Diagnostic value of serum erythropoietin level in patients with absolute erythrocytosis

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A B S T R A C T

Background and Objectives. The diagnosis of polycythemia vera (PV) is based on clinical and biological criteria defined by either the Polycythemia Vera Study Group (PVSG) or the World Health Organization (WHO). Both the PVSG and WHO PV criteria have proven helpful and are extensively used, yet diagnostic strategies and scheduling of biological investigations vary. We assessed the value of measuring serum erythropoietin (Epo) as a first intention diagnostic test in patients with absolute erythrocytosis.

Design and Methods. Serum and bone marrow (BM) samples of 241 patients with a suspicion of erythrocytosis were collected in 8 hospital centers. One hundred and ninety had an absolute erythrocytosis (116 had PV, 66 had secondary erythrocytosis and 4 had idiopathic erythrocytosis). Serum Epo was assayed (ELISA) in 186. Statistical analysis (ROC curves) was used to define serum Epo thresholds that were specific for PV and secondary erythrocytosis and to analyze the diagnostic value of a low or high serum Epo level.

Results. A large majority of PV patients (87% or 101/116) had a serum Epo level below the normal range found in healthy patients (3.3 IU/L), giving this value a specificity of 97% with a 97.8% positive predictive value for the diagnosis of PV. Statistical analysis (ROC curves) defined two thresholds allowing a specific and direct diagnosis of 65.6% (65/99) of patients with untreated PV (Epo < 1.4 IU/L) and 19.7% (13/66) of those with secondary erythrocytosis (Epo >13.7 IU/L).

Interpretation and Conclusions. Based on these data, we propose that measurement of serum Epo level, a simple, reliable and inexpensive test, should be considered as a first intention diagnostic test for patients with absolute erythrocytosis.

Key words: erythropoietin (Epo), polycythemia vera (PV) myeloproliferative disorders, red cell.

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Polycythemia vera (PV) is a myeloproliferative disorder characterized by overproduction of red blood cells (RBC) although white blood cells (WBC) and/or platelets can also be increased in many patients. Bone marrow erythroid hyperplasia, growth factor independent-erythroid colony formation *in vitro* and decreased serum erythropoietin (Epo) form a frequent and specific triad of biological markers of PV. For many years, most physicians have based the diagnosis of PV on clinical and biological criteria proposed by the Polycythemia Vera Study Group (PVSG).[1,2] Recently the Word Health Organization[3] proposed slightly modified PV criteria, which gave more weight to biological investigations such as culture of hematopoietic progenitor cell cultures [assay of endogenous erythroid colonies (EEC)] and serum Epo dosage. Yet there is no consensus on the optimal diagnostic strategy, nor on scheduling of biological investigations, probably because of the scarcity of large, convincing statistical studies. A positive EEC assay is specific to myeloproliferative disorders and is, therefore, a very powerful diagnostic indicator for PV, but its use has been limited by technical difficulties, lack of standardization and absence of statistical validation. The recent standardization of the serum-free EEC assay[4-6] has led this to becoming either a minor (PVSG) or a major (WHO) PV criterion. We recently showed on a large group of patients that a standardized EEC assay performed with either blood or bone marrow progenitors is by itself a major biological diagnostic marker positive in 80% of PV patients.[7] Kralovics *et al.* recently compared the diagnostic value of molecular markers (PRV-1, c-mpl) in myeloproliferative disorders and concluded that EEC remained the most reliable diagnostic test for PV.[8] Sim-

ilarly, low levels or absence of serum Epo, which is not found in patients with secondary erythrocytosis, is now considered as a minor PV criterion in both the PVSG and WHO guidelines.[2,3] Proposed by some authors as a diagnostic test for PV,[9-12] the use of serum Epo dosage remains limited and controversial.[13-15] Indeed, technical problems and lack of standardization have impeded identification of reliable thresholds discriminating patients with PV from those with secondary erythrocytosis. In the present large multi-center study, we recruited a cohort of 241 patients diagnosed according to the WHO criteria, independently of Epo level. Serum Epo level was assessed with a standardized and commonly used commercial ELISA kit and two Epo thresholds specific for PV and secondary erythrocytosis were defined by statistical analysis of ROC curves. The individual diagnostic value of the Epo thresholds thus defined was also analyzed.

