A novel method utilizing markers of altered erythropoiesis for the detection of recombinant human erythropoietin abuse in athletes

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ABSTRACT

The use of recombinant human erythropoietin (r-HuEPO) is officially prohibited by the International Olympic Committee and other major sporting bodies. However, currently available tests cannot readily differentiate between exogenous and endogenous EPO. Therefore the aim of our study was to investigate possible indirect detection of r-HuEPO abuse through its effect on other blood characteristics.

Background and Objectives. The use of recombinant human erythropoietin (r-HuEPO) to enhance athletic performance is prohibited. Existing tests cannot readily differentiate between exogenous and endogenous EPO. The concept of using multivariate statistical models may become redundant with the emergence of EPO mimetics that also stimulate erythropoiesis. Suitable indirect markers of r-HuEPO administration may include the numbers and physical properties of erythrocytes and reticulocytes, and the serum concentration of soluble transferrin receptor. Whereas most studies have proposed an upper limit for a single variable to identify r-HuEPO use, we hypothesised that combining multiple indirect markers of altered erythropoiesis would have greater discriminative power. Specifically, we aimed to evaluate the concept of using multivariate statistical models as an approach to developing a test battery for detection of r-HuEPO during current use (ON-models), as well as after recent use (OFF-models).

Subjects

Thirty healthy volunteers underwent medical assessment and signed statements of informed consent to the experimental procedures, which had been approved by the Ethics Committee of the Australian Institute of Sport. Twenty-seven recreational athletes were assigned to three groups prior to a 25 day drug administration phase, with the following protocols: EPO+IM group (n = 10), 50 U kg⁻¹ r-HuEPO at a frequency of 3 wk⁻¹, 100 mg intramuscular (IM) iron 1 wk⁻¹ and a sham iron tablet daily; EPO+OR group (n = 8), 50 U kg⁻¹ r-HuEPO 3 wk⁻¹, sham iron injection 1 wk⁻¹ and 105 mg of oral elemental iron daily; placebo group (n = 9), sham r-HuEPO injections 3 wk⁻¹, sham iron injections 1 wk⁻¹ and sham iron tablets daily. Each group was monitored during and for 4 weeks after drug administration.

Results. Models incorporating combinations of the variables reticulocyte hematocrit (RetHct), serum EPO, soluble transferrin receptor, hematocrit (Hct) and % macrocytes were analyzed by logistic regression. One model (ON-model) repeatedly identified 94-100% of r-HuEPO group members during the final 2 wk of the r-HuEPO administration phase. One false positive was recorded from a possible 189. Another model (OFF-model) incorporating RetHct, EPO and Hct was applied during the wash-out phase and, during the period of 12-21 days after the last r-HuEPO injection, it repeatedly identified 67-72% of recent users with no false positives.

Interpretation and Conclusions. Multiple indirect hematologic and biochemical markers used simultaneously are potentially effective for identifying current or recent users of r-HuEPO.

Key words: r-HuEPO, reticulocytes, athletes, soluble transferrin receptor, macrocytes.
Institute of Sport in accordance with the Helsinki Declaration. No subject was a member of a national sporting squad, but all had been in regular training during the year preceding the study (4-22 hr wk\(^{-1}\)) and continued to train throughout. Two subjects suffered training accidents during the study and another developed mild hypertension with r-HuEPO administration and had to be withdrawn. The remaining 27 subjects completed all experimental procedures and their baseline characteristics are presented in Table 1.

Study design
This double-blind study comprised 5 weeks of preliminary training followed by 25 days of r-HuEPO (or placebo) administration and a 4 week wash-out during which time subjects were monitored but injections had ceased (Figure 1). Venous blood was collected on 17 occasions. A baseline was determined from the first 2 samples, which were collected 14 days apart before the first injection. Blood was also collected on days 1, 3, 10, 15, 22 and 24 during r-HuEPO administration, and on days 5, 7, 12, 14, 19, 21, 26 and 28 of wash-out. All blood was collected in the morning at the same times to control for diurnal variations. Posture was standardized with each subject seated for 5 minutes before assuming a supine position for venipuncture. Total Hb mass (Hbmass) and \(\text{VO}_{2\text{max}}\) were determined 1 week prior to r-HuEPO administration, within 3 days after the last injection and at the end of wash-out.

r-HuEPO administration
After initial testing, subjects were divided into 3 equal groups, matched for Hct, [Hb] and reticulocyte parameters. During the administration phase subjects received injections and oral supplements as follows:
- EPO+IM group: r-HuEPO injections, iron injections, sham iron tablets
- EPO+OR group: r-HuEPO injections, sham iron injections, iron tablets
- Placebo group: sham r-HuEPO injections, sham iron injections, sham iron tablets.

