A Rational Approach to *JAK2* Mutation Testing in Patients with Elevated Hemoglobin: Results from the *JAK2* Prediction Cohort (JAKPOT) Study

Benjamin Chin-Yee[1,2], Pratibha Bhai[3,4], Ian Cheong[5], Maxim Matyashin[1,2], Cyrus C. Hsia[1,2,5], Eri Kawata[6,7], Jenny M. Ho[1,2], Michael A. Levy[3,4], Alan Stuart[3,4], Hanxin Lin[3,8], Ian Chin-Yee[1,2,5], Mike Kadour[3,4,5], Bekim Sadikovic[3,4,5], and Alejandro Lazo-Langner[1,2,8]

[1]Division of Hematology, Department of Medicine, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada; [2]Division of Hematology, Department of Medicine, London Health Sciences Centre, London, Ontario, Canada; [3]Molecular Diagnostic Division, London Health Sciences Centre, London, Ontario, Canada; [4]Verspeeten Clinical Genome Centre, London Health Sciences Centre, London, Ontario, Canada; [5]Department of Pathology and Laboratory Medicine, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada; [6]Division of Hematology, Panasonic Health Insurance Organization, Matsushita Memorial Hospital, Moriguchi, Osaka, Japan; [7]Division of Hematology and Oncology, Department of Medicine, Kyoto Prefectural University of Medicine, Kyoto, Kyoto, Japan; [8]Department of Epidemiology and Biostatistics, Schulich School of Medicine and Dentistry, Western University, London, Ontario, Canada.

BACKGROUND: Erythrocytosis, most often measured as an increase in hemoglobin and/or hematocrit, is a common reason for referral to internal medicine and hematology clinics and a rational approach is required to effectively identify patients with polycythemia vera while avoiding over-investigation.

AIM: We aimed to develop and validate a simple rule to predict *JAK2* mutation positivity based on complete blood count parameters to aid in the diagnostic approach to patients referred for elevated hemoglobin.

SETTING: Internal medicine and hematology clinics at an academic tertiary referral center.

PARTICIPANTS: The *JAK2* Prediction Cohort (JAKPOT), a large retrospective cohort (n = 901) of patients evaluated by internal medicine and hematology specialists for elevated hemoglobin.

DESIGN: *JAK2* mutation analysis was performed in all patients and clinical and laboratory variables were collected. Patients were randomly divided into derivation and validation cohorts. A prediction rule was developed using data from the derivation cohort and tested in the validation cohort.

KEY RESULTS: The JAKPOT prediction rule included three variables: (i) red blood cell count >6.45×10^{12}/L, (ii) platelets >350×10^{9}/L, and (iii) neutrophils >6.2×10^{9}/L; absence of *all* criteria was effective at ruling out *JAK2*-positivity with sensitivities 94.7% and 100%, and negative predictive values of 98.8% and 100% in the derivation and validation cohorts, respectively, with an overall low false negative rate of 0.4%. The rule was validated for three different methods of *JAK2* testing. Applying this rule to our entire cohort would have resulted in over 50% fewer tests.

CONCLUSION: In patients with elevated hemoglobin, the use of a simple prediction rule helps to accurately identify patients with a low likelihood of having a *JAK2* mutation, potentially limiting costly over-investigation in this common referral population.

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INTRODUCTION

Erythrocytosis is defined as an increase in concentration of red blood cells (RBCs) above age- and sex-specific reference ranges, most often measured as an increase in hemoglobin and/or hematocrit, both common reasons for referral to internal medicine or hematology specialists for further investigations and management. The differential diagnosis of erythrocytosis is broad and includes an apparent (or relative) increase in RBCs due to decreased plasma volume or a true increased red cell mass (polycythemia). Absolute erythrocytosis or polycythemia can be secondary to underlying conditions such as hypoxia, medications, and erythropoietin secreting tumors or the result of primary bone marrow disorders, namely myeloproliferative neoplasms such as polycythemia vera (PV).

