Red Cells & Iron

Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis

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Background and Objectives. The detection of recombinant human erythropoietin (r-HuEPO) abuse by athletes remains problematic. The main aim of this study was to demonstrate that the five indirect markers of altered erythropoiesis identified in our earlier work were reliable evidence of current or recently discontinued r-HuEPO use. A subsidiary aim was to refine weightings of the five markers in the initial model using a much larger data set than in the pilot study. A final aim was to verify that the hematologic response to r-HuEPO did not differ between Caucasian and Asiatic subjects.

Design and Methods. Recreational athletes resident in Sydney, Australia (Sydney, n = 49; 16 women, 33 men) or Beijing, China (Beijing, n=24; 12 women, 12 men) were randomly assigned to r-HuEPO or placebo groups prior to a 25 day administration phase. Injections of r-HuEPO (or saline) were administered double-blind at a dose of 50 IU/kg three times per week, with oral iron (105 mg) or placebo supplements taken daily by all subjects. Blood profiles were monitored during and for 4 weeks after drug administration for hematocrit (Hct), reticulocyte hematocrit (RetHct), percent macrocytes (%Macro), serum erythropoietin (EPO) and soluble transferrin receptor (sTfr), since we had previously shown that these five variables were indicative of r-HuEPO use.

Results. The changes in Hct, RetHct, %Macro, EPO and sTfr in the Sydney trial were qualitatively very similar to the changes noted in our previous administration trial involving recreational athletes of similar genetic origin. Statistical models developed from Fisher's discriminant analysis were able to categorize the user and placebo groups correctly. The same hematologic response was demonstrated in Beijing athletes also administered r-HuEPO.

Interpretation and Conclusions. This paper confirms that r-HuEPO administration causes a predictable and reproducible hematologic response. These markers are disturbed both during and for several weeks following r-HuEPO administration. This work establishes an indirect blood test which offers a useful means of detecting and deterring r-HuEPO abuse. Ethnicity did not influence the markers identified as being able to detect athletes who abuse r-HuEPO.

Key words: r-HuEPO, athletes, ethnicity, reticulocytes, erythropoiesis

There is persuasive evidence to suggest that even during ancient times athletes relied upon exotic substances to improve athletic performance.[1,2] Given the unsophisticated nature of these elixirs, it is equivocal whether their intentions were met. But the development of amphetamines around the time of the Second World War, and subsequent pharmacologic advances during the 1960s,[1] provided athletes with an indisputable means of obtaining a performance advantage. Due to associated health risks and ethical concerns, such drugs were banned from competition, and tests were implemented by governing bodies to detect the presence of banned chemical agents in urine samples. However tests are less effective against the next generation of performance-enhancing substances, such as recombinant protein hormones, that cannot be readily differentiated from the homologous, naturally occurring hormone. One such drug that has achieved prominence in recent years is recombinant human erythropoietin (r-HuEPO). Injecting r-HuEPO increases the number of red blood cells in circulation, and dramatically improves the oxygen-carrying capacity of the blood and therefore endurance performance.[3,4] Abnormally accelerated erythropoiesis also leads to a measurable change in the number and physical property of reticulocytes[5-9] and the serum concentration of soluble transferrin receptor.[4,10,11] This prompted investigation of whether an indirect approach relying on markers of altered erythropoiesis, rather than the presence of the drug itself, was a feasible alternative to detect r-HuEPO abusers. A pilot study was undertaken in which recreational athletes were given r-HuEPO injections over a four-week period.[12] Serial blood samples were collected and numerous hematologic and biochemical characteristics monitored. Five parameters were found to deviate substantially from typical levels during the period of drug administration, whilst three of these were altered for several weeks afterward. The five parameters were hematocrit (Hct), reticulocyte hematocrit (RetHct), percent macrocytes (%Macro), serum erythropoietin (EPO) and soluble transferrin receptor (sTfr). This led to the development of two models, an ON-

model for discriminating between r-HuEPO users and placebo subjects *during* a period of drug administration, and an OFF-model for discriminating between r-HuEPO users and placebo subjects *following* a period of drug administration.

Based on these promising initial results, a worldwide collaborative effort was funded by the International Olympic Committee (IOC) and the Australian Government to develop further the concept of an indirect blood test to detect athletes who had used r-HuEPO. The first step in this process was to confirm that the hematologic changes associated with r-HuEPO use demonstrated during the pilot study[12] could be replicated in a separate subject group. New statistical modelling using the data from this larger subject group was undertaken to refine the weightings of variables in each model. Since it was plausible (but unlikely) that different ethnic groups responded differently to r-HuEPO, this trial also examined the response of a different ethnic group to r-HuE-PO administration.

Design and Methods

Subjects

Two separate groups of recreational athletes were studied. One group was resident in Sydney, Australia (Sydney, n = 49; 16 women, 33 men, including one East Asian, one Central/South African, and three Australian Aboriginal/Melanesian/Papuan subjects). The other group was resident in Beijing, China (Beijing, n=24; 12 women, 12 men) and was entirely of Asiatic origin. All subjects 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

Table 1. Age and body mass of recreational Sydney and Beijing athletes who received three injections per week of either 50 U/kg r-HuEPO or saline. Values are mean and standard deviation.

