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original paper

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

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ABSTRACT

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. Therefore the aim of our study was to investigate possible indirect detection of r-HuEPO use via blood markers of altered erythropoiesis.**

Design and Methods. **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 Ukg^{-1} r-HuEPO at a frequency of $3wk^{-1}$, 100 mg intramuscular (IM) iron $1wk^{-1}$ and a sham iron tablet daily; EPO+OR group (n = 8), 50 $U.kg^{-1}$ r-HuEPO $3wk^{-1}$, sham iron injection $1wk^{-1}$ and 105 mg of oral elemental iron daily; placebo group (n = 9), sham r-HuEPO injections $3wk^{-1}$, sham iron injections $1wk^{-1}$ 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

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The use of recombinant human erythropoietin (r-HuEPO) is officially prohibited by the International Olympic Committee and other major sporting bodies.[1] However, currently available tests cannot readily differentiate between exogenous and endogenous EPO. Since r-HuEPO is demonstrably effective in increasing hemoglobin concentration [Hb], maximum oxygen consumption ($\dot{V}O_{2max}$) and physical work capacity,[2,3] the lack of a test to confirm its use may have induced many athletes to experiment with the drug.

To dissuade the use of r-HuEPO and to minimize the associated risks of hyperviscosity, thrombosis and hypertension,[4-7] some sports have imposed upper limits on hematocrit (Hct, 50%) or [Hb], (18.5 g/dL). Disadvantages of using these thresholds include: large natural variation between individuals (unpublished Hct data from our laboratory: male athletes n=3069 =0.46, SD=0.03; female athletes n=2235 =0.42, SD=0.03), postural effects,[8] and ease of manipulation through interventions such as saline infusion. Nevertheless, the concept of indirectly detecting r-HuEPO abuse through its effect on other blood characteristics shows promise.[9-14] An indirect method may even be the most robust approach, since attempts to detect the pharmacologic agent itself may become redundant with the emergence of EPO mimetics[15,16] that also stimulate erythropoiesis. Suitable indirect markers of r-HuEPO administration may include the numbers and physical properties of erythrocytes and reticulocytes,[17-20] and the serum concentration of soluble transferrin receptor (sTfr).[3,9,21]

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).

Design and Methods

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 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, 17, 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 (Hb_{mass}) and $\dot{V}O_{2max}$ were determined 1 week prior to r-HuEPO administration, within 3 days after the last injection and at the end of wash-out.

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

Group	*Sex F/M*	*Age years*	*Mass kg*	*$\dot{V}O_{2max}$ $mL.kg^{-1}.min^{-1}$*	*Hb_{mass} g*
EPO+IM n=10	2/8	24 (1.0)	71 (2.7)	62 (1.9)	840 (46)
EPO+OR n=8	1/7	28 (1.5)	73 (4.7)	61 (2.8)	891 (77)
Placebo n=9	2/7	23 (1.6)	67 (3.6)	57 (3.1)	758 (65)

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 ($\dot{V}O_{2max}$), hemoglobin mass (Hb_{mass}). Data are reported as mean with standard error of mean in parenthesis.