PV has an incidence of 2.7 per 100,000 and is associated with high morbidity and mortality if untreated,[1] making it critical to differentiate PV from other causes of erythrocytosis.[2] Concerns over underdiagnosis of early-stage PV[3] motivated the 2016 revision of the World Health Organization (WHO) classification, which decreased the hemoglobin thresholds to 160

g/L for females and 165 g/L for males.[4] These revised thresholds, however, overlap with the normal reference range for healthy adults, raising opposite concerns of potential over-investigation of patients with elevated hemoglobin.[5] Indeed, since 2016 our center has seen an increase in referrals for patients with elevated hemoglobin, accompanied by a significant rise in the volume of molecular testing for *JAK2* mutations and associated decline in test positivity rate.[6, 7] This trend may reflect a combination of factors including adherence to the revised WHO hemoglobin thresholds, improved access to molecular testing, and lack of discriminatory function of conventional tests such as serum erythropoietin (EPO).[8]

JAK2V617F or exon 12 mutations are present in over 99% of patients with PV.[9] However, the frequency of *JAK2* mutations in patients with erythrocytosis undergoing molecular testing ranges from 1 to 5% depending on the referral population,[10] indicating that the majority of patients investigated for elevated hemoglobin do not have PV. There is currently a lack of consensus on appropriate criteria for molecular testing, which in practice often translates into over-investigation, creating the need for a rational approach to molecular testing.[8] Attempts to develop prediction rules to guide *JAK2* mutation testing have largely focused on population-based cohorts and have proposed over-inclusive testing criteria, limiting their applicability in clinical practice.[13, 14] In this article, we outline a diagnostic approach to patients referred for elevated hemoglobin that uses a simple prediction rule based on complete blood count and white blood cell differential (CBC) parameters to guide *JAK2* mutation testing, which we developed and validated in a large retrospective clinical cohort of patients evaluated by internal medicine and hematology specialists (the *JAK2* Prediction Cohort).

METHODS

The *JAK2* Prediction Cohort (JAKPOT) study was conducted at London Health Sciences Centre, a tertiary referral center which serves a population of approximately 2 million in Southwestern Ontario, Canada. All adult patients referred for elevated hemoglobin (≥160 g/L for females, or ≥165 g/L for males) between January 1, 2015, and May 12, 2021, who underwent *JAK2* mutation testing were included. This referral population included patients evaluated by internal medicine and hematology specialists who also performed investigations for possible secondary causes of erythrocytosis. Molecular testing was performed by quantitative polymerase chain reaction (qPCR) using the Roche 480 LightCycler (La Roche AG, Switzerland), single nucleotide polymorphism (SNP) allelotyping using the Agena MassARRAY system (Agena Biosciences, CA, USA), or next-generation sequencing (NGS) using the Oncomine Myeloid Research Assay (ThermoFisher Scientific, MA, USA). qPCR and SNP allelotyping assays tested for *JAK2V617F* mutations; the NGS assay tested for both *JAK2V617F* mutations and exon 12 mutations. Age, sex, and CBC parameters at the time of *JAK2* mutation testing, including hemoglobin, hematocrit, erythrocytes, leukocytes, neutrophils, platelets, and mean corpuscular volume values were extracted. CBCs were performed on a Sysmex XN Analyzer (Sysmex Corporation, Japan). The JAKPOT study was approved by the Research Ethics Boards at Western University (118139).

Patients were randomly divided into derivation and validation cohorts including all methods of *JAK2* mutation testing. *JAK2*-positive and -negative groups were compared using Student's *t*-tests or χ^2 tests, as appropriate. Continuous variables were dichotomized at optimal cut-off points using receiver operating characteristic curves. Variables tested included age, sex, hematocrit, and RBC, leukocyte, neutrophil, and platelet counts. Potentially significant predictors were evaluated using multiple variable stepwise logistic regression analysis with *JAK2* positivity as the dependent variable with probability for inclusion and removal from the model at 0.05 and 0.10, respectively. The models were evaluated using Hosmer–Lemeshow tests and pseudo-R^2 measures. A dichotomous score was derived based on the presence or absence of significant variables and subsequently evaluated and internally validated using logistic regression and χ^2 tests using non-parametric bootstrapping with 1000 samples. The final model was tested in the validation cohort and sub-analyses for each method were conducted in a similar fashion. Test accuracy for the criteria was evaluated in both cohorts and all subgroups. Analyses were performed using SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA) and MedCalc® Statistical Software version 20.026 (MedCalc Software Limited, Ostend, Belgium). Further details on analysis are available from the authors upon request.