Group	Sex	Age (years)	Body mass (kg)
Sydney athletes			
EPO	Female (n=9)	28.9 ± 4.8	65.7 ± 5.1
	Male (n=20)	27.6 ± 5.7	83.6 ± 11.1
Placebo	Female (n=7)	26.4 ± 4.4	58.6 ± 7.2
	Male (n=13)	27.8 ± 4.4	85.0 ± 16.0
Both groups combined	N = 49	27.7 ± 4.9	77.1 ± 15.3
Beijing athletes			
EPO	Female (n=6)	20.8 ± 0.4	57.0 ± 7.6
	Male (n=6)	20.3 ± 0.8	67.8 ± 5.6
Placebo	Female (n=6)	21.7 ± 2.9	55.3 ± 6.7
	Male (n=6)	21.7 ± 2.0	70.0 ± 6.3
Both groups combined	N = 24	21.1 ± 1.8	62.5 ± 9.0

was a member of a national sporting squad.

All 73 subjects who commenced the current study completed an 8-week protocol (25 days of injections + 4 weeks of washout) and their characteristics are shown in Table 1. In accordance with health and safety guidelines imposed by the Ethics Committee, one Caucasian subject was given a saline injection instead of r-HuEPO on two occasions since his hematocrit exceeded 0.55. This occurred at the time of the last two doses of r-HuEPO in the fourth week of administration.

Study design

We used a double-blind design to characterize the changes in hematologic and biochemical indices of

Table 2. Outline of the study design to examine changes in blood parameters associated with r-HuEPO administration. The design mimicked the initial pilot trial undertaken in 1999, and was duplicated in Australia (Sydney athletes) and China (Beijing athletes). Subjects received three r-HuEPO injections per week at a dose of 50U/kg for 25 days.

	Time		
	Weeks 1-2	*Weeks 3-6*	*Weeks 7-10*
Overview	Medical and hemoglobinopathy screening° of subjects	Experimental period. (r-HuEPO administration).	Follow-up blood collection.
r-HuEPO injection		Three per week (day 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23 & 25)	
Blood collection	Two baseline samples. 1999: Day –14 and day 0; Sydney: Day –25 and 0; Beijing: Day –8 and 0; relative to first r-HuEPO injection.	Twice per week: 1999: day 1, 3, 10, 15, 17, 22, 24 Sydney: day 1, 3, 8, 10, 15, 17, 22, 24 Beijing: day 1, 4*, 8, 11*, 15, 18*, 22, 25*	Twice per week: 1999: day 5, 7, 12, 14, 19, 21, 26, 28 after last r-HuEPO injection Sydney Beijing: day 4, 6, 11, 13, 18, 20, 25 and 27 after last r-HuEPO injection Beijing: day 4, 7, 11, 14, 18, 21, 25 and 28 after last r-HuEPO injection

When blood collection and r-HuEPO injection occurred on the same day, blood collection preceded the injection. °Subjects in the 1999 trial did not undergo hemoglobinopathy screening.

increased red cell production in response to injecting r-HuEPO at a dose of 50 U/kg three times per week for 25 days. After medical screening for illness, injury, blood pressure and blood disorders a blood sample from each subject was screened for hemoglobinopathies using a Bio-Rad Variant Analyzer (Bio-Rad, Hercules, California, USA), the subjects were randomly assigned to receive either r-HuEPO or placebo (Table 2). A baseline was determined from two samples collected before the first r-HuEPO injection. Subsequent blood sample collection and r-HuEPO injections were scheduled to coincide with the administration protocol of the original study.[12] Details of the current study design are shown in Table 2.

Injections of r-HuEPO (EPREX 4000, Janssen-Cilag, Australia) or saline (NaCl 0.9% BP) were given subcutaneously (buttock). Oral iron tablets (Ferrogradumet, Abbott, Australia) were taken daily by the r-HuEPO subjects (~105 mg of elemental iron derived from 350 mg of dried ferrous sulphate) and the placebo subjects received sham iron tablets.

Blood analysis

All blood was collected by trained phlebotomists in the morning at a similar time to control for diurnal variation, and was sampled from an antecubital vein after 5 min of supine rest. Collection procedures at both sites were standardized and supervised by members of the research team. Samples were drawn into one 8 mL serum separation Vacuette tube with clot activator (Greiner Labortechnik, Frikenhaussen, Germany) and two 2 mL K_3EDTA Vacuette tubes (Greiner Labortechnik). Whole blood samples (analyzed within 8 hours of collection) provided erythrocyte and reticulocyte parameters measured with an ADVIA Hematology Analyzer (Bayer Diagnostics, Tarrytown, NY, USA). One ADVIA was located in Australia at the Australian Institute of Sport and the second was located in Beijing, China. This analyzer superseded the Bayer H*3 analyzer used in the pilot study. Although both analyzers employ the same flow cytometric techniques, the ADVIA permits automated reticulocyte analysis, and software modifications report a greater range of cellular characteristics. The ADVIA makes direct measurements of the number of erythrocytes per unit of blood volume, and of the number of reticulocytes as a percentage of all red cells. It also measures the size and hemoglobin concentration of erythrocytes and reticulocytes, as well as the percentages of macrocytic, microcytic, hypochromic and hyperchromic cells. Numerous additional parameters can be calculated from the basic information, including both Hct and RetHct, which represents the fractional volume of the reticulocyte pool in the bloodstream and is calculated as mean corpuscular volume of the reticulocytes (MCVr, fL) multiplied by the number of reticulocytes (x10^9/L).

