufficient for the diagnosis of PV due to the low sensitivity of hemoglobin, hematocrit, and EPO in our cohort of the patient.[11] In this situation, the study of RCM might be necessary to confirm the diagnosis.[17] Recently, an increased attention is drawn toward the molecular testing owing to its diagnostic and prognostic significance. Despite that, the identification of JAK2 mutation is a powerful predictive marker for the diagnosis of PV, the discrimination between PV, ET, and PMF is still a challenge since they share the same point mutation.[18]

The presence of JAK2 mutations together with the hemoglobin and hematocrit in PV patients had dramatically increased the sensitivity and specificity for the diagnosis of PV. It is worth noting that the sensitivity of hemoglobin and hematocrit in the diagnosis of PV was equal. These results are in line with the latest WHO criteria for the diagnosis of MPN published in 2016,[3] which depend on hemoglobin or hematocrit equally in the diagnosis of PV. In contrast, the BCSH criteria depend on the hematocrit alone as a diagnostic marker of PV with the qualitative measurement of JAK2 mutations.[19] Despite the update in the WHO criteria, discrimination between PV and other MPN is still difficult in a proportion of patients when the RCM cannot be measured.[11]

Our results showed that there were gender differences in the accuracy of diagnostic criteria of PV defined by the WHO. We could not find an explanation for these gender differences, but we might learn from a bigger sample size.

In the current work, we evaluated the precision of different combinations of diagnostic criteria of PV. We demonstrated that the different combinations of hemoglobin, hematocrit, WBC count, and platelet levels in the presence or absence of JAK2 mutations had low sensitivity in the diagnosis of PV. However, patients presented with erythrocytosis, leukocytosis, and thrombocytosis reach specificity of 100% in the presence or absence of JAK2 mutations; this may indicate that in such group of patients, the diagnoses as PV might be reached without the need for the molecular study.

Conclusion

The current study implied that low EPO levels are not a good predictive marker for PV. Depending on EPO might cause misdiagnoses of PV patients who have comorbidities that increase the serum EPO levels. The use of hemoglobin or hematocrit as major diagnostic criteria gave an equal predictive validity in the diagnosis of PV. Evaluating JAK2V617F mutation is very convenient in the diagnosis of PV.

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Conflicts of interest
There are no conflicts of interest.

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