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Development of reference ranges in elite athletes for markers of altered erythropoiesis

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Background and Objectives. Our previous research developed two statistical models that are useful indicators of current (ON-model) or recently discontinued (OFF-model) recombinant human erythropoietin (rHuEPO) use by athletes. The component variables of the ON-model are hematocrit (Hct), reticulocyte hematocrit (RetHct), serum erythropoietin (EPO), percent macrocytes (%Macro), and soluble transferrin receptor (sTfr), whilst the OFF-model uses only the first three variables. Genetics and training modalities of elite athletes may conceivably produce unusual values for blood parameters related to erythropoiesis. The aims of this study were to develop reference ranges in elite athletes for key hematologic parameters as well as ON- and OFF-models scores, and to evaluate the effect of ethnicity, gender, residence at moderate altitude (~2000 m) and within-individual variation on the variables and model scores.

Design and Methods. Over a period of three weeks, 413 female and 739 male elite athletes from 12 countries visited laboratories to provide three blood samples for analysis of blood parameters sensitive to erythropoiesis. For each parameter and for the ON- and OFF-model scores, we used mixed modeling to establish the range within which we could be 95% certain that the value for a randomly chosen athlete would fall, taking into account various random effects (variation within and between subjects and laboratories) and fixed effects (means for different levels of ethnicity, age, sport, altitude of residency). We performed similar analyses for changes in the ON- and OFF-model scores between the three visits.

Results. Most fixed effects were accompanied by clear-cut, small to moderate differences in several parameters. However, residency at moderate altitude was accompanied by a much higher hematocrit than residency nearer sea level, with the mean (and 95% confidence limits) for the difference being 2.3 (0.9 to 3.7) and 1.8 (0.1 to 3.5) units for males and females, respectively. Males at altitude also demonstrated a moderately higher ON-model score. Otherwise the influence of these effects was small for ON-, OFF- and changes in model scores.

Interpretation and Conclusions. Assessment of an athlete's blood parameters and ON- and OFF-model scores may need adjustment for training modalities and other characteristics of the subject. Changes in model scores (together with monitoring of urine samples for the presence of rHuEPO) provide a promising approach to detection of rHuEPO abuse, because they are less sensitive to subject characteristics and less variable than raw model scores.

Key words: rHuEPO, athletes, ethnicity, reference range, erythropoiesis.

Although the *International Olympic Committee* (IOC) officially prohibited the use of recombinant human erythropoietin (rHuEPO) in 1990, its detection is complicated by the fact that erythropoietin (EPO) occurs naturally in the body. In 2000, Lasne and de Ceaurriz demonstrated that the isoform pattern of rHuEPO in urine was quite different to that of endogenous erythropoietin.[1] At the same time, we demonstrated that a statistical model using five blood parameters, which are altered substantially during and after a period of rHuEPO administration, was capable of discriminating between subjects administered either rHuEPO or placebo.[2] This blood test, in combination with the urine test, was approved and implemented by the IOC for use during the Sydney 2000 Olympic Games.

Our approach using the blood matrix established two statistical models. The ON-model, indicative of accelerated erythropoiesis, was developed to discriminate between current rHuEPO users and placebo subjects using the component variables hematocrit (Hct), reticulocyte hematocrit (RetHct), percent macrocytes (%Macro), serum EPO concentration and serum soluble transferrin receptor (sTfr)

concentration.[2] The OFF-model, which relies on indicators of depressed erythropoiesis (abnormally low RetHct and EPO) juxtaposed with high Hct, was developed to identify subjects who had recently stopped receiving rHuEPO. In addition, because of the marked acceleration in erythropoiesis during the early stages of rHuEPO use, and the decrease in erythropoiesis after cessation of use, there is a resultant variation in ON- and OFF-model scores as a function of time. We propose that *changes* in these model scores could be another potentially useful indicator of rHuEPO use.

However the erythropoietic-dependent parameters included in our models could potentially be confounded by numerous factors. These include biological factors such as disease,[3-6] physiologic factors such as exercise,[7] environmental factors such as altitude exposure[8] and also the biological variation of blood parameters over time.[9,10] Consequently identifying rHuEPO users amongst the elite athlete population will entail differentiating between the fluctuations associated with exposure to such influences, and the atypical variation of hematologic parameters caused by rHuEPO use.