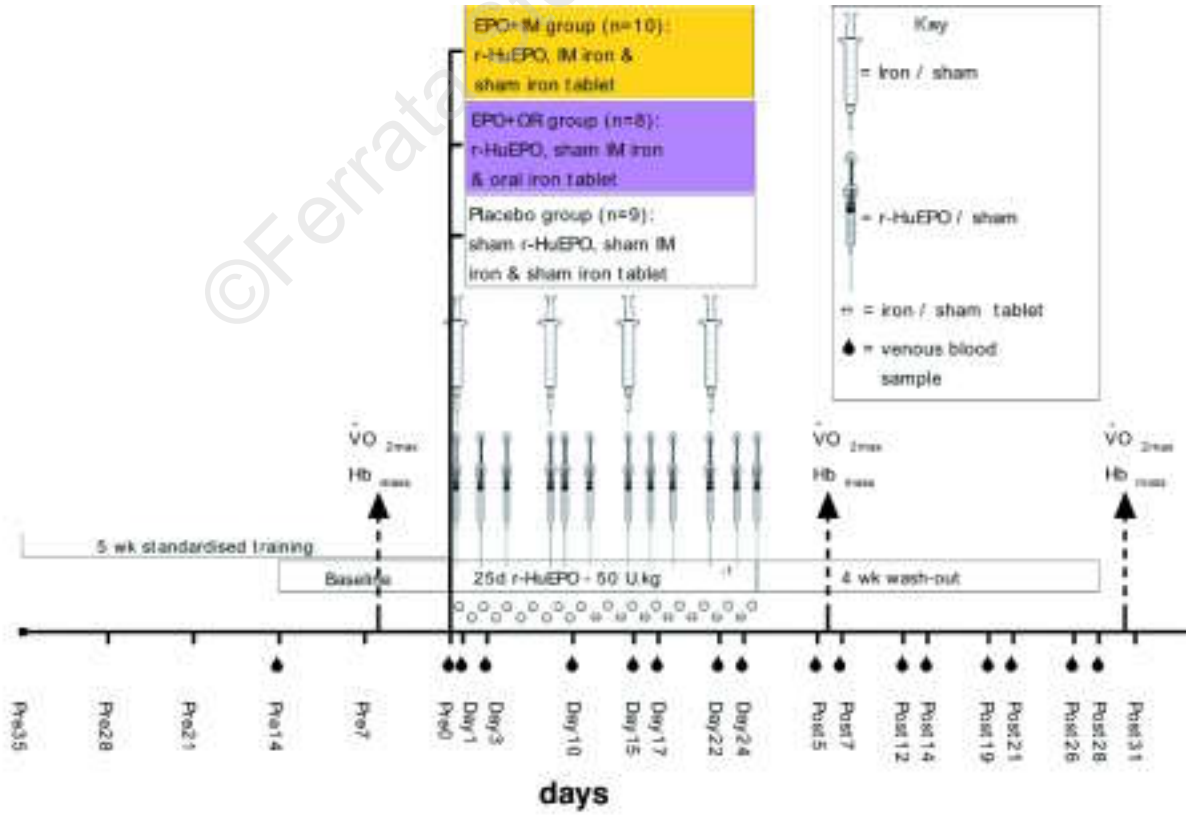
r-HuEPO administration

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.



All injections of r-HuEPO (EPREX 4000, Janssen-Cilag, Australia) were subcutaneous at 50 U kg^{-1} body mass 3 wk^{-1} (day 0, 2, 4, 8, 9, 11, 14, 16, 18, 21, 23, and 25). Iron injections were intramuscular (IM) and administered once weekly. They contained 100 mg of iron in a complex of iron polymaltose (Ferrum H ampoules, Sigma Company Ltd., Clayton, Australia). Iron tablets were taken daily and provided ~105 mg of elemental iron derived from 350 mg of dried ferrous sulphate (Ferrogradumet, Abbott, Australia). Saline (NaCl 0.9% BP, Astra Pharmaceuticals, Australia) was used for sham injections of r-HuEPO and iron. Placebo tablets were lactose. The design and drug administration protocols of the study are shown in Figure 1.

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.

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.[17,18] The direct measures performed by the H*3 on erythrocytes include: count (RBC), size (mean cell volume - MCV) and cell mean hemoglobin concentration (CHCM), as well as [Hb]. The analyzer indicates percentage of cells with MCV > 120 fL (%Macro) and percentage of cells with CHCM < 28 gdL (%Hypo). Direct reticulocyte measurements include: percent reticulocytes (%retic), mean cell volume of reticulocytes (MCVr) and cell mean hemoglobin concentration of reticulocytes (CHCM r). Calculated parameters include; Hct (RBC $\times$ MCV), absolute reticulocyte count (#retic = RBC $\times$ %retic), content of hemoglobin per reticulocyte (CHr = MCVr $\times$ CHCM), MCVratio (MCVr/MCV mature erythrocytes) and total reticulocyte hemoglobin (RetHb = CHr $\times$ #retic).[17,18] In addition a novel parameter, 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 erythropoeisis,[10] 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, IL, USA) and data analyzed using Mulitical Software (Wallac, Oy, Finland). 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%; %Macro, 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 Hb_{mass} and $\dot{V}O_{2max}$