Serum sample tubes were allowed to stand at room temperature and when clotting of the sample was complete, the tubes were centrifuged at 3,500-4,000 rpm for 10 minutes to separate serum from the cells. Serum was then drawn off and aliquoted into 2 mL cryotubes (Nalgene Cryoware, Interpath Services, Sydney, Australia). Frozen serum aliquots from Beijing were freighted on dry ice to Australia, where measurements were made of serum concentrations of erythropoietin (EPO) and soluble transferrin receptor (sTfr) for both the Sydney and Beijing samples. The EPO concentrations were determined using an automated solid-phase, sequential chemiluminescent Immulite EPO assay (Diagnostic Products Corporation, Los Angeles, CA, USA). The sTfr concentrations were measured by means of an automated immunonephelometric assay (Dade Behring GmBH, Marburg, Germany). The ferritin concentrations were measured using a 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 storage disk containing the Beijing control data was found to be corrupted and the raw data were not recoverable. Therefore only one set of control data can be presented. For this ADVIA, the mean values and coefficients of variation (CV) for 20 repeated measures on a whole blood sample were as follows: Hct, 0.45 and 1%; percent reticulocytes, 1.4% and 5.6%; MCVr, 98.2 fL and 0.6%; percent macrocytes, 3.2% and 6.0%. Using three levels of EPO controls (mean 15.2, 30.4 and 62.3 mU/mL), the within-assay CVs were 4.7, 7.1 and 5.1%, and between-assay CVs were 7.3, 7.2 and 9.5% respectively. Using two levels of sTfr controls (mean 0.63 and 1.45 mg/L), the within-assay CVs were 0.0 and 2.2%, and between-assay CVs were 3.4 and 2.8%, respectively.

Methodologic differences between the 1999 study and the current study

To facilitate valid replication of the initial trial undertaken in 1999, whenever practical, methodologies and treatments were kept identical. It was considered important to evaluate the effect of the interval between r-HuEPO injection and blood collection on the blood variables included in our models. The objective was to verify whether the models could still identify r-HuEPO users when serum EPO levels decay due to the short half-life of this hormone in the bloodstream. Therefore in the Beijing group, samples were alternately obtained 24 and 48 hours after r-HuEPO injection as opposed to a 24-hour interval in both the 1999 and Sydney trials. Whereas the initial trial was merely a pilot study into the usefulness of hematologic markers of erythropoiesis to detect r-HuEPO abuse, the current studies were undertaken with the aim that the resulting test procedures might be adopted by sport drug testing laboratories. Therefore it was necessary to incorporate assay techniques that were compatible with IOC guidelines. These guidelines require serum measures of peptide hormones to be confirmed with a different immunoassay in the event of a *suspect sample*. We elected to use the Dade Behring sTfr and Immulite EPO assays as the initial screen and for quantitative data since they used automated analyzers that

permitted a high throughput. The more labor-intensive, and expensive R&D assays described in the original study[12] were considered a better choice as the confirmation assay techniques. It must be noted that the R&D sTfr assays express results in nM whilst the Dade Behring assay expresses results in mg/L. There was a strong correlation in both cases between the R&D Systems kits used in the original study and the Immulite EPO and Dade Behring sTfr methods used in the current study, as indicated by the following equations (data set augmented with additional samples).

$$EPO (n=1898):$$
$$[R\&D] = 0.73 \times [Immulite] + 0.39, R^2 = 0.88$$

$$sTfr (n=1898):$$
$$[R\&D] = 14.14 \times [DB] + 3.31, R^2 = 0.89$$

In the case of hematologic parameters, instead of the H*3 analyzer used in the 1999 trial we used the next generation of this analyzer, the ADVIA, since the ADVIA is capable of reporting additional detailed hematologic information that could conceivably assist in positively identifying r-HuEPO abusers. Software modifications within the ADVIA meant that one of the parameters, %Macro, was calculated using a different, but directly comparable, algorithm. Consequently, results derived from the H*3 analyzer in the 1999 trial were higher than the same parameter calculated by the ADVIAs in Australia and China (Figure 1) as follows:

$$\%Macro (n=30):$$
$$[H*3] = 3.23 \times [ADVIA] + 0.29, R^2 = 0.55$$

Only data from the current studies, using the newly adopted analytical techniques, were used to derive the ON- and OFF-model equations.

Statistical analysis

Overview. One approach to demonstrate that the hematologic response to r-HuEPO was the same in the Sydney trial as that in the pilot trial was to apply a repeated measures analysis of variance to the raw data. However statistical comparisons between the two trials were confounded by the different assays used for serum measures, and different analyzers used for whole blood measures. Since there was no statistical approach to overcome the confounding effect, we relied on qualitative analysis to confirm that the five parameters changed in a similar manner in both the Sydney and the original subject groups. This qualitative comparison was augmented with data from the Beijing trial to elucidate the consistency of the hematologic response to r-HuEPO further.