### Table 1. Physical characteristics of r-HuEPO and placebo groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Mass (kg)</th>
<th>(\text{VO}_{2\text{max}}) (mL,kg,-1,min,-1)</th>
<th>Hbmass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO+IM n=10</td>
<td>2/8</td>
<td>24 (1.0)</td>
<td>71 (2.7)</td>
<td>62 (1.9)</td>
<td>840 (46)</td>
</tr>
<tr>
<td>EPO+OR n=8</td>
<td>1/7</td>
<td>28 (1.5)</td>
<td>73 (4.7)</td>
<td>61 (2.8)</td>
<td>891 (77)</td>
</tr>
<tr>
<td>Placebo n=9</td>
<td>2/7</td>
<td>23 (1.6)</td>
<td>67 (3.6)</td>
<td>57 (3.1)</td>
<td>758 (65)</td>
</tr>
</tbody>
</table>

EPO+IM received intramuscular iron injections while EPO+OR received oral iron tablets. These are baseline data collected after 5 weeks of standardized training. Abbreviations: maximum oxygen consumption (\(\text{VO}_{2\text{max}}\)), hemoglobin mass (Hbmass). Data are reported as mean with standard error of mean in parenthesis.
Blood analysis

Erythrocyte and reticulocyte parameters were analyzed within 4 hours of blood collection using an H*3 Hematology Analyzer (Bayer Diagnostics, Tarrytown NY, USA) which performs flow cytometric measurements. The direct measures performed by the H*3 include: percent reticulocyte (CHCM), MCV ratio of reticulocytes (CHCMr), and total reticuloocyte (MCVr). Calculated parameters include: Hct (RBC x MCVr), absolute reticulocyte count (%retic x MCVr), content of hemoglobin per reticulocyte (CHM = MCVr x CHCMr), MCV ratio (MCV/M Cv mature erythrocytes) and total reticulocyte hemoglobin (RetHb = CHMr x #retic). In addition to the above parameters, the reticulocyte Hct (RetHct = #retic x MCVr) was used to quantify the fractional volume of the reticulocyte pool. Since both #retic and MCVr are known to be influenced by changes in erythropoiesis, we hypothesized that RetHct would contribute to the detection of subjects with an artificially induced change in the rate of red cell production. Serum was stored at −80°C. EPO and sTfr concentrations were determined through standard ELISA techniques (R&D Systems, Quantikine IVD Kits, Minneapolis, MN). Ferritin and total protein concentrations were measured using an Hitachi 911 Biochemistry Analyzer (Roche Diagnostics, Rotkreuz, Switzerland) which employs photometric methods. 

All analyzers were calibrated against appropriate reference materials and checked daily against internal and external quality controls. The coefficients of variation (CV) for erythrocyte and reticulocyte parameters during the analysis period were as follows: RBC, 1.1%; MCV, 0.2%, %Mac, 3.0%; %Hypo, 4.2%; CHCM, 0.2%; [Hb], 0.5% and %retic, 5.5%. Using 3 fixed and 2 random controls, the inter-assay and intra-assay CVs for EPO were 4.4% and 6.5%, respectively. The corresponding CVs for sTfr were 5.7% and 5.8%.

Total Hbmass and VO2max

Subjects underwent measurement of VO2max on either a treadmill (AusTredEx, Australia) or cycle ergometer (Lode, Groningen, The Netherlands), with the mode of testing for individual subjects kept constant throughout the study. The VO2 system has been described elsewhere. Two of the subjects reached a predetermined Hct limit of 0.55 in response to r-HuEPO. According to protocol, r-HuEPO injections were substituted by saline injections on two occasions (days 18 and 21 of the administration period). Thereafter the subjects resumed the standard r-HuEPO regimen.