Subjects underwent measurement of $\dot{V}O_{2max}$ 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 $\dot{V}O_2$ system has been described elsewhere.[22] Subjects were also assessed for total Hb_{mass} using carbon monoxide rebreathing.[23] Our CV for determining $\dot{V}O_{2max}$ is 3.3%, while that for total Hb_{mass} is 2.7%.

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_{mass} and $\dot{V}O_{2max}$. 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)[24] 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 (>1.10) 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

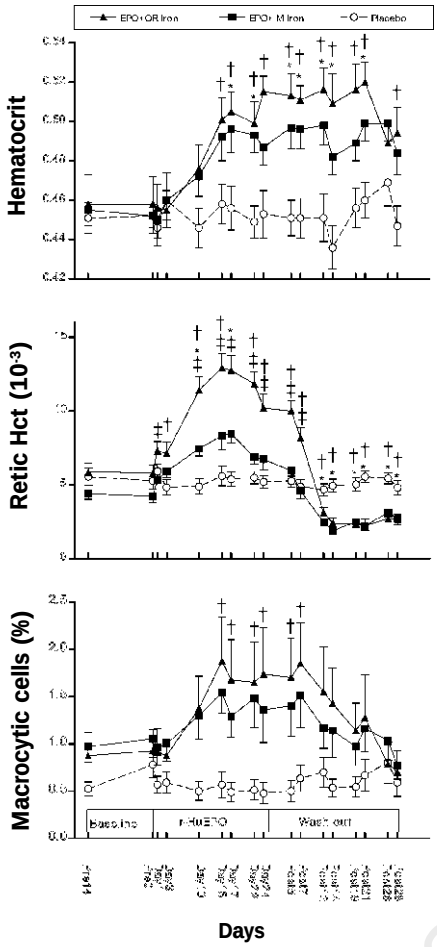


Figure 2. Effect of human recombinant erythropoietin (r-HuEPO) administration over 25 days on hematocrit, reticulocyte hematocrit and % macrocytes. Values are mean with vertical bars to show standard error of the mean. EPO+OR group received r-HuEPO and oral iron, EPO+IM received r-HuEPO and intramuscular iron. Statistically significant differences ($p<0.05$) are: † = EPO+OR versus placebo group. * = EPO+IM versus placebo group. ‡ = EPO+OR versus EPO+IM group.

(StatSoft, Tulsa, OK, USA), except for logit, which was performed using GLIM 4 software (Royal Statistical Society, London, UK).

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 MCVr during treatment and a return to baseline during wash-out (Table 2). The combined effect of increases in #retic and MCVr during treatment markedly raised RetHct, particularly in the EPO+OR group (Figure 2). From weeks 2-4 of wash-out, depressed #retic and normalized MCVr 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 CHCMr and CHr, and values were never significantly above those of the placebo group (Table 2). MCVratio 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 $\dot{V}O_{2max}$

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,

$\dot{V}O_{2max}$ 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 $\dot{V}O_{2max}$ 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 MCVratio (-1.60), EPO (-1.58),

Table 2. Hematologic variables before, during and after 25 days of r-HuEPO administration or placebo treatment. To facilitate presentation, indicative values are provided for 5 key stages of the study.