Although numerous large-scale studies of blood parameters have been performed with athletes[11-15] we could not locate any conducted on athletes from multiple countries which also reported each of the five parameters included in our model. Therefore, in order to extend our previous research, which demonstrated that the erythropoietic changes associated with rHuEPO use were repeatable and consistent across different ethnic groups, we sought to quantify the reference ranges for these hematologic parameters under different training, environmental and physiologic circumstances. We anticipate that generating these reference ranges within an athlete population will assist readers to better appreciate the discriminative power of hematologic parameters to highlight rHuEPO use, and also provide a reference source for institutions who collect and analyze blood samples from athletes.

Our research group undertook a large-scale, international blood profiling study of elite athletes and this paper presents data on two important goals. First, our aim was to develop 95% reference ranges (the range within which we could be 95% certain that the value for a randomly chosen athlete would fall) in elite athletes for our component hematologic parameters (as well as ON- and OFF-model scores). Second, we sought to quantify the effects of gender, ethnicity, exercise, sporting discipline, altitude as well as biological and analyzer variation on the hematologic variables and detection models.

Design and Methods

Subjects

There were 1152 (739 males, 413 females) state/national level athletes from 12 countries recruited as volunteers to provide blood samples (Table 1). All athletes signed statements of informed consent, and completed a detailed case report form regarding factors such as: sport, ethnic group, tobacco use, injury or illness, iron and other dietary supplementation. Athletes were classified into one of the four major ethnic groups: Caucasian, Asian, African or Oceanian (subjects with mixed ethnic background were noted). The experimental procedure was approved by the Ethics Committee of the Australian Institute of Sport in accordance with the Helsinki Declaration.

Suitability for inclusion in the study was based on three criteria (age, performance level and drug history). Athletes included in the study were required to be at least 14 years of age at the commencement of the study, and had to have competed at state/regional level (or higher) during the previous 12 months. Athletes were excluded from the study if they reported that they had received a blood transfusion during the previous month, or if they admitted taking growth hormone, insulin-like growth factor, erythropoietin or any substance known to enhance red blood cell formation during the previous two months. All information was gathered by questionnaire but there was no expedient means to verify whether athletes answered truthfully.

Study design

Three venous blood samples were collected from each athlete over an extended period (day 1, days 3-9 and days 10-21). The day and time of blood collection were recorded in each case, together with the time elapsed since the athlete had last exercised. At the end of the study, athletes completed the final section of a case report form which detailed any injury, illness, variations in medication, alterations in living altitude or other relevant changes that had occurred during the study period. In 32 cases (0.01%) the day of collection was not recorded, and in a total of 66 cases (0.02%) the sample was collected outside the specified day range, though seldom by more than two days. In some cases, only one (n=140, 0.04%) or two (n=162, 0.05%) blood samples were collected from an athlete due to logistical reasons. In 100 cases (0.03%) whole blood samples were analyzed but not serum measures, whilst in 40 cases (0.01%) serum assays were performed but whole blood analyses were

Table 1. Ethnicity and sport group of international athletes.

Ethnicity	*Country of residence during study*	*Male (n)*	*Female (n)*
Caucasian	Australia, France, Italy, Mexico, New Zealand, Norway, South Africa, USA	378	242
Asian	China, Malaysia, Singapore	221	126
African	Kenya, South Africa	87	27
Oceanian	Australia, New Zealand	19	7
Other		34	11
Totals		739	413

Group	*Sporting Category*	*Example*	*Male (n)*	*Female (n)*
1	Esthetic sports	Gymnastics, Synchronized swimming, Diving	11	19
2	Athletics	Track and Field	65	27
3	Combat Sports	Boxing, Wrestling, Judo, Karate, Fencing, Taekwondo	149	58
4	Endurance Sports	Marathon running; Cycling, Orienteering; Triathlon, Nordic Ski	84	41
5	Multiple	Modern pentathlon, Decathlon, Heptathlon, Biathlon	6	1
6	Power Sports	Weightlifting, Athletic field events such as hammer, discus	36	13
7	Power/ Endurance	Rowing, Canoeing, Swimming, Track cycling	133	84
8	Racquet Sports	Tennis, Badminton, Squash	33	20
9	Skill Sports	Table tennis, Shooting, Archery	16	14
10	Team Ball Games	Basketball, Netball, Volleyball, Hockey, Soccer	201	136
11	Not stated		5	0

Athletes self-identified to which ethnic group they belonged. Caucasian includes European, North African, South West Asian, Arabian, Persian and Indians; Asian includes Chinese, Korean, Japanese, Siberian, American Indian and Eskimos; African includes both Central and South Africans; Oceanian refers to Australian Aboriginals, Melanesians and Polynesians; Other includes those who did not state their ethnic background or athletes of mixed ethnic background.

incomplete, so that different numbers of observations were available for different variables.