Table 1 Characteristics of the Derivation and Validation Cohorts

Cohort characteristics	Derivation cohort (*n* = 616)	Validation cohort (*n* = 285)
Age, mean (SD) (years)	58 (15)	58 (15)
Male sex, number (%)	449 (72.9)	205 (71.9)
Hemoglobin, mean (SD) (g/L)	177 (11)	177 (11)
Hematocrit, mean (SD) (L/L)	0.53 (0.04)	0.53 (0.04)
RBCs, mean (SD) ($\times 10^{12}$/L)	6.11 (0.597)	5.90 (0.607)
Platelets, mean (SD) ($\times 10^9$/L)	266 (162)	281 (187)
Leukocytes, mean (SD) ($\times 10^9$/L)	9.2 (4.0)	9.1 (3.3)
Neutrophils, mean (SD) ($\times 10^9$/L)	6.0 (3.7)	5.7 (2.5)
JAK2 mutation positive, number (%)	75 (12.2)	31 (10.9)
Testing method, number (%)		
Next-generation sequencing	358 (58.1)	173 (60.7)
Polymerase chain reaction	155 (25.2)	70 (24.6)
SNP allelotyping	103 (16.7)	42 (14.7)

Abbreviations: SD, standard deviation; RBCs, red blood cells; JAK2, Janus Kinase 2; SNP, single nucleotide polymorphism. P-values for all variables were >0.05

RESULTS

The total study cohort included 901 patients (derivation n = 616, validation n = 285). Population characteristics (Table 1) were similar between derivation and validation cohorts. A positive *JAK2* mutation was found in 11.8%, 12.2%, and 10.9% of the total, derivation, and validation cohorts, respectively. Of the variables tested, the final model included the following: RBC count >6.45×10^{12}/L, platelets >350×10^{9}/L, and neutrophils >6.2×10^{9}/L. Patients with *none* of these criteria were considered low-risk; patients with *any* of these criteria were considered high-risk. In the derivation cohort, the percentages of *JAK2*-positive results in low- and high-risk patients were 1.2% and 24.9%, respectively. The model had a sensitivity of 94.7% and a negative predictive value of 98.8%. Results were consistent in the validation cohort, where the model had a sensitivity of 100% and negative predictive value of 100%. Table 2 shows contingency tables for each cohort. Overall, the false negative rate in our study was 0.4%. Results were also consistent across each testing method (Supplemental Table 1). Based on this analysis, we developed a testing algorithm to guide *JAK2* mutation testing using our model (Fig. 1). Applying this rule to our entire cohort of patients would have resulted in over 50% fewer tests.

DISCUSSION

PV is a myeloproliferative neoplasm associated with high morbidity and mortality if untreated. PV has a range of presentations, with classic clinical features including aquagenic pruritus, splenomegaly, and unexplained thrombosis; however, initial referral to internal medicine or hematology is most commonly triggered by an elevated RBC count, elevated hemoglobin and/or elevated hematocrit. Comprehensive evaluation of patients with erythrocytosis can include extensive investigations for secondary causes (Fig. 2) in addition to more costly, specialized testing such as *JAK2* mutation analysis. The high sensitivity and specificity of *JAK2* mutation analysis makes it the preferred test in patients with suspected PV resulting in this test being performed up front in many settings.[9] However, there remains a need for tools to more effectively utilize *JAK2* testing in patients referred for erythrocytosis in order to reduce indiscriminate use of costly molecular testing.[8]

Table 2 Contingency Tables for the Derivation and Validation Cohorts

Cohort	JAKPOT criteria	*JAK2* mutation	
		Positive	Negative
Derivation (n = 616)	0	4 (0.6%)	326 (52.9%)
	≥1	71 (11.5%)	215 (34.9%)
Validation (n = 285)	0	0 (0%)	153 (53.7%)
	≥1	31 (10.9%)	101 (35.4%)
Total (n = 901)	0	4 (0.4%)	479 (53.2%)
	≥1	102 (11.3%)	316 (35.1%)

The initial diagnostic approach should include evaluation for absolute versus relative erythrocytosis and clinical assessment for common secondary causes such as smoking, hypoxic lung disease, and medications (Fig. 2). If identified, management should be directed at the secondary cause with monitoring for resolution of erythrocytosis. In patients where no clear secondary cause is identified, the next step is to determine the likelihood of PV and whether *JAK2* molecular testing may be necessary. Traditionally, serum EPO has been used to assist in decision-making, with low EPO being associated with PV versus high EPO in secondary erythrocytosis; however, recent evidence suggests serum EPO measurement is often unreliable and can be normal in over a third of patient with PV.[8] Moreover, EPO levels are rarely available to guide decision-making at the time of initial consultation and measurement can further contribute to diagnostic delays.