Variable	*Group*	*Baseline (pre 14 & pre 0)*	*Week 2 of r-HuEPO (days 15&17)*	*End of r-HuEPO (days 22&24)*	*Week 2 of wash-out (post 12&14)*	*End of wash-out (post 26&28)*
[Hb]	EPO+IM	14.8 (0.3)	15.8 (0.5)°	15.9 (0.4)°	15.9 (0.3)*°	15.8 (0.4)°
g.dL	EPO+OR	15.0 (0.4)	16.3 (0.3) °	16.6 (0.3)*°	16.8 (0.4)*°	16.3 (0.4)°
	Placebo	14.8 (0.2)	15.0 (0.3)	15.0 (0.3)	14.7 (0.3)	15.0 (0.3)
#retic	EPO+IM	40 (4)	73 (6)°	58 (4)°	21 (2)*°	27 (3)*°
$x10^9/L^{-1}$	EPO+OR	54 (5)	114 (8)*°	98 (8)*°	27 (3)*°	25 (3)*°
	Placebo	51 (6)	52 (6)	50 (4)	46 (4)	49 (1)
MCVr	EPO+IM	108.0 (1.1)	115.1 (1.6)*°	116.1 (1.1)*°	106.1 (1.1)	106.6 (1.1)
fL	EPO+OR	106.4 (1.5)	113.2 (1.7)°	113.4 (1.9)*°	103.0 (1.1)	107.1 (2.3)
	Placebo	105.8 (1.4)	107.4 (1.9)	107.6 (1.1)	106.4 (2.1)	106.4 (1.5)
RetHb	EPO+IM	1.17 (0.11)	2.02 (0.21)°	1.63 (0.14)°	0.60 (0.06)*°	0.81 (0.10)*°
g/L	EPO+OR	1.59 (0.14)	3.29 (0.24)*°	2.85 (0.24)*°	0.78 (0.09)*°	0.75 (0.09)*°
	Placebo	1.47 (0.16)	1.48 (0.16)	1.44 (0.12)	1.33 (0.12)	1.39 (0.11)
CHCMr	EPO+IM	27.7 (0.4)	24.8 (0.5)*°	24.9 (0.6)*°	28.7 (0.3)	28.6 (0.3)
g/dL	EPO+OR	28.2 (0.4)	26.5 (0.5)°	26.8 (0.3)°	29.2 (0.5)	29.0 (0.4)
	Placebo	28.0 (0.3)	27.7 (0.5)	27.9 (0.3)	28.4 (0.4)	28.0 (0.4)
CHr	EPO+IM	29.5 (0.4)	27.3 (0.7)°	27.7 (0.8)°	29.5 (0.4)	29.4 (0.4)
pg	EPO+OR	29.6 (0.3)	28.9 (0.5)	29.2 (0.4)	29.2 (0.4)	30.0 (0.5)
	Placebo	29.0 (0.3)	28.5 (0.2)	28.8 (0.2)	29.2 (0.3)	28.7 (0.1)
MCV ratio	EPO+IM	1.25 (0.04)	1.31 (0.03)*°	1.32 (0.03)*°	1.21 (0.04)°	1.25 (0.03)
	EPO+OR	1.25 (0.04)	1.29 (0.03)°	1.29 (0.03)	1.16 (0.02)*°	1.24 (0.06)
	Placebo	1.25 (0.02)	1.27 (0.04)	1.28 (0.03)	1.25 (0.04)	1.26 (0.03)
%Hypo	EPO+IM	1.1 (0.2)	3.9 (0.8)*°	4.5 (0.9)*°	4.7 (1.1)*°	3.9 (1.1)°
	EPO+OR	0.9 (0.3)	1.9 (0.3)°	1.7 (0.3)	2.1 (0.6)°	1.0 (0.3)
	Placebo	1.2 (0.3)	1.1 (0.3)	0.9 (0.3)	1.4 (0.4)	1.8 (0.6)
Ferritin	EPO+IM	84.5 (15.8)	44.4 (11.9)*°	45.6 (9.9)*°	115.7 (17.7)	136.6 (22.9)°
ng/mL	EPO+OR	119.7 (25.6)	42.6 (8.1)°	35.6 (5.6)*°	116.0 (19.1)	158.2 (25.0)
	Placebo	131.9 (26.8)	111.2 (29.8)	110.1 (24.9)	116.3 (23.1)	97.2 (21.9)°