Statistical analysis

Values are reported as mean and standard error unless otherwise specified. Using data from all 17 blood measurements, repeated measures analysis of variance (ANOVA) was conducted for each variable to determine whether changes over time differed between groups. ANOVA was also used to examine changes in Hb, VO2max, and %Hypo. Where a significant (p<0.05) group by time interaction was observed, one-way ANOVA was performed at each time point, followed by Tukey post-hoc comparisons.

Combining data from days 22 and 24, effect sizes (ES) were calculated for all variables to quantify the magnitude of change induced by r-HuEPO relative to placebo. Variables that were not significantly intercorrelated but had large ES (>1.0) were included in models designed to detect current r-HuEPO use (ON-models). The models were analyzed by logistic regression (logit) and the coefficients applied to the raw data of all subjects to determine whether those from the r-HuEPO and placebo groups could be correctly identified at the end of r-HuEPO administration. Because of the possible consequences of false positive results in sporting situations, we classified subjects as r-HuEPO users only if the probability indicated by logit was >0.999999. Models including each of the independent variables singly and in all possible combinations were analyzed. The logit coefficients for every model were subsequently applied at each of the 17 sampling points to evaluate model sensitivity (correct identification of r-HuEPO users) and specificity (correct identification of non-users). The models were also tested by application to data from 556 blood samples collected from 66 male and 18 female athletes (athlete reference group) who had participated in studies involving exposure to natural altitude (1,800-2,700 m, n=6), simulated altitude (2,500-3,100 m, n=35), a 6-day cycling race (n=23), or normal training (n=20).

A similar process was used to develop and test models for application during the wash-out period to detect those subjects who had recently used, but were no longer receiving, r-HuEPO (OFF-models). Data from the middle of wash-out (post days 12 and 14) were used to determine ES and the logit coefficients for all possible models, which were then assessed at each of the 17 blood sampling points and tested on the athlete reference group as above.

Analyses were conducted with Statistica software.
Results

Hematocrit and hemoglobin concentration

In both r-HuEPO groups Hct was significantly greater than that in the placebo group by the third week of r-HuEPO administration and remained elevated 21 days after injections ceased (Figure 2). Findings for [Hb] were similar to those for Hct (Table 2).

Reticulocyte parameters

During treatment, both groups receiving r-HuEPO approximately doubled their baseline #retic, whereas this did not occur in the placebo group (Table 2). The increase was greater and more prolonged in the EPO+OR group. During wash-out, #retic of both r-HuEPO groups fell to abnormally low levels. In both r-HuEPO groups, but not the placebo group, there was a significant increase in M Cv r during treatment and a return to baseline during wash-out (Table 2). The combined effect of increases in #retic and M Cv r during treatment markedly raised RetHct, particularly in the EPO+OR group (Figure 2). From weeks 2-4 of wash-out, depressed #retic and normalized M Cv r resulted in RetHct levels of both r-HuEPO groups falling significantly below those of the placebo group and their own baseline values. Changes in RetHb were qualitatively similar to those of RetHct but less pronounced (Table 2). The increase in RetHb for the EPO+IM group during treatment was limited by reduction in CHCr and CHr, and values were never significantly above those of the placebo group (Table 2). M Cv ratio was elevated only in the EPO+IM group during r-HuEPO injections but was significantly depressed for both groups at the midpoint of wash-out (Table 2).

Erythrocyte parameters

During r-HuEPO administration, %Macro increased in both r-HuEPO groups (Figure 2). By the end of wash-out it had returned to the original level. At baseline, %Hypo averaged ~1% for each group (Table 2). At the end of treatment, the EPO+IM group reached a mean value of 4.5±0.9% and the values for this group were significantly higher than those of the other groups throughout much of the study. There were no significant differences in %Hypo between the EPO+OR and placebo groups.