To assist in the decision to order *JAK2* mutation testing, we propose a simple prediction rule, the JAKPOT rule, which uses CBC parameters to predict the likelihood of *JAK2* mutation positivity in patients presenting with elevated hemoglobin. We developed and validated this prediction rule in a large cohort of patients referred to internal medicine and hematology specialists for erythrocytosis where it demonstrated a high negative predictive value (>98.8%). The proposed rule demonstrates improved sensitivity and negative predictive value compared to low serum EPO in ruling out a diagnosis of PV in a similar patient population.[8] This rule could help limit indiscriminate over-investigation of patients referred for erythrocytosis and help optimize the diagnostic yield of molecular testing. In our cohort, implementation of this rule could have significantly reduced the number of tests for *JAK2* mutations with minimal risk of missing diagnoses of *JAK2*-positive PV.

The three parameters included in the JAKPOT rule are RBC count, platelet count, and neutrophil count, which have well-established pathophysiologic rationale given that elevations in all of these values (i.e., panmyelosis) are a recognized biological hallmark of PV.[11] If *either* the RBC count, platelet count, or neutrophil count meet their respective thresholds as outlined in the JAKPOT rule, then molecular diagnostic testing for *JAK2* mutations is recommended. Whereas hemoglobin and hematocrit are independently affected by cell size, plasma volume, and iron stores, RBC count provides a more accurate indicator of erythropoiesis and red cell mass.[12] Despite this, RBC count has not been incorporated in other prediction rules or diagnostic criteria for PV. It is also known that patients with so-called masked PV—"masked" due to concurrent iron deficiency—can initially present with thrombocytosis.[3] Platelet count has been used in other algorithms;[13] however, our model uses a higher threshold of 350×10^{9}/L than other algorithms, increasing the discriminatory power.

Our approach has several strengths. First, it uses readily available CBC parameters making it easy to apply at initial

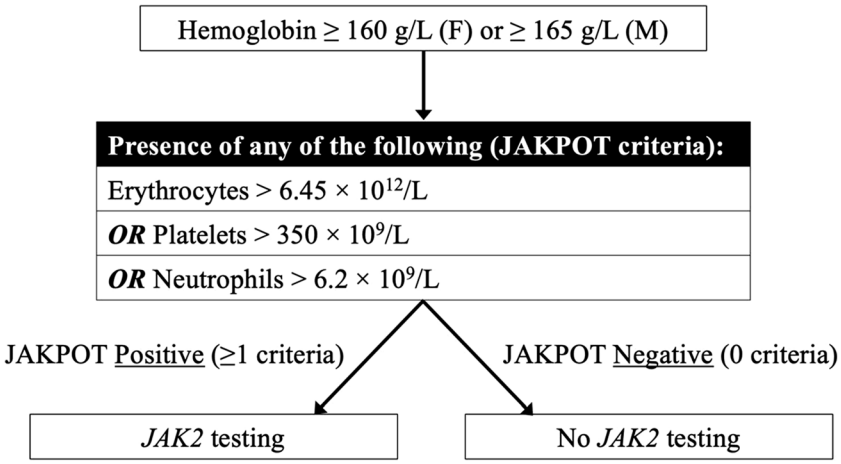

Figure 1 Flowchart illustrating the JAKPOT rule to guide *JAK2* mutation testing using CBC parameters. Abbreviations: JAKPOT, *JAK2* Prediction Cohort; CBC, complete blood count; F, female; M, male; RBC, red blood cell; *JAK2*, Janus Kinase 2.

clinic visits without introducing delays required for other assays such as serum EPO. Importantly, these parameters are objective and not subject to interpretation with its inherent bias. Additionally, this prediction rule was derived from a real-world cohort of patients referred for elevated hemoglobin as opposed to a population-based cohort,[14] and validated across three different assays for *JAK2* mutation testing, including qPCR, SNP allelotyping, and NGS platforms. Most importantly, our approach uses a Bayesian decision framework that increases the diagnostic accuracy and safety by applying this rule only to patients with a higher pre-test probability of having PV as defined by the hemoglobin threshold accepted by the WHO 2016 diagnostic criteria. The use of the rule allows to identify those patients with a very low posterior probability of having a positive *JAK2* mutation with a very high level of accuracy. This strategy has proven to be highly successful in other areas of medicine. The best example is the use of the Wells' clinical prediction rules for deep vein thrombosis and pulmonary embolism that, in conjunction with the results of a D-dimer, allow clinicians to safely rule out these potentially serious diagnoses with a very high level of accuracy.[15, 16]