*EPO+IM received intramuscular iron injections while EPO+OR received oral iron tablets. Abbreviations: hemoglobin concentration [Hb], number of reticulocytes (#retic), mean cell volume of reticulocytes (MCVr), reticulocyte hemoglobin (RetHb), mean cell hemoglobin concentration of reticulocytes (CHCMr), hemoglobin content of reticulocytes (CHr), mean cell volume ratio (MCVratio) and % hypochromic erythrocytes (%Hypo). Value is mean of two consecutive measures and SEM is shown in parenthesis. * = significant difference (p<0.05) from placebo group at matched time. ° = significant difference (p<0.05) within a group compared to baseline.*

sTfr (1.49), Hct (1.44) and RetHct (-1.39). 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[2,3] we found that r-HuEPO administration elevated $\dot{V}O_{2max}$ by 6-7% and our observation of a 7-12% increase in Hb_{mass} confirms the potential for enhanced oxygen delivery. Previous research has not serially monitored Hb_{mass} after cessation of r-HuEPO, but we found that both Hb_{mass} and $\dot{V}O_{2max}$ returned to baseline levels within one month. Ekblom and Berglund[2] concluded that

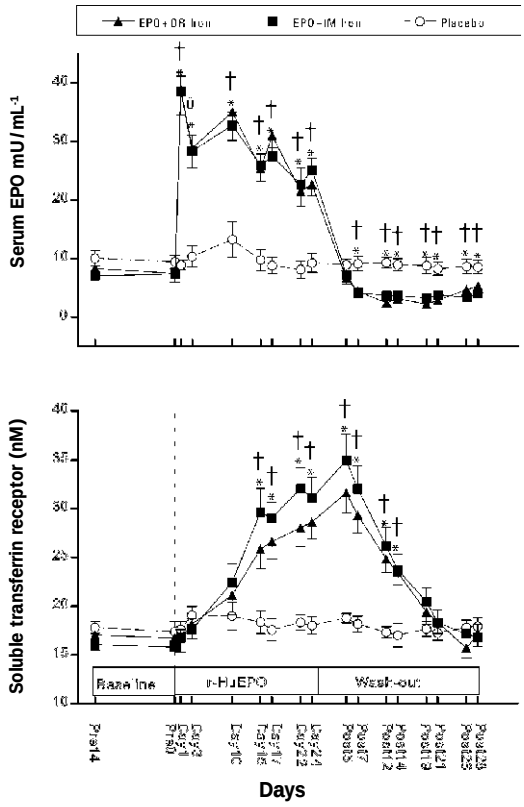


Figure 3. Effect of human recombinant erythropoietin (r-HuEPO) administration over 25 days on serum EPO and soluble transferrin receptor. Values are mean with vertical bars to show standard error of the mean. The three groups and doses of r-HuEPO are described in the Methods. Statistically significant differences ($p<0.05$) are: †= EPO+OR versus placebo group; *= EPO+IM versus placebo group.

$\dot{V}O_{2max}$ 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 were each at the 97.5th percentile, a positive result was not obtained (Table 4). Thus identification of current r-HuEPO use generally required substantial elevation of several relatively independent markers of accelerated erythropoiesis, and an isolated abnormality in any one marker was buffered.

Gareau[9] was the first to suggest using multiple indi-

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.

	Constant	Beta coefficient				
Model variables		RetHct	EPO	sTfr	Hct	%Macro
ON-model						
RetHct+EPO+sTfr+Hct+%Macro	-76.96	299.3	0.946	0.892	78.67	2.08
OFF-model						
Hct+RetHct+EPO	-12.40	-1566	-0.765	–	50.90	–

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.