Blood collection and analysis

All blood was collected by trained phlebotomists and was sampled from an antecubital vein after five minutes of supine rest. To minimize inter-group variation, the collection procedures at all sites were standardized (a detailed protocol for collection and processing of the blood and serum was sent to each institution participating in the study). Samples were drawn into one 8 mL serum separation tube with clot activator (Vacuette, Greiner Labortechnik, Frikenhaussen Germany) and two 2 mL K3EDTA tubes (Vacuette, Greiner Labortechnik). All blood collection tubes, cryogenic vials and other items required for the study were purchased in Australia and transported to each location (with the exception of Norway and Italy).

Erythrocyte and reticulocyte parameters were analyzed using the ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Tarrytown, NY, USA) which performs flow cytometric measurements. When possible, analysis was completed within 8 hr of collection. A total of 13 ADVIAs located in 11 countries (two each in France and Italy) were used during the study. Each ADVIA was calibrated against appropriate reference materials, and controlled daily using Bayer ADVIA *TEST*point Haematology Low, Normal and High controls and Bayer ADVIA *TEST*point Reticulocyte Low and High controls. The average coefficient of variation (CV) for the parameters used in the models (or those used to derive the parameters) analyzed on all the ADVIAs were as follows: Hct 2.0%; percent reticulocytes (%Retic) 11%; mean cell volume of reticulocytes (MCVr) 1.6%; red blood cell count (RBC) 1.6%; mean cell volume (MCV) 1.3%; hemoglobin concentration (Hb) 1.4%. The relatively high CV for reticulocytes (the range for all laboratories was 3.6-17.5%) was due to high values recorded in two laboratories that had no previous experience with reticulocyte analysis prior to our study. All serum samples were separated and aliquoted into cryotubes as previously described,[2] stored at –20°C or –80°C, packed on dry-ice, then shipped to Australia for analysis. The EPO and sTfr concentrations were determined using an automated solid-phase chemiluminescent immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) and an automated immunonephelometric assay (Dade Behring, Germany), respectively. The automated immunoassays for EPO and sTfr were controlled using three and two levels of controls, respectively. 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 <1.0% and 2.2%, and between-assay CVs were 3.4 and 2.8%, respectively. Ferritin concentration was measured on the first serum sample collected from each athlete using a single Hitachi 911 Biochemistry Analyzer (Roche Diagnostics, Rotkreuz, Switzerland). The mean CV for ferritin assays throughout the study was 5.5% over the range of 15-500 ng/mL.

A blood sample from each subject was screened for hemoglobinopathies using a Bio-Rad Variant Analyzer (Bio-Rad, Hercules, California, USA) and a Variant Beta-Thalassemia Short Program Kit (Bio-Rad, Hercules, California, USA). This enables detec-

tion and quantification of hemoglobins A, A_2, F, S and E. For hemoglobinopathy screening, the between assay CV for HbA_2 and HbF quantification was established using two control levels for each. For HbA_2 levels 2.6% and 5.3% the CV was 6.9% and 4.9%, respectively; for HbF levels 1.8% and 9.1% the CV was 10.9% and 2.9%, respectively.

For reference, the models that will be used are those previously published.[2]

ON-model score = $3.721Hct + 30.45RetHct + 0.1871\log_e(EPO) + 0.1269\log_e(sTfr) + 0.1115\log_e(\%macrocytes + 0.1)$

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

Statistical analysis

Estimating reference ranges for hematologic parameters and model scores. For each of the hematological parameters and model scores, means, standard deviations and 95% reference ranges (defined herein as the range within which we could be 95% certain that the value for a randomly chosen athlete would fall, taking into account various random and fixed effects) were estimated using the mixed modeling procedure (Proc Mixed) in the Statistical Analysis System (SAS Version 8.1, SAS Institute, Cary, NC, USA). The fixed effects were ethnicity, sport, altitude, age, visit, time since last exercise, and time of day of the visit. Levels of some of these effects were chosen in an iterative process of analysis to identify those for which there was a substantial difference and for which there was a reasonable number of athletes on each level. The levels for the first three fixed effects are shown in the tables, whilst data for the remaining fixed effects have been included in a comprehensive online appendix.