Serum erythropoietin, soluble transferrin receptor and ferritin

Serum EPO was significantly elevated in the r-HuEPO groups throughout administration (Figure 3). During wash-out, EPO concentrations in the r-HuEPO groups became significantly lower than those in the placebo group. The sTfr concentration was significantly elevated in the r-HuEPO groups relative to the placebo group throughout the last 2 weeks of treatment and the first 2 weeks of wash-out (Figure 3). For both r-HuEPO groups serum ferritin concentration decreased to ~40 ng/mL during r-HuEPO administration and returned to baseline after 2 weeks of wash-out (Table 2). Total Hb mass and VO₂max

Relative to baseline (Table 1), the EPO+IM and EPO+OR groups showed significant increases (6.9±0.6% and 12.0±0.7%, respectively) in Hb mass at the end of treatment, while the placebo group showed no significant change (0.8±0.8%). At the end of administration, VO₂max of the EPO+IM and EPO+OR groups was 6.3±1.8% and 6.9±1.1%, respectively, above baseline. The corresponding change for the placebo group was 0.4±1.5%. After 4 wk of wash-out, Hb mass and VO₂max of the r-HuEPO groups had returned to baseline.

Prediction of r-HuEPO use

The five variables with the largest ES for the ON-models were RetHct (1.65), EPO (1.59), sTfr (1.57), Hct (1.41) and %Macro (1.11), and those for the OFF-models were M Cv ratio (-1.60), EPO (-1.58),
Of 31 possible ON-models, that which combined all 5 variables showed outstanding ability to differentiate between users and non-users of r-HuEPO during treatment (best ON-model). During the wash-out phase, a 3 variable model combining Hct, RetHct and EPO was the most effective for detecting recent r-HuEPO use (best OFF-model). The constants and beta coefficients for these models are shown in Table 3, while Table 4 illustrates the relative effect of changes in each component of the 5-variable model.

For simplicity, the best ON- and best OFF-model are hereafter referred to as the ON-model and the OFF-model. The ON-model was able to correctly identify 94-100% of r-HuEPO group members during the final 2 weeks of drug administration, and produced a single false positive measurement in a member of the placebo group (Figure 4). The ON-model lost sensitivity rapidly once r-HuEPO administration was ceased. In the athlete reference group, the ON-model produced one false positive result, which occurred following the first night of a simulated altitude program. While the OFF-model had zero sensitivity during r-HuEPO administration it correctly classified 67-72% of r-HuEPO users at days 12-21 of wash-out and identified 33% at 28 days post (Figure 4). Importantly, it produced no false positives in the placebo or athlete reference groups.

Discussion

The major finding of this study is that when multiple indirect markers of altered erythropoiesis are used simultaneously they can identify current or recent r-HuEPO use. In agreement with others we found that r-HuEPO administration elevated VO\text{2max} by 6-7% and our observation of a 7-12% increase in Hb\text{mass} confirms the potential for enhanced oxygen delivery. Previous research has not serially monitored Hb\text{mass} after cessation of r-HuEPO, but we found that both Hb\text{mass} and VO\text{2max} returned to baseline levels within one month. Ekblom and Berglund concluded that...
VO$_{\text{max}}$ decreases within 2 weeks of finishing r-HuEPO. Consequently, to gain a competitive advantage, athletes would need to continue using r-HuEPO until a late stage of preparation for an event. A test for accelerated erythropoiesis in the 2-6 weeks before major competition would therefore have a high likelihood of detecting r-HuEPO abuse using our ON-model. If athletes ceased using r-HuEPO 2-3 weeks before competition, our OFF-model would detect approximately two-thirds of them. However, on day 7 of wash-out the sensitivity of the OFF-model was 6% and that of the ON-model was 11%. Notwithstanding the low sensitivity of both models on this one occasion, analyzing blood data using both ON- and OFF-models may be a more effective deterrent than merely detecting current r-HuEPO use. Our ON-model incorporated RetHct, sTfr, EPO, Hct and %Macro for prediction of current r-HuEPO use. These 5 variables not only showed the largest ES in our study but with the exception of RetHct have also been reported by others to change with r-HuEPO use.3,11 The ON-model was repeatedly able to identify subjects receiving r-HuEPO while also providing very few (<0.6%) false positives. Using multiple variables may attenuate the relative effect of an abnormal level of any single variable, decreasing the chance that a particular pathology or physiological disturbance could either cause a false positive or mask a true positive. For any one of the components of the 5-variable ON-model to influence the outcome sufficiently to alone cause a positive result in our subjects, it had to reach a value above the 99.9 percentile of the group at baseline. Even when 4 variables are at the 97.5th percentile for our group (shaded values), the threshold probability (0.999999) for identification of r-HuEPO use is not attained.