	RetHct	*EPO*	*STfr*	*Hct*	*%Macro*	*Y'*	*Probability*
Baseline mean	0.005	7	18	0.45	0.9	-15.5216	0.000000
Change 1 var	0.0093	7	18	0.45	0.9	-14.23461	0.000000
	0.005	14	18	0.45	0.9	-8.8975	0.000000
	0.005	7	26	0.45	0.9	-8.3896	0.000000
	0.005	7	18	0.50	0.9	-11.5881	0.000000
	0.005	7	18	0.45	3.4	-10.3291	0.000000
Change 3 var	0.0093	14	26	0.45	0.9	-0.47851	0.249401
	0.005	14	26	0.50	0.9	2.168	0.993254
	0.005	7	26	0.50	3.4	0.7364	0.844962
Change 4 var	0.0093	14	26	0.50	0.9	3.45499	0.999649
Change 5 var	0.0093	14	26	0.50	3.4	8.64749	1.000000

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').

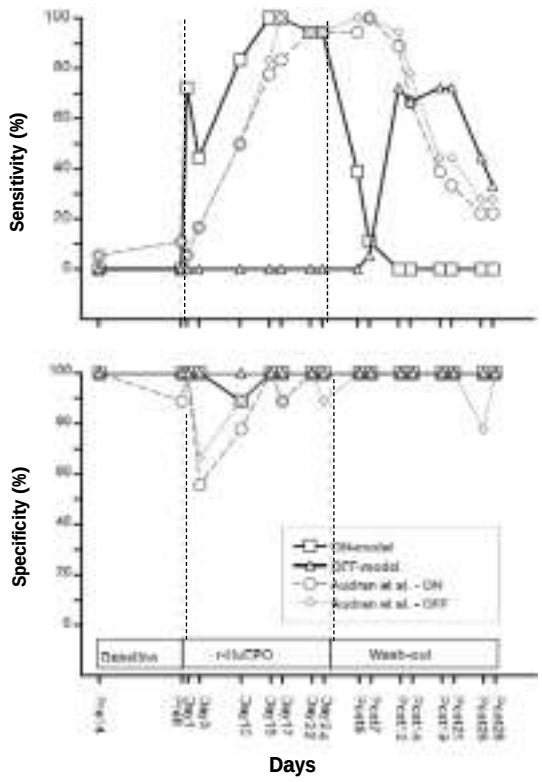


Figure 4. Comparison of the sensitivity and specificity of a 5-variable ON-model (RetHct, EPO, sTfr, Hct, %Macro) and 3-variable OFF-model (RetHct, EPO, Hct) with the 3 variables suggested by Audran *et al.*[3] (Hct, sTfr, and sTfr: protein ratio) as suitable for detecting human recombinant erythropoietin (r-HuEPO) use. ON- and OFF-models were developed for Audran *et al.*[3] by using data from our subjects both at the end of r-HuEPO administration (days 22-24) and middle of wash-out (post 12-14), respectively. For each model, sensitivity was calculated for 18 subjects given r-HuEPO, while specificity was calculated for 9 placebo subjects. Identification of r-HuEPO use was based on a probability >0.999999.

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.*[3] 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.*[3] 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.*[3] proposed using the sTfr:protein ratio was to circumvent the possibility that hemoconcentration could cause false positives by artificially elevating blood markers such as sTfr and Hct. Hemoconcentration 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%.[8,25] 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[17] and it has been reported that only intravenous iron injection can prevent functional iron deficiency during r-HuEPO administration.[26] 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.[27] However, in contrast to previous research[17,18,28] 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,[29] 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 %Macro 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 %Macro 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,[30-33] as has an increase in Hct due to plasma volume loss.[34] With a few days of continued hypoxia, the serum EPO of this athlete fell substantially, which is consistent with the available literature.[35] Despite tests on successive days, no further false positive results were obtained. In contrast, the ON-model identified all r-HuEPO users repeatedly, suggesting that serial testing might be appropriate following an initial positive result. The OFF-model, which relies on elevated Hct at the same time as depressed RetHct and serum EPO, yielded no false positives after hypoxic exposure.