Model derivation. The five parameters reported here were previously identified as being potentially useful indicators of r-HuEPO use due to the large changes over time associated with r-HuEPO administration.[12] Whereas previously logit analysis had been used to identify r-HuEPO users, advice from a statistician recruited to the project suggested that Fisher's discriminant analysis methodology was a more effective approach in this con-
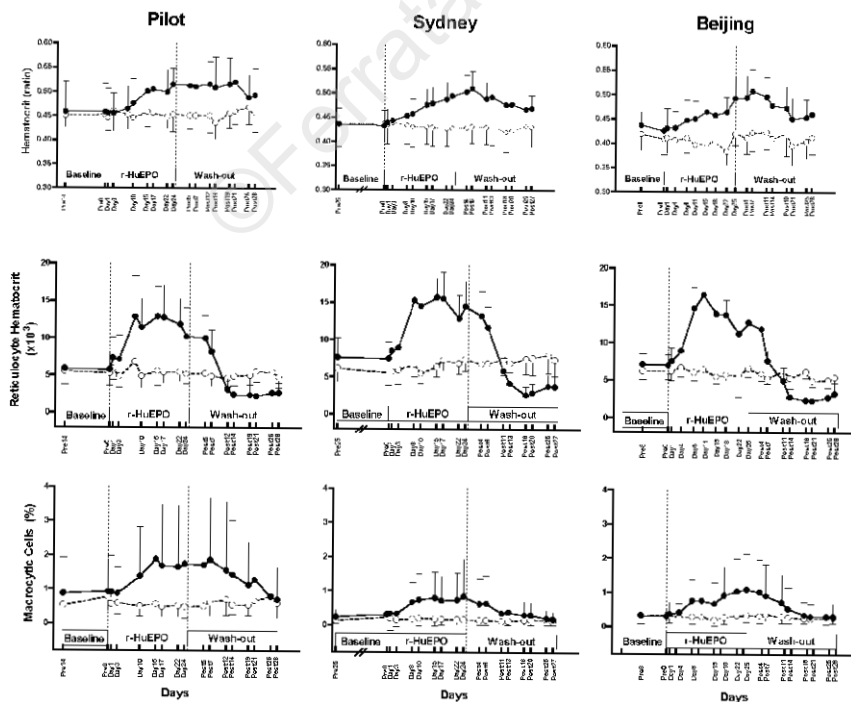

Figure 1. Response of the three variables measured in whole blood to 25 days of r-HuEPO injections (50 U/kg three times per week) and a 4-week wash-out period: Pilot trial 1999 (n=18 r-HuEPO, n=9 placebo); Sydney trial 2000 (n=29 r-HuEPO, n=20 placebo); Beijing trial 2000 (n=12 r-HuEPO, n=12 placebo). All groups were recreational athletes. The three panels illustrate hematocrit, reticulocyte hematocrit and percent macrocytes. Values are mean and standard deviation.

text. One of the assumptions required for Fisher's method to be optimal is that the variables used be normally distributed. The normality of each of the five variables was evaluated using data not only from this study but also from a trial involving more than 1,000 athletes from around the world (these data will be presented in a subsequent manuscript). This resulted in three variables being transformed, using natural logarithms. The five variables used for the present analysis were: Hct, RetHct, $\log_e$(EPO), $\log_e$(sTfr) and $\log_e$(%Macro + 0.1). It was necessary to add 0.1 to the values of %Macro to allow for zero values.

Statistical models were developed using data from the Sydney administration trial. In order to enhance the reliability of the discriminant models, placebo group data were augmented with data from Caucasian placebo subjects who participated in a subsequent r-HuEPO administration trial. Data from the r-HuEPO users in this subsequent trial were not included in this analysis due to differences in the r-HuEPO administration protocol. Since the normal ranges of some of the variables such as Hct are known to depend on gender,[12] separate discriminant models were developed for males and females. The resultant models were sufficiently similar that a gender-independent model was found to be equally effective for discriminating between the r-HuEPO users and the placebo group. However, to account for gender-specific differences in the parameters, the cut-off score for a model (that is, the value used to discern between

the user and placebo groups) was lower for females than for males. Two distinct models were developed: an ON-model that used all five variables to identify individuals during a period of drug administration, and an OFF-model that employed just three variables to identify individuals who had recently stopped using r-HuEPO.

Analyses were conducted using Statistica (StatSoft, Tulsa, OK, USA), GLIM 4 (Royal Statistical Society, London, UK) and Minitab (Minitab Inc., State College, PA, USA) software.

Results

Blood markers of erythropoiesis

The changes in Hct, RetHct, %Macro, EPO and sTfr induced by r-HuEPO administration were qualitatively similar for the pilot, Sydney, and Beijing trials, notwithstanding the different assays and analyzers used between trials.