Design and Methods

Patient samples

Serum and bone marrow samples from 241 patients with suspected erythrocytosis were collected in 8 hospital centers during 3 years (2001-2004). One hundred and ninety patients had an absolute erythrocytosis (red cell mass (RCM) over 2 standard deviations above the mean of controls (RCM > 1.25), 51 had an apparent erythrocytosis (RCM < 1.25). Serum Epo level was assayed in 186 of the patients with an absolute erythrocytosis. Taking into account the result of the EEC assay but not the serum Epo level, these patients were classified according to WHO guidelines as follows: 116 with PV, 66 with secondary erythrocytosis and 4 with idiopathic erythrocytosis (no cause of secondary erythrocytosis identified). Of the 116 PV patients, 99 were newly diagnosed cases and 17 had been treated (phlebotomy and/or chemotherapy) prior to the Epo assay. No patient had familial polycythemia. The study was approved by the institutional ethics committee on human experimentation, Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB), of the Région Bourgogne.

Dosage of serum Epo

Blood tubes were centrifuged at 1400 g for 15 min. The serum was then aliquoted into fresh polypropylene tubes and kept frozen at -80°C until shipping on dry ice to the CHU de Grenoble (France) where Epo was assessed in duplicate by enzyme-linked immunosorbent assay (ELISA) using Quantikine IVD Erythropoietin ELISA (R & D Systems Inc., Abingdon, UK) according to the manufacturer's instructions. Values (average of duplicates) are expressed as IU/L. Normal serum Epo values provided by the manufacturer are 3.3 to 16.6 IU/L. Iron deficiency was excluded by measuring ferritin levels (normal values: 10 to 150 μg/L in females, 50 to 350 μg/L in males) and serum iron concentration (normal values: 7.0 to 31 μmol/L).

EEC assays

Standardized EEC assays were performed in each center on serum-free, collagen-based semisolid medium with lipids (MegaCult, StemCell Technologies, Meylan, France) without cytokines, on fresh bone marrow samples as described elsewhere.[7] Briefly, 10^5 fresh bone marrow mononuclear cells were plated in 1 mL of serum-free, collagen-based medium (Megacult with lipids, ref 4850, Stem Cell Technologies, Meylan, France) in triplicate without cytokines and grown for 7 days. In order to evaluate sample quality, CFU-GM and BFU-E were grown in parallel in collagen-based medium supplemented with serum and cytokines. EEC assays were considered positive when CFU-E-derived colonies were observed after 7 days of culture in serum-free, cytokine-free medium. Conversely, in order to eliminate false negatives, we concluded that an EEC assay was negative only when BFU-E growth in bone marrow cultures stimulated with cytokines was greater than 50 BFU-E/10^5 bone marrow mononuclear cells.[7]

Statistical analysis

Analysis was performed by the Departement de Biostatistique et Informatique Médicale of the CHU de Dijon (France), using Stata version 7.0 software. Receiver operating characteristics (ROC) curves were used to determine thresholds of serum Epo levels. Sensitivity, specificity and predictive values were calculated for each identified Epo cut-off.

Results

Characteristics of patients

Table 1 gives the major characteristics of the different groups of patients with absolute erythrocytosis. The mean Epo level was significantly lower ($p < 0.01$) in patients with PV (2.27±2.61) than in those with secondary erythrocytosis (10.6±6.69) or idiopathic erythrocytosis (11.1±2.16). As previously published,[16] the mean Epo level was significantly higher (2.85±3.13, p=0.002) in treated (phlebotomy, with or without chemotherapy) than in untreated PV patients (1.69±2.09) with respectively 23.5% of treated and 11.1% of untreated PV patients having a normal Epo level. This highlights the importance of assaying Epo prior to any treatment. The diagnostic accuracy of the serum Epo level was confirmed by its correlation with results of the standardized EEC assay performed for all patients with absolute erythrocytosis. The EEC assay was positive for 81% of PV

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Table 1. Characteristics of patients.