Our models and logit coefficients cannot be extrapolated to the general athlete population until a much larger study is conducted. The relatively short duration of our program may limit the ability to apply the results if athletes are using r-HuEPO for extended periods. Our subjects included very few women and gender-specific models may be required. There is also a need to study athletes from Asian, African and Mediterranean locations where hematologic abnormalities are relatively prevalent.[36] We are uncertain as to whether intravenous iron injection might change the sensitivity of our current models. The continued development of a test to detect banned r-HuEPO use 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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RP, CJG, KRE, MJA, CB, CH, DTM, GJT, AGH participated in the conception/design, data collection, analysis and interpretation and drafting of the manuscript, and saw and approved the final version of the manuscript.

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Disclosures

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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

1. Sawka MN, Joyner MJ, Miles DS, Robertson RJ, Spriet LL, Young AJ. American College of Sports Medicine Position Stand. The use of blood doping as an ergogenic aid. Med Sci Sports Exerc 1996; 28:I-VIII.
2. Ekblom B, Berglund B. Effect of erythropoietin administration on maximal aerobic power. Scand J Med Sci Sports 1991; 1:88-93.
3. Audran M, Gareau R, Matecki S, et al. Effects of erythropoietin administration in training athletes and possible indirect detection in doping control. Med Sci Sports Exerc 1999; 31:639-45.
4. Berglund B, Ekblom B. Effect of recombinant human erythropoietin treatment on blood pressure and some haematological parameters in healthy men. J Intern Med 1991; 229:125-30.
5. Carlini RG, Reyes AA, Rothstein M. Recombinant human erythropoietin stimulates angiogenesis in vitro. Kidney Int 1995; 47:740-5.
6. Maschio G. Erythropoietin and systemic hypertension. Nephrol Dial Transplant 1995; 10 (Suppl 2):74-9.
7. Raine AE. Hypertension, blood viscosity, and cardiovascular morbidity in renal failure: implications of erythropoietin therapy. Lancet 1988; 1:97-100.
8. Harrison MH. Effects of thermal stress and exercise on blood volume in humans. Physiol Rev 1985; 65: 149-209.
9. Gareau R, Gagnon MG, Thellend C, et al. Transferrin soluble receptor: a possible probe for detection of erythropoietin abuse by athletes. Horm Metab Res 1994; 26:311-2.
10. Breymann C, Bauer C, Major A, et al. Optimal timing of repeated rh-erythropoietin administration improves