In all groups injected with r-HuEPO, Hct rose markedly in response to injections of 50 U/kg r-HuEPO three times per week for 25 days (Figure 1). In each study Hct rose by ~0.06 over the administration period. Four weeks after injections ceased, Hct remained elevated by ~0.03. RetHct more than doubled by day 8 of the injection period in all treated groups (Figure 1). Like in the 1999 trial, values then remained relatively constant during r-HuEPO administration in both the Sydney and Beijing trials, but declined markedly thereafter and were depressed to abnormally low levels (approximately half
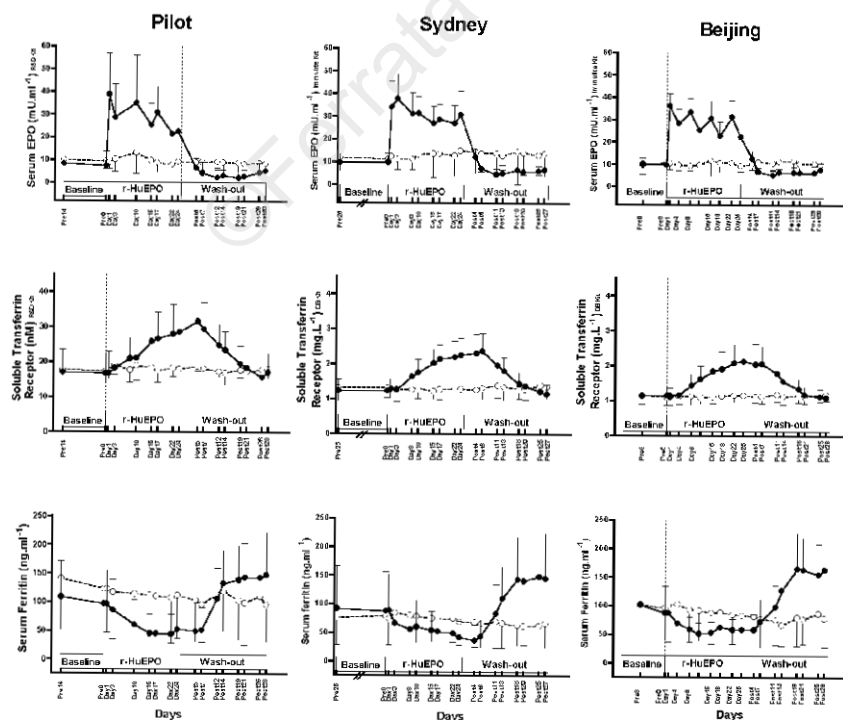

Figure 2. Response of the three variables measured in serum to 25 days of r-HuEPO injections (50 U/kg three times per week) and a 4-week wash-out period: Pilot trial 1999 (n=18 r-HuEPO, n=9 placebo); Sydney trial 2000 (n=29 r-HuEPO, n=20 placebo); Beijing trial 2000 (n=12 r-HuEPO, n=12 placebo). All groups were recreational athletes. The three panels illustrate serum EPO, serum sTfr and serum ferritin. Note that for serum EPO and serum sTfr, the 1999 study values were obtained using R&D Systems assays, while the two latter studies used Immulite EPO and Dade Behring sTfr. Values are mean and standard deviation.

of the baseline levels) during the washout period. Values were still depressed when serial blood sampling ceased four weeks after the final injection.

There was an increase in %Macro that mirrored the increase in RetHct during the administration period (Figure 1). The approximate doubling of baseline levels that was evident during the 1999 trial was also apparent in both the Sydney and Beijing groups, albeit from different baseline levels attributable to the use of a different analyzer in the pilot study. There was a gradual fall in %Macro during washout, but in contrast to RetHct values, %Macro did not fall below pre-injection levels at any stage.

Serum EPO was approximately three-fold elevated in the r-HuEPO groups throughout the administration period, reflecting the capacity of the assay to detect both endogenously produced EPO and exogenous r-HuEPO (Figure 2). In all three groups there was a gradual decline in EPO during the administration period, despite athletes receiving constant doses of r-HuEPO and providing blood samples at consistent intervals. Immediately after injections ceased, EPO levels declined, and within one week were below baseline values. Abnormally low values persisted during much of the washout, although after four weeks the values were similar to baseline for the 1999 and Beijing groups, but remained subnormal for the Sydney group.

There was a consistent and uniform increase in sTfr concentration that was evident within 8-10 days of the first r-HuEPO injection (Figure 2). The relative magnitude of the increase was similar, in percentage terms, between the three groups notwithstanding the change in scale associated with the change in assay between studies. The increase persisted after injections ceased, and had not declined markedly in any of the three groups by at least 11 days after the final injection. However after four weeks of washout, values had returned to pre-injection levels in each of the groups administered r-HuEPO.

EPO values for the Beijing group 48 hours post-injection were moderately lower than values recorded only 24 hours after injection (Figure 2). This reflects the additional clearance of r-HuEPO from the circulation during the extra 24-hour period. However the magnitude of the difference was not sufficient to affect the ON-model scores.

There was a consistent decline of approximately 40-70% in serum ferritin for subjects administered r-HuEPO during the administration phase in each of the separate trials (Figure 2). For a small percentage of subjects with low ferritin values at the commencement of the trial (four females in the Sydney trial, one male in the Pilot trial), this decrease resulted in nadir values of 10-15 ng/mL during stages of the administration phase. Without exception ferritin values increased markedly during the washout phase and exceeded baseline levels in all individuals administered r-HuEPO.

ON- and OFF-model equations

One factor that had to be considered when deriving the equations to detect r-HuEPO users was gender, since the normal ranges of some of the parameters, particularly Hct, are known to differ between males and females. This violates the assumption of discriminant analysis that the parameters are normally distributed amongst the subject population. We initially developed separate discriminant models for males and females. However it became apparent that it was just as effective to use the same model, but with different cut-offs.