Our prediction rule offers several advantages over others in the literature.[13, 14] Piris-Villaespesa et al.[13] recently proposed an algorithm for PV screening using platelet and neutrophil counts but lacked *JAK2* status for all patients in the validation cohort. Mahe et al.[14] developed a decision tree using CBC parameters to guide *JAK2* mutation testing based on data from the Copenhagen General Population Study. While this algorithm had a high negative predictive value of 92% and a false-negative rate of only 1.2%, it recommended *JAK2* mutation testing in any patient with a hemoglobin >160 g/L (>165 g/L in men), a platelet count $>350\times10^9$/L, or white blood cell count $>7\times10^9$/L and thus has limited ability to reduce molecular testing in patients with abnormal CBC parameters. Given that this rule recommends testing in all patients with elevated hemoglobin level (>160 g/L in women and >165 g/L in men), nearly all patients in our cohort would have been eligible for testing, limiting its applicability in clinical practice.

There are several limitations to our study. Our prediction rule was derived and validated retrospectively using data from a single referral center, which may limit its generalizability to other centers and populations. Further studies to prospectively validate this prediction rule in different populations at other centers are planned. While our cohort included all patients with elevated hemoglobin who underwent *JAK2* mutation analysis, it did not capture patients who did not have *JAK2* testing performed. Nonetheless, during the study period it was standard practice at our center to perform *JAK2* mutation testing in patients referred for elevated hemoglobin, and, moreover, this cohort better reflects the more clinically relevant, potentially higher risk population to whom our prediction rule is most applicable. The different methods for *JAK2* mutation testing used in our study had varying sensitivities, with qPCR, SNP allelotyping, and NGS detecting allele frequencies as low as 0.01%, 5%, and 5% respectively and detection of *JAK2* exon 12 mutations was limited to the NGS assay. Despite these differences, the model showed consistent performance across all methods of *JAK2* testing suggesting it may be generalizable to centers using different testing platforms, although this remains to be demonstrated. Additional clinical variables, such as medications, history of arterial or venous thrombosis, and splenomegaly, were not considered in our model but could potentially further improve to predictive value of this rule. Given this study's retrospective nature, we were unable to evaluate follow-up CBCs and their impact on the likelihood of *JAK2* positivity; however, such variables might also be used in future prediction rules to further improve sensitivity.

In conclusion, the differential diagnosis for patients presenting with erythrocytosis is broad; PV, albeit rare, must be considered due to its high morbidity and mortality. We outlined a rational diagnostic approach to erythrocytosis (Fig. 2), which includes a simple prediction rule to guide *JAK2* mutation testing using readily available CBC parameters