	PV (untreated) (n=99)	*PV (treated) (n=17)*	*Secondary Erythrocytosis (n=66)*	*Idiopathic Erythrocytosis (n=4)*
Males/Females	63/36	9/8	57/9	4/0
Age, median (range)	72 (26-93)	63 (39-78)	60 (19-86)	58 (25-70)
EEC	83.8% (83/99)	64.7% (11/17)	0% (0/66)	0% (0/4)
Serum Epo (IU/L) mean±SD median (range)	 1.69±2.09 0.9 (0.6-13.7)	 2.85±3.13 2.12 (0.6-13.7)	 10.6±6.69 8.65 (1.4-33.9)	 11±2.16 10.4 (8.8-14.4)
WBC ($\times 10^9$) mean±SD	 11.76±4.45	 11.61±3.72	 7.18± 2.81	 7.82±0.63
RBC ($\times 10^9$) mean±SD	 6.81±0.91	 7.05±0.91	 5.66±0.72	 5.82±0.53
Hb (g/L) mean±SD	 18.69±1.70	 18.55±1.76	 17.59±2.54	 18.07±0.83
Ht (%) mean±SD	 57.37±5.72	 57.93±5.26	 52.59±9.12	 53.72±2.54
MCV (μ^3) mean±SD	 84.66±8.11	 86.66±10.65	 91.77±10.13	 92.4±5.12
Platelets ($\times 10^9$) mean±SD	 450±206	 382±179	 215±67	 221±26
Splenomegaly	39.6% (38/96)	61.5% (8/13)	0%	0%
Cytogenetic abnormality	8.7% (4/46)	0%	0%	0%

Patients with absolute erythrocytosis were diagnosed according to the WHO classification of tumors of hematopoietic and lymphoid tissues, into four groups: untreated polycythemia vera (PV), treated PV (prior to Epo assay), and secondary and idiopathic erythrocytosis (SE and IE). Endogenous erythroid colony (EEC) formation and serum Epo level were assessed as described in Methods. WBC: white blood cells; RBC: red blood cells; Hb: hemoglobin; Ht: hematocrit; MCV: mean corpuscular volume.

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patients (83.8% [83/99] of untreated patients vs. 64.7% [11/17] of treated patients). For untreated PV patients, correlations between Epo and RCM (n = 99, r = -0.29), Epo and hematocrit (n=99, r=-0.33) and Epo and hemoglobin (n=99, r =-0.29) were significant ($p < 0.01$); this was not true for treated PV patients ($p > 0.10$). The highest values of hematocrit, hemoglobin and RCM were found in patients with the lowest Epo values, suggesting that the Epo production and release correlated with disease extent. Interestingly, among patients with a low RCM (< 1.25) at inclusion, only one patient (out of 51) had an Epo level under 1.4 IU/L (0.9 IU/L).

Diagnostic value of low serum Epo (< 3.3 IU/L)

Most untreated PV patients (88/99 or 88.8%) had a serum Epo level below the 3.3 IU/L lower value of healthy controls (Figure 1). The proportion of PV patients with normal Epo levels (Epo > 3.3 IU/L) was low (9.5%; 11/116) and all had an Epo level below the 13.7 IU/L upper normal limit. In contrast, 96.8% (61/63) of patients with secondary or idiopathic erythrocytosis had an Epo level over 3.3 IU/L, with only two patients with secondary erythrocytosis having a value below this lower normal level, giving this Epo limit a very good specificity (97%) and predictive value (97.8%) for the diagnosis of PV. Re-examination of clinical and laboratory data of the two patients with secondary erythrocytosis and low Epo levels did not suggest that a diagnosis of a PV had been missed.

Definition of diagnostic thresholds for PV and secondary erythrocytosis by ROC curve analysis

Taking advantage of our large cohort, we looked for statistically validated thresholds with a 100% predictive value for the diagnosis or exclusion of PV and secondary erythrocytosis. ROC curve analysis of Epo values in patients with PV and secondary erythrocytosis defined 1.4 IU/L and 13.7 IU/L as the thresholds giving Epo level alone a 100% specificity and a 100% predictive value for the direct diagnosis of PV (< 1.4 IU/L) and secondary erythrocytosis (> 13.7 IU/L). Using these thresholds, 65.6% (65/99) of untreated PV patients, 15% (9/60) of those with secondary erythrocytosis, and finally 45.4% of untreated patients with absolute erythrocytosis were directly diagnosed by Epo dosage alone (Figure 1). Among the 25 untreated patients with a serum Epo level between 1.4 and 3.3 IU/L, 23 had PV and 2 had secondary erythrocytosis according to the WHO criteria. Twenty (86.9%) displayed EEC vs. 89.2% (58/65) for patients with Epo <1.4 IU/L (difference not significant). Because the reduction of serum volume in patients with polyglobulia likely increases serum Epo concentration,