### Table 3. Determining probability of r-HuEPO use and discontinued use: the constants and beta coefficients for the 5-variable ON-model and the 3-variable OFF-model.

<table>
<thead>
<tr>
<th>Model variables</th>
<th>RetHct</th>
<th>EPO</th>
<th>sTfr</th>
<th>Hct</th>
<th>%Macro</th>
<th>Y'</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON-model</td>
<td>RetHct+EPO+sTfr+Hct+%Macro</td>
<td>-76.96</td>
<td>299.3</td>
<td>0.946</td>
<td>0.892</td>
<td>78.67</td>
<td>2.08</td>
</tr>
<tr>
<td>OFF-model</td>
<td>Hct+RetHct+EPO</td>
<td>-12.40</td>
<td>-1566</td>
<td>-0.765</td>
<td>-50.90</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: reticulocyte hematocrit (RetHct), erythropoietin (EPO), soluble transferrin receptor (sTfr), hematocrit (Hct) and % macrocytes (%Macro).

### Table 4. Effects of increasing 1, 3, 4 and 5 components of the 5-variable ON-model on the calculated probability of r-HuEPO use. Even when 4 variables are at the 97.5th percentile for our group (shaded values), the threshold probability (0.999999) for identification of r-HuEPO use is not attained.

<table>
<thead>
<tr>
<th>RetHct</th>
<th>EPO</th>
<th>sTfr</th>
<th>Hct</th>
<th>%Macro</th>
<th>Y'</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline mean</td>
<td>0.005</td>
<td>7</td>
<td>18</td>
<td>0.45</td>
<td>0.9</td>
<td>-15.5216</td>
</tr>
<tr>
<td>Change 1 var</td>
<td>0.0093</td>
<td>7</td>
<td>18</td>
<td>0.45</td>
<td>0.9</td>
<td>-14.23461</td>
</tr>
<tr>
<td>Change 3 var</td>
<td>0.0093</td>
<td>14</td>
<td>26</td>
<td>0.45</td>
<td>0.9</td>
<td>-0.4785</td>
</tr>
<tr>
<td>Change 5 var</td>
<td>0.0093</td>
<td>14</td>
<td>26</td>
<td>0.50</td>
<td>0.9</td>
<td>3.45499</td>
</tr>
</tbody>
</table>
| Baseline means are for 111 athletes (27 r-HuEPO or placebo athletes and 84 from the athlete reference group). The abbreviations in the first column indicate the number of variables manipulated to the 97.5th percentile with the remainder held constant at the mean value. Y' is calculated from the linear combination of the coefficients in the 5-variable model (see Table 3). The probability of being on r-HuEPO is calculated as antilog Y/(1+antilog Y).

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**Detection of r-HuEPO use by athletes**

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rect markers for detecting current r-HuEPO abuse, and no-one has previously proposed an OFF-model for detecting recently discontinued r-HuEPO use. Audran et al. proposed that elevated Hct and sTfr together with an increased sTfr:protein ratio could constitute an initial screening step for current r-HuEPO use. They did not specify a cut-off for Hct nor attempt to combine the proposed markers into a fully integrated model. In our study, sTfr and sTfr:protein ratio were highly correlated (r=0.96) and therefore could not be regarded as independent indicators of erythropoiesis. Nevertheless, we used our data from days 22-24 and post days 12-14 to develop ON- and OFF-models based on the parameters of Audran et al. Both of these models were evaluated at each of the 17 blood sampling points, and each identified 78-94% of r-HuEPO recipients on days 15-24 of administration, and 89-100% during the first 12 days of wash-out. However, they produced 14 (Audran ON-model) and 9 (Audran OFF-model) false positives among members of the placebo group (Figure 4). The Union Cycliste Internationale (UCI) currently excludes athletes from competition if Hct exceeds 0.50 for men or 0.47 for women. During our study, there was no sampling point at which these criteria would have excluded more than 56% of members of the r-HuEPO groups. Subjects not receiving r-HuEPO recorded a total of 8 Hct readings above the UCI limits. The need for a more discriminative test is evident. At this stage, only the UCI and the Federation Internationale de Ski have imposed limits on blood parameters. Athletes from other sports could be using large and potentially dangerous doses of r-HuEPO without scrutiny.