ON-model scores were based on blood samples collected at the end of the administration phase in the Sydney trial. The model derived for identifying current r-HuEPO users was:

$$ON\text{-model score} =$$
$$3.721Hct + 30.45RetHct + 0.1871\log_e(EPO)$$
$$+ 0.1267\log_e(sTfr)$$
$$+ 0.115 \log_e(\%macro + 0.1)$$

The ON-model yielded complete separation between the placebo group and r-HuEPO users for both males and females in the Sydney trial (Figure 3). This illustrates how 100% correct classification can be achieved for these data using appropriate cut-offs. This figure also illustrates why the cut-off for females should be lower than that for males.

OFF-model scores were derived from the Sydney trial samples taken approximately two weeks after the final r-HuEPO injection. The model derived for identifying subjects who have recently stopped taking r-HuEPO was:
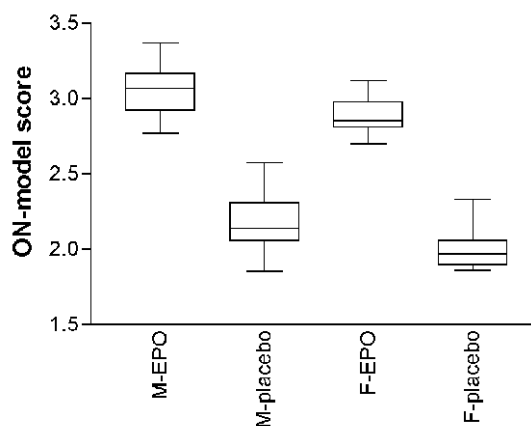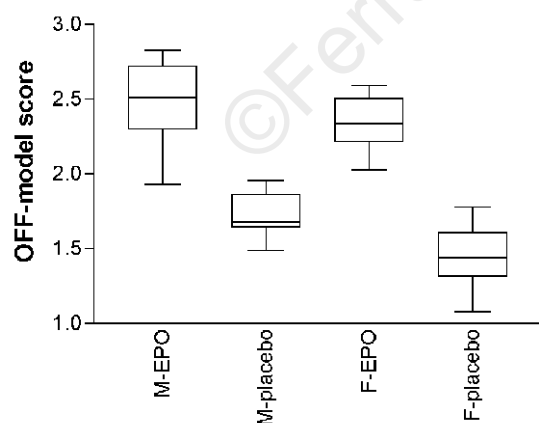

Figure 3. Boxplot of ON-model values from the Sydney r-HuEPO administration trial (n=25 r-HuEPO, n=28 placebo). Four subjects from the Sydney trial had missing data at the time point used to calculate these ON-model scores. The line across the middle of each box indicates median score. The box itself shows the inter-quartile range (25th-75th percentile), while vertical lines show the absolute range of scores. M denotes males, F denotes females.

OFF-model =
$6.149Hct - 92.87RetHct - 0.1463\log_e(EPO)$.

Figure 4 illustrates how the OFF-model was able to discriminate between r-HuEPO users and subjects taking placebo. Among the females there was complete separation between the placebo group and r-HuEPO users in the Sydney trial. There was almost complete separation among the males, although one male user gave a smaller value for the OFF-model than one of the placebo subjects.

ON- and OFF-model scores during and after r-HuEPO use

Figures 3 and 4 represent a snapshot of how the equations performed at the time point from which the models were derived. To understand better how the equations performed throughout the administration and washout phases, ON- and OFF-model scores were retrospectively calculated for each blood collection point during both the Sydney and Beijing trials. None of the male subjects who received placebo injections during the Sydney study recorded an ON-model score greater than 2.66 (range of peak scores 2.18-2.66), and the highest score recorded in male members of the Beijing placebo group was 2.47 (range of peak scores 2.21-2.47). Toward the end of the administration period all male members of the r-HuEPO group, in both the Sydney and Beijing trials, reached values above 2.66 at some stage. Within the female cohort, the score of all subjects given placebo in Sydney remained below 2.34 (range of peak scores 1.97-2.33), whilst that of their counterparts in Beijing remained

below a value of 2.20 (range of peak scores 1.87-2.19). All females treated with r-HuEPO in the Sydney and Beijing studies attained scores of more than 2.40 toward the end of the administration period.

None of the male subjects who received placebo injections during the Sydney trial had an OFF-model score in excess of 2.47 (range of peak scores 1.52-2.47), and the highest score recorded among male members of the Beijing placebo group was 2.29 (range of peak scores 1.86-2.29). In the 2-4 weeks following cessation of injections, 16 of the 20 male members of the Sydney r-HuEPO group, and five of the six male members of the Beijing r-HuEPO group, obtained an OFF-model score above 2.47. Within the female cohort, the score of all Sydney subjects given placebo treatment remained below 2.10 (range of peak scores 1.46-2.09) whilst that of the Beijing females given placebo treatment remained below a value of 1.88 (range of peak scores 1.51-1.87). All nine female members of the Sydney r-HuEPO group, and five of the six female members of the Beijing r-HuEPO group, registered an OFF-model score above 2.10 during the washout phase.

Discussion

The major finding from this study was that changes in the hematologic parameters shown to be sensitive to r-HuEPO use in the 1999 pilot study[12] were replicated in a larger subject group. These changes were also demonstrated in a group of Asiatic ethnic origin. Equations utilizing these hematologic parameters were able to discriminate between r-HuEPO users and subjects taking placebo. The ON-model equation forms the basis of an indirect approach which, in combination with the detection of r-HuEPO in urine,[13] has been accepted by the IOC for detecting r-HuEPO abuse amongst athletic populations.