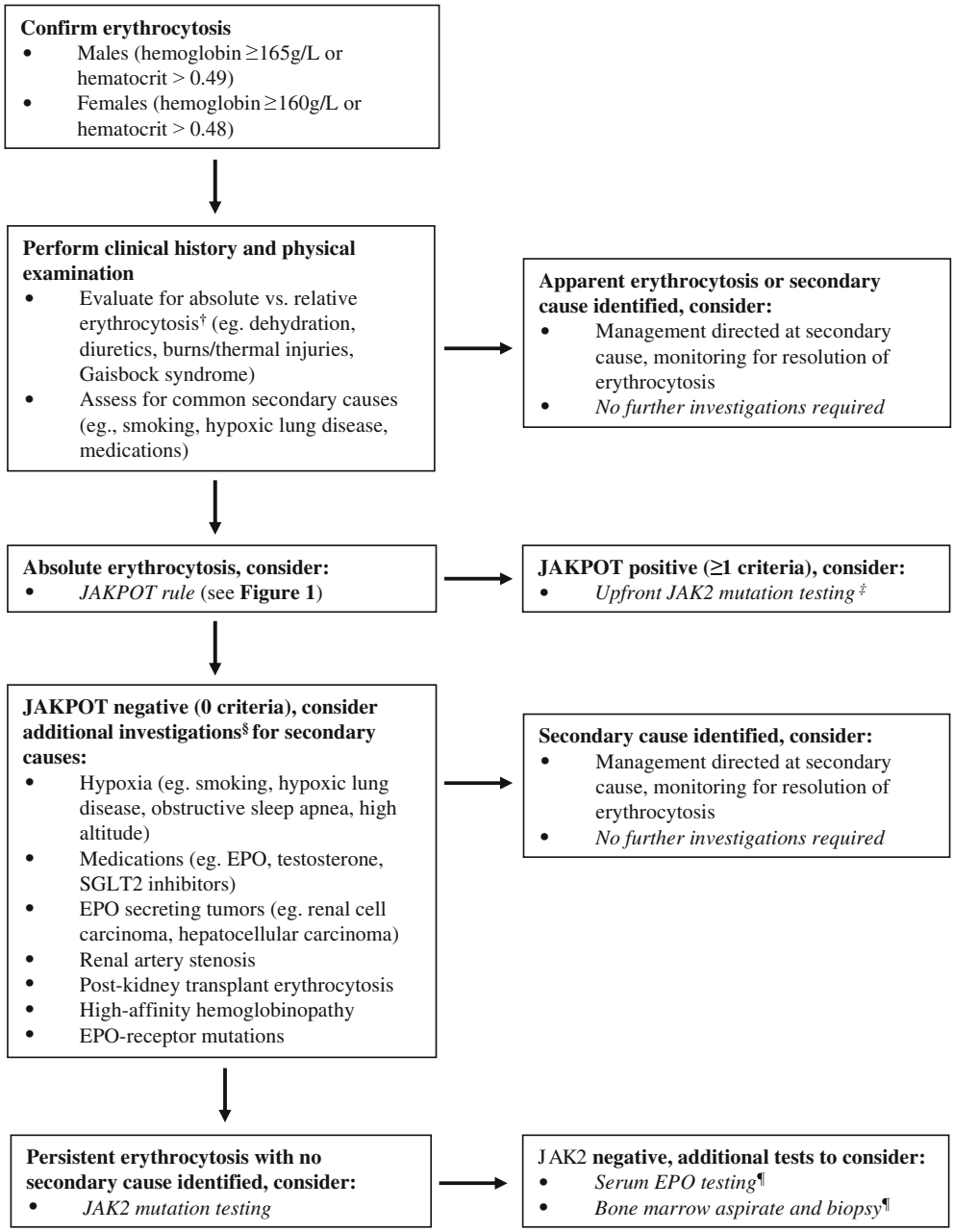

Figure 2 Proposed rational diagnostic approach to the investigation of patients referred for erythrocytosis. Note: this is a proposed approach to the investigation of erythrocytosis demonstrating how the JAKPOT rule might be used to identify low-risk patients; this approach is provisional and requires prospective validation before implementation in routine clinical practice. †Some advocate red cell mass studies to establish absolute vs. relative erythrocytosis, although this test is not widely available and not routinely performed. ‡*JAK2* mutation testing can be performed polymerase chain reaction (qPCR), single nucleotide polymorphism (SNP) allelotyping, or next-generation sequencing (NGS). §Serum EPO measurement can be considered an ancillary test to evaluate for primary vs. secondary causes of erythrocytosis, although results are often unavailable at time of initial consultation and can be normal in patients with PV; EPO likely has limited added diagnostic value in settings where *JAK2* mutation testing is available. ¶Note: bone marrow biopsy demonstrating "panmyelosis" is a major criterion and "subnormal" serum EPO level a minor criterion in the World Health Organization 2016 diagnostic criteria for polycythemia vera. Abbreviations: EPO, erythropoietin; SGLT2, sodium-glucose cotransporter-2; *JAK2*, Janus Kinase 2; *JAKPOT*, *JAK2* Prediction Cohort.

(Fig. 1). As with all clinical prediction rules, this rule is not meant to replace clinical judgment but could be used as a guide to help limit over-investigation in a common referral population. Such a rule could help improve resource stewardship by avoiding unnecessary and costly testing, in line with other Choosing Wisely initiatives.[13]

Corresponding Author: *Alejandro Lazo-Langner, Division of Hematology, Department of Medicine, London Health Sciences Centre, London, Ontario, Canada (e-mail: alejandro.lazolangner@lhsc.on.ca).*

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Author Contribution *This study was designed by B.C.Y., A.L.L., I.C.Y., and C.C.H. Molecular diagnostic data were provided by B.S., P.B., M.A.L., A.S., and H.L. Clinical and laboratory data were collected by I.C., M.M, E.K. B.C.Y., C.C.H, and I.C.Y. Data analysis and interpretation was performed by A.L.L., I.C., B.C.Y., M.K., and J.M.H. B.C.Y. wrote the paper with input from all authors who approved the final manuscript.*

Declarations:

Conflict of interest: *The authors declare that they do not have a conflict of interest.*

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