The main reason that Audran et al. proposed using the sTfr:protein ratio was to circumvent the possibility that hemocoagulation could cause false positives by artificially elevating blood markers such as sTfr and Hct. Hemocoagulation can be caused by dehydration associated with prolonged exercise or by posturally-induced shifts in plasma volume, and may be in the order of 10-20%. We standardized postural factors in the collection of blood samples. Furthermore the samples were obtained in the early morning, and not immediately after exercise. This minimized any acute dehydration. Our approach to detect r-HuEPO use by athletes is not currently based on immediate post-exercise blood sampling. Bioavailability of iron is crucial in determining the efficacy of r-HuEPO and it has been reported that only intravenous iron injection can prevent functional iron deficiency during r-HuEPO administration. This strategy was not adopted in our study, resulting in the EPO+IM group showing a high %Hypo and low CHCMr and CHr, which indicates iron-deficient erythropoiesis. However, in contrast to previous research such changes did not occur in the EPO+OR group, perhaps because of our comparatively low r-HuEPO doses. The difference in response of the EPO+IM and EPO+OR groups occurred despite serum ferritin falling to ~40 ng/mL in both cases. It has been recommended that serum ferritin should be maintained above 100 or even 300 ng/mL to support accelerated erythropoiesis induced by r-HuEPO therapy, but our findings suggest that this may not apply when low r-HuEPO dosages are used. Since athletes are likely to ensure adequate iron availability during administration of r-HuEPO, hematologic markers of iron-deficient erythropoiesis (%Hypo, CHCMr, CHr) may be of limited value in detecting their use of r-HuEPO. These markers did not qualify for inclusion in our models.

We cannot exclude the possibility that specific pathologies might affect the components of our models in a manner similar to r-HuEPO use, although preliminary indications are encouraging. One female member of our placebo group was subsequently found to be heterozygous for hemoglobin C. This subject had elevated baseline #retic, sTfr and EPO, and might have been considered at risk of registering a false positive. However, her reticulocytes were small, and consequently RetHct levels were normal. Her values for Hct and %M acro were always low. Thus both our ON- and OFF-models were able to differentiate
the effects of the hemoglobinopathy from those of r-HuEPO administration. Similarly, clinical iron deficiency (serum ferritin 12 ng/mL; transferrin saturation 12%) in a male runner from the athlete reference group was not confused with r-HuEPO abuse, despite elevated sTfr. Low levels of Hct and %M acro again proved protective against a false positive for the ON-model and low Hct combined with a normal EPO concentration prevented a false positive for the OFF-model.

The only false positive result produced by our ON-model in a member of the placebo group was caused by transient increases in EPO and sTfr the day after a knee injury severe enough to require modification of training for the ensuing 2 weeks. We do not know whether the alteration in the blood variables was due to the injury, but the same subject did not register a false positive on any of 16 other measurements. The effect of acute injury on the variables comprising our models requires further evaluation.

The ON-model developed from this study may occasionally produce a false positive for an athlete at the beginning of natural or simulated altitude exposure. We studied 41 athletes (as part of the athlete reference group) exposed to hypoxic environments and our ON-model yielded 1 false positive after the first night. This was due to elevated EPO, RetHct and Hct. An initial increase in EPO in response to hypoxia has been described previously, as has an increase in Hct due to plasma volume loss. With a few days of continued hypoxia, the serum EPO of this athlete fell substantially, which is consistent with the few days of continued hypoxia, the serum EPO of this athlete fell substantially, which is consistent with the continued development of a test to detect banned r-HuEPO abuse requires future research to address these issues and refine the predictor variables. Nevertheless, our data clearly support the concept of combining multiple variables to detect r-HuEPO abuse by athletes. The prospect of developing accurate tests based on this approach now appears to be strong.

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Potential implications for clinical practice
♦ Some of the new markers of altered erythropoiesis could be used for differential diagnosis of erythrocytosis in clinical settings.

References