Our indirect approach to detecting r-HuEPO abuse relies upon the ability to measure markers of abnormal red blood cell production, and to demonstrate that the results fall outside the physiologic range. Although previous studies have documented the effectiveness with which r-HuEPO administration is able to increase red blood cell production,[9,14,15] it was essential for us to demonstrate that this response occurred in a predictable manner. To facilitate ease of interpretation, we shall delineate the response into changes occurring during the r-HuEPO administration period, and those occurring after injections ceased.

During the administration period, in both the Sydney and Beijing groups, markers of elevated erythropoiesis increased in a manner consistent with that recorded in our pilot trial. The ensuing reticulocytosis was compatible with the findings of numerous papers that have quantified the release of reticulocytes during accelerated red cell production.[5-8,16] Unfortunately the inter-individual variation in many of these reticulocyte characteristics[17-19] is sufficiently large to obscure the perturbations due to accelerated red cell production, rendering the majority of these derived values ineffective as

Figure 4. Boxplot of OFF-model values from the Sydney r-HuEPO administration trial (n=27 r-HuEPO, n=28 placebo). Two subjects from the Sydney trial had missing data at the time point used to calculate these OFF-model scores. The line across the middle of each box indicates median score. The box itself shows the interquartile range (25th-75th percentile), while vertical lines show the absolute range of scores. M denotes males, F denotes females.

potential indicators of r-HuEPO abuse. However, there is a substantial increase in the number of circulating reticulocytes, and in the cell volume of reticulocytes. The product of these two parameters, RetHct, is therefore also above normal levels during r-HuEPO administration. As well as stimulating increased reticulocyte release, we found that EPO and sTfr levels were higher than normal during the administration phase. Such increases in EPO[4,14,20,21] and sTfr[4,11] have been reported previously in healthy populations injected with r-HuEPO. The consistency of the responses across different subject groups, in both magnitude and temporal characteristics, confirmed their suitability as potential markers of r-HuEPO abuse in athletes. By adding together the (weighted) scores for each of the five parameters, r-HuEPO users have a resultant ON-model score that is demonstrably higher than the corresponding scores of control subjects with normal rates of red blood cell production.

In the Beijing study, we found no significant difference in ON-model scores whether blood was collected 24 or 48 hours after r-HuEPO injection. Since clinical studies indicate that to obtain optimal results it is necessary to inject r-HuEPO at least every 2-3 days to maintain a substantially elevated rate of erythropoiesis,[9,15] our data suggest there is a high likelihood that an athlete requested to provide a blood sample during a period of r-HuEPO use will record an elevated ON-model score.

Following cessation of artificially-induced accelerated red cell production, the total red cell mass of r-HuEPO users must regress back to the normal homeostatic level. Little research has been undertaken on the kinetics of hematologic parameters in the weeks after ceasing r-HuEPO administration. However previous studies[4,21] have indicated that a reduction of red cell mass is achieved by a virtual 'shutdown' of red cell production, as evidenced by a sharp reduction in serum EPO and the number of circulating reticulocytes within days of ceasing r-HuEPO administration.

Souillard et al.[21] administered 200 U/kg r-HuEPO on five occasions over a 10-day period, and reported a 0.04 increase in Hct and approximate tripling of reticulocyte count. During the 14 days after injections ceased, reticulocyte counts fell to below baseline levels on days 11 and 14, whilst the elevated Hct was sustained within 0.01 of peak levels that occurred four days post-injections. Serum EPO levels also fell below baseline on these post-injection days. In a study that more closely resembles the design of our own, Audran et al.[4] presented data on all parameters except %Macro in athletes who had received daily 50 U/kg r-HuEPO injections for 26 days. By the end of the four-week washout period, there had been a decline of 0.04 in Hct from peak values, reticulocyte counts were below baseline levels, serum EPO was below original values and sTfr had regressed to the levels recorded at baseline. It is not clear why a study involving relatively high doses of r-HuEPO (1,200 U/kg over a 10-day period) that increased Hct by around 0.06, found

that reticulocyte levels decreased back to normal levels rather than below baseline 14 days after injections ceased.[14] Perhaps this relates to the markedly higher doses of r-HuEPO used in that study, and/or to the shorter period of drug administration. Although no mean data were presented in tables, the graphs presented by the researchers indicate that Hct regressed partially toward normal levels but was still substantially higher than baseline after 14 days, which agrees with our observation of a slight regression of Hct by day 12-14.

Our research confirms the existence of a post-administration phase of reduced erythropoiesis. Assuming a normal rate of red cell removal from the circulation, this would return the total red cell mass to baseline over several weeks. Our pilot study demonstrated that markers of accelerated erythropoiesis rapidly lose their ability to discriminate between r-HuEPO users and controls during this period. Therefore, in order to detect athletes who had recently ceased taking r-HuEPO, a different combination of erythropoietic markers was used to develop an OFF-model[12] that is sensitive to decreased erythropoiesis in the presence of an unusually high Hct. Although disease states such as myelodysplastic syndromes, aplastic anemias, pure red cell aplasias and anemias of chronic renal disease may depress erythropoiesis,[22] this only occurs in association with markedly depressed Hct. In the medical literature we cannot locate any physiologic disturbance that is known to depress erythropoiesis to the extent noted in our studies whilst normal Hct levels are maintained. Therefore, the OFF-model score can be used to differentiate between a subject who has used r-HuEPO in the recent past and subjects with normal rates of red cell production.