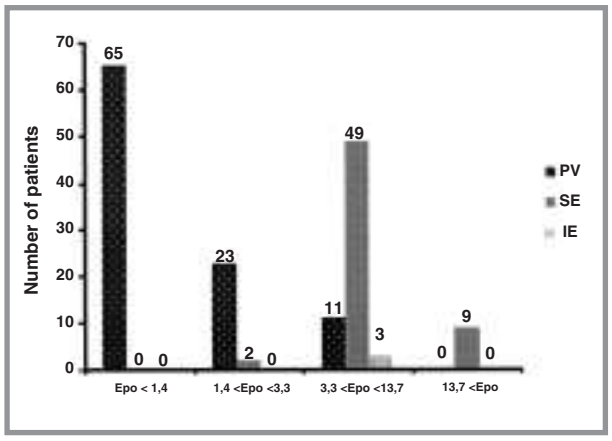

Figure 1. Distribution of untreated patients according to serum Epo level and the etiology of polycythemia (WHO classification). Patients (PV, secondary erythrocytosis (SE) and idiopathic erythrocytosis (IE), were distributed into 4 groups according to 3 Epo thresholds (1.4; 3.3 and 13.7 IU/L). 3.3 IU/L represents the low normal value of healthy controls. The 1.4 and 13.7 IU/L thresholds were calculated by statistical analysis (ROC curves).

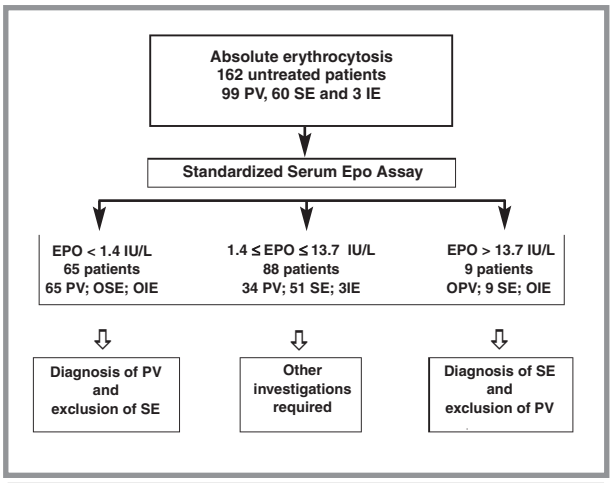

Figure 2. Efficiency of serum Epo level at first diagnostic test in untreated patients with absolute erythrocytosis. The figure represents the efficiency of Epo thresholds 1.4 and 13.7 IU/L (as defined by ROC analysis) at diagnosing PV and secondary erythrocytosis among untreated patients with absolute erythrocytosis. *Polycythemia vera (PV), secondary erythrocytosis (SE), idiopathic erythrocytosis (IE).*

which may decrease the diagnostic sensitivity of a low serum Epo value, we re-calculated Epo levels taking hematocrit (ht) into account. Results of Epo dosage were calculated for PV and secondary erythrocytosis patients as [Epo serum level (IU/L) / Ht (%)]×100]. Using this method, the mean Epo level of PV patients was 3.10±4.07 IU/L, with a median of 1.45 (0.99-24.9) IU/L, compared to a mean of 19.83±12.42 IU/L and a median of 17.11 (2.8-65.19) IU/L for patients with secondary erythrocytosis. The cut-off allowing direct diagnosis of PV and exclusion of secondary erythrocytosis with 100% specificity was 2.8 IU/L. Using this threshold we observed that 72 untreated PV patients (out of 99) were identified, giving this value a sensitivity of 72.72%.