its effectiveness in stimulating erythropoiesis in healthy volunteers. Br J Haematol 1996; 92:295-301.
11. Casoni I, Ricci G, Ballarin, E et al. Hematological indices of erythropoietin administration in athletes. Int J Sports Med 1993; 14:307-11.
12. Conconi F, Casoni I, Manfredini F, et al. Detection of erythropoietin administration in sports. Proceedings of the Second International Symposium on Drugs in Sport. Towards the use of blood samples in doping control? Lillehammer, Norway, August 29-31 1993. Blood samples in doping control.
13. Rutherford CJ, Schneider TJ, Dempsey H, Kirn DH, Brugnara C, Goldberg MA. Efficacy of different dosing regimens for recombinant human erythropoietin in a simulated perisurgical setting: the importance of iron availability in optimizing response. Am J Med 1994; 96:139-45.
14. Souillard A, Audran M, Bressolle F, Gareau R, Duvallet A, Chanal J-L. Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin in athletes. Blood sampling and doping control. Br J Clin Pharmacol 1996; 42:355-64.
15. Johnson DL, Farrell FX, Barbone FP, et al. Identification of a 13 amino acid peptide mimetic of erythropoietin and description of amino acids critical for the mimetic activity of EMP1. Biochemistry 1998; 37:3699-710.
16. Wrighton NC, Balasubramanian P, Barbone FP, et al. Increased potency of an erythropoietin peptide mimetic through covalent dimerization. Nat Biotechnol 1997;15: 1261-5.
17. Brugnara C, Colella GM, Cremins J, et al. Effects of subcutaneous recombinant human erythropoietin in normal subjects: development of decreased reticulocyte hemoglobin content and iron deficient erythropoiesis. J Lab Clin Med 1994; 123:660-7.
18. Brugnara C, Zelmanovic D, Sorette M, Ballas SK, Platt O. Reticulocyte hemoglobin: an integrated parameter for evaluation of erythropoietic activity. Am J Clin Pathol 1997; 108:133-42.
19. Major A, Bauer C, Breymann C, Huch A, Huch R. rh-erythropoietin stimulates immature reticulocyte release in man. Br J Haematol 1994; 87:605-8.
20. Major A, Mathez-Loic F, Rohling R, Gautschi K, Brugnara C. The effect of intravenous iron on the reticulocyte response to recombinant human erythropoietin. Br J Haematol 1997; 98:292-4.
21. Bressolle F, Audran M, Gareau R, Baynes RD, Guidicelli C, Gomeni R. Population pharmacodynamics for monitoring epoetin in athletes. Clin Drug Invest 1997; 14:233-42.
22. Gore CJ, Hahn G, Burge CM, Telford RD. VO2max and haemoglobin mass of trained athletes during high intensity training. Int J Sports Med 1997; 18:477-82.
23. Burge CM, Skinner SL. Determination of hemoglobin mass and blood volume with CO: evaluation and application of a method. J Appl Physiol 1995; 79:623-31.
24. Aron A, Aron EN. Statistical power and effect size. Statistics for psychology. Englewood Cliffs, New Jersey: Prentice Hall; 1994. p. 206-47.
25. Gore CJ, Scroop GC, Marker JD, Catcheside PG. Plasma volume, osmolarity, total protein and electrolytes during treadmill running and cycle ergometer exercise. Eur J Appl Physiol 1992; 65:302-10.
26. Macdougall IC. Strategies for iron supplementation: oral versus intravenous. Kidney Int Suppl 1999; 69:S61-S66.
27. Brugnara C. Use of reticulocyte cellular indices in the diagnosis and treatment of hematological disorders. Int J Clin Lab Res 1998; 28:1-11.
28. Drueke TB, Barany P, Cazzola M, et al. Management of iron deficiency in renal anemia: guidelines for the optimal therapeutic approach in erythropoietin-treated patients. Clin Nephrol 1997; 48:1-8.
29. Tarng DC, Huang TP, Chen TW, Yang WC. Erythropoietin hyporesponsiveness: from iron deficiency to iron overload. Kidney Int Suppl 1999; 69:S107-S118.
30. Gunga HC, Kirsch K, Rocker L, Schobersberger W. Time course of erythropoietin, triiodothyronine, thyroxine, and thyroid- stimulating hormone at 2,315 m. J Appl Physiol 1994; 76:1068-72.
31. Chapman RF, Stray-Gundersen J, Levine BD. Individual variation in response to altitude training. J Appl Physiol 1998; 85:1448-56.
32. Piehl Aulin K, Svedenhag J, Wide L, Berglund B, Saltin B. Short-term intermittent normobaric hypoxia - haematological, physiological and mental effects. Scand J Med Sci Sports 1998; 8:132-7.
33. Rusko HK, Penttinen JTT, Koistinen PO, Vähäsöyrinki PI, Leppäluoto JO. A new solution to simulate altitude and stimulate erythropoiesis at sea level in athletes. In: Viitasalo J, Kujala U, eds. The way to win. Helsinki: International Congress on Applied Research in Sports; 1994. p. 287-9.
34. Jung RC, Dill DB, Horton R, Horvath SM. Effects of age on plasma aldosterone levels and hemoconcentration at altitude. J Appl Physiol 1971; 31:593-7.
35. Jelkmann W. Renal erythropoietin: properties and production. Rev Physiol Biochem Pharmacol 1986; 104:139-215.
36. Hoffbrand AV, Pettit JE. Essential haematology. Oxford: Blackwell Scientific; 1993.