As illustrated in Figures 1 and 2, the response of subjects from each ethnic group to r-HuEPO administration was virtually identical. This was expected, since the EPO molecule is highly specific to progenitor cells within bone marrow, stimulating committed stem cells to proliferate and differentiate and enhancing survival.[15,22] Although EPO can also decrease cell maturation time, increase the rate of hemoglobin synthesis, and stimulate release of immature reticulocytes,[22] these processes are not under direct genetic control and are therefore unlikely to be influenced by variations in genome. Our data suggest that it is unnecessary to derive separate equations for athletes of different ethnic origin.

However the concept of a common erythropoietic response to the r-HuEPO stimulus must be differentiated from the assumption that a given dose of r-HuEPO will stimulate a predictable increase in total hemoglobin mass for an individual. The type and quantity of globin chain synthesized during the polychromatophilic stage, which occurs after the stem cell has proliferated under the influence of r-HuEPO, is under genetic control[22] and thus can be expected to show individual variation. This will be particularly apparent in instances of genetic abnormalities, and more specifically in some hemoglobinopathies, that are relatively prevalent in Asiatic, Mediterranean and African populations. A subsequent

paper will describe the typical distribution of ON- and OFF-model scores in athletic populations around the world, and will address the incidence of hematologic abnormalities. This population data is essential for nominating an appropriate cut-off score that correctly identifies an r-HuEPO user, but does not falsely identify an athlete who possesses an unusual blood profile.

In conclusion, this paper confirms that r-HuEPO administration causes a predictable and reproducible hematologic response. Ethnicity did not influence the markers identified as being able to detect athletes who abuse r-HuEPO. These markers are disturbed both during and for several weeks following r-HuEPO administration, and provide a valid and reliable basis for indirect detection and deterrence of r-HuEPO abuse.

Potential implications for clinical practice

We have shown that the use/abuse of r-HuEPO is a potentially significant medical issue. The depression of erythropoietic activity after EPO use appears to be unique to previous EPO use. This may have implications in the future when assessing normal hematopoietic responses, or the lack of them, in "long-term" r-HuEPO users (and abusers). We have confirmed that the unique parameter of RetHct provides a robust but sensitive indicator of erythropoietic activity (accelerated or depressed production).

References

1. Mottram DR, Gunnell S. Drugs in sport. 2nd ed. Great Britain:E & FN Spon, 1996.
2. Voy R. Drugs, sport, and politics. Champaign, Ill:Leisure Press, 1991.
3. Ekblom B, Berglund B. Effect of erythropoietin administration on maximal aerobic power. Scand J Med Sci Sports 1991; 1:88-93.
4. 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.
5. 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.
6. 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.
7. 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.
8. 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.
9. Breyman C, Bauer C, Major A, et al. Optimal timing of repeated rh-erythropoietin administration improves its effectiveness in stimulating erythropoiesis in healthy vol-

unteers. Br J Haematol 1996; 92:295-301.

10. Bressolle F, Audran M, Gareau R, Baynes R, Guidicelli C, Gomeni R. Population pharmacodynamics for monitoring epoetin in athletes. Clin Drug Invest 1997; 14:233-42.

11. Gareau R, Gagnon M, Thellend C, et al. Transferrin soluble receptor: a possible probe for detection of erythropoietin abuse by athletes. Horm Metab Res 1994; 26:311-2.

12. Parisotto R, Gore C, Emslie K, et al. A novel method for the detection of recombinant erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. Haematologica 2000; 85:564-72.

13. Lasne F, de Ceaurriz J. Recombinant erythropoietin in urine. Nature 2000; 405:635.

14. Rutherford C, Schneider T, Dempsey H, Kirn D, Brugnara C, Goldberg M. 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.

15. Cazzola M, Mercuriali F, Brugnara C. Use of recombinant human erythropoietin outside the setting of uremia. Blood 1997; 89:4248-67.

16. Macdougall I, Cavill I, Hulme B, et al. Detection of func-

tional iron deficiency during erythropoeitin treatment: a new approach. Br Med J 1992; 304:225-6.

17. Tarallo P, Humbert JC, Mahassen P, Fournier B, Henny J. Reticulocytes: biological variations and reference limits. Eur J Haematol 1994; 53:11-5.

18. Buttarello M, Bulian P, Venudo A. Laboratory evaluation of the Miles H*3 automated reticulocyte counter. Arch Pathol Lab Med 1995; 119:1141-8.

19. d'Onoforio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995; 85:818-23.

20. Casoni I. Hematological indices of erythropoietin administration in athletes. Int J Sports Med 1993; 14:307-11.

21. Souillard A, Audran M, Bressolle F, Gareau R, Duvallet A, Chanal JL. Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin in athletes. Blood sampling and doping control. Br J Clin Pharmacol 1996; 42:355-64.

22. McKenzie S. Textbook of Hematology. 2nd ed. Baltimore:Williams & Wilkins, 1996.