Discussion

The diagnosis of PV remains difficult since it is based on a set of indirect, time-consuming, costly and often non-standardized techniques. Clonal markers are restricted to women, clonogenic assays require technical skills and equipment and bone marrow biopsy is painful and requires careful examination by a trained pathologist. In this context the recent improvement and standardization of several key biological tests such as Epo dosage and EEC assay, as well as the discovery of novel markers such as PRV-1 and c-Mpl, call for statistical validation in order to up-date diagnostic strategies for patients with absolute erythrocytosis. The usefulness of measuring serum Epo level has long been controversial because of conflicting results in the literature about the reliability and specificity of a low Epo value. This controversy is related to three often associated problems: i) the absence, until recently, of a standardized, reliable and commercially available Epo determination kit, ii) the lack of large multicenter studies mandatory to validate methods of Epo dosage and iii) the heterogeneity in diagnostic and inclusion procedures between series (for instance, no distinction between treated and untreated patients). Taking in account the limits of previous studies, our cooperative group was formed with the aim to evaluate diagnostic tests and procedures in myeloproliferative disorders in large multicenter studies. The diagnostic specificity and sensitivity of EEC assays based on commercial culture kits in PV and essential thrombocythemia were the first studies published. [6,7] The present multicenter study describes the individual impact of serum Epo level for the diagnosis of PV.

We used a standardized commercial ELISA kit that gives reliable, reproducible results whatever the Epo level in serum. A large majority of the patients included in the study were at diagnosis and untreated. Indeed, only 17 PV patients had received treatment (phlebotomy and/or chemotherapy) before inclusion. As expected, a normal Epo level (3.3 to 13.7 IU/L) was more frequent in treated (23.5%) than in untreated (11.1%) PV patients. This indicates that decreasing RCM rapidly influences serum Epo level and therefore, absence of previous treatment including phlebotomy is of major importance if serum Epo level is to be used as a diagnostic criterion of PV. Our study clearly shows that untreated PV patients typically have a serum Epo level below the normal range found in healthy patients. These data strengthen results

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published by Messinezy *et al.*[12] and Shih *et al.*[18] who found that a low serum Epo level has a high specificity (92%) and a sensitivity of over 60% for diagnosing PV. ROC curve analysis defined low (1.4 IU/L) and high (13.7 IU/L) thresholds specific, respectively, for PV and secondary erythrocytosis that gave the Epo dosage a good sensitivity (65.6%) for PV. Interestingly, when Epo levels were calculated taking the hematocrit into account, the sensitivity increased to 72.72%, suggesting that it could be interesting to express Epo levels in terms of whole blood.

Altogether these data emphasize the value of serum Epo dosage in the diagnosis of polycythemias (17). Hence, for a patient with elevated RCM (> 1.25) with a serum Epo level < 1.4 IU/L, one can diagnose PV without further investigation, whereas with a serum Epo value above 13.7 IU/L, the diagnosis of PV can be eliminated and the diagnosis of secondary erythrocytosis made (Figure 2). Moreover, we observed that only one patient (out of 51) displayed an Epo level < 1.4 and a red cell mass < 1.25, giving this value (1.4 IU/L) a very good specificity for the diagnosis of absolute erythrocytosis. It suggests that combining Epo dosage with hemoglobin and/or hematocrit determination should allow the diagnosis of absolute erythrocytosis in a significant percentage of patients, without red cell mass needing to be determined. This point is now under investigation by our group. A serum Epo level between the two thresholds did not discriminate between PV and secondary erythrocytosis. In our study EEC allowed the diagnosis of 76.5% (26/34) of these untreated PV patients, leading us to propose the EEC assay as the next biological investigation to establish or exclude a diagnosis of PV. Moreover, we observed that bone marrow histology allowed diagnosis of 80.7% (42/52) of PV patients with Epo < 3.3 IU/L, including 5 out of 6 PV patients with a serum Epo between 1.4 and 3.3 IU/L and a negative EEC assay. This confirms the value of bone marrow biopsy when WHO criteria are not fulfilled concluusively. Further studies should test whether the combination of other biological parameters (especially PRV-1 mRNA determination) could improve diagnostic efficiency.

In conclusion, our data demonstrate that a standardized, easy to perform commercial serum Epo dosage provides a reliable and accurate biological criterion which alone can allow the definitive diagnosis of a significant proportion of patients with PV (65.6% in our study). We suggest that serum Epo measurement should become a first intention test in the schedule of biological exploration of absolute erythrocytosis and that a serum Epo level below the statistically validated threshold of 1.4 IU/L can now be considered as a major biological marker of PV.

All the authors participated in the conception and the design of the study, the analysis and the interpretation of data and revised the article critically for important intellectual content. They all approved theversion to be submitted. The authors reported no potential conflicts of interest.

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