While a small number of athletes in other locations recorded their residence during the study as being at moderate altitude, the majority resided in Kenya, Mexico, South Africa and the USA, and the effect of altitude was evaluated as a comparison between these four nations (altitude) and all of the others (non-altitude). For sport the only comparison we considered was that between endurance (groups 4 and 7 in Table 1) and non-endurance.

The first visit of the sub-group of athletes with the following characteristics was nominated as the reference group: Caucasian, non-endurance athletes of age 19-24 years, who resided less than 610 m above sea level, and whose blood sample was collected between 07:30AM and 4:00PM at least 12 hours after exercise. The levels chosen for the reference group were those with the largest number of athletes. To avoid possible confusion with reference ranges, this group is subsequently referred to as the *modal* group. Findings for other levels of the fixed effects are expressed as departures from this modal group. The use of a different instrument to analyze the blood of athletes in each country and the analysis of three blood samples from each athlete permitted estimation of between- and within-instrument variation as well as between- and within-athlete variation. Although beyond the scope of the current paper, a comprehensive account of the statistical procedures used and the results obtained are included in the online appendix.

Transformations. The reference ranges are based on the assumption that the different values of the parameter for different athletes are normally distributed. The distributions of the raw values of several parameters were highly skewed, so these parameters were transformed appropriately. Square-root transformations were used for RetHct, reticulocyte count and %Retics, while log transformations were used for EPO, and sTfr. The transformation of macrocyte counts was log(count + 0.1), to accommodate observations with zero counts.

Changes in model scores over time. Changes in the ON- and OFF-model scores were comprehensively analyzed for the subgroup of 995 athletes (366 females and 629 males) who had at least two visits (see online appendix for details of the statistical approach and results). The data reported here are mean and standard deviations, as well as 95% reference ranges, for changes in the modal group on their first two laboratory visits within 2 hours of the same time of day, with an unknown change in time since exercise, and with 7-8 days between visits.

Results

Subject demographics

Athletes have been categorized into groups of similar training/motor skill basis (Table 1), and these athletes trained on average for 3.3 hours per day. For statistical purposes, *endurance* was a combination of *endurance sport* and *power/endurance* categories (217 males, 125 females in total). Two hundred and forty of the athletes who trained/resided at moderate altitude (~1730–2220 m) were permanent residents, whilst 48 resided temporarily for the duration of the study. Around 6.7% of the athletes were current smokers (55 males, 22 females) whilst an additional 2.7% (26 males, 5 females) had smoked at some time previously. Approximately 18% of the athletes stated that they had taken oral creatine supplementation or some form of iron supplementation either during or in the four weeks prior to the study.

Table 2. 95% reference ranges, means and standard deviations for the five component hematologic and serum parameters, as well as ON- and OFF-model scores, for the modal group (Caucasian non-endurance athletes aged 19-24 years, measured <610 m above sea level on their first laboratory visit between 07:30 and 16:00 at least 12 h after exercise). Reference ranges for other groups are shown only when they are substantially different from those of the modal group.

Females	*Hematocrit (%)*	*Reticulocyte hematocrit (%)*	*Macrocytes[a] (%)*	*EPO[b] (mU/L)*	*sTfr[b] (mg/L)*	*ON-model score*	*OFF-model score*
Range - Modal group	34.3–45.0	0.30–1.08	0.0–1.9	4.4–23.5	0.76–2.02	1.63–2.42	1.01–2.01
Mean (SD)	39.6 (2.6)	0.79 (0.12)	0.29 (*2.24)	10.1 (*1.53)	1.24 (*1.28)	2.03 (0.19)	1.51 (0.25)
Ethnicity							
Asian					0.83–2.21		
African	33.4–44.5	0.34–1.20			0.93–2.53		0.85–1.88
Oceanian			0.0–1.1	3.6–21.0	0.65–1.84	1.47–2.30	
Sport							
Endurance	35.3–46.1		0.0–2.5			1.68–2.47	1.08–2.08
Altitude							
1730 to 2220 m	35.9–46.8	0.36–1.20	0.0–1.1		0.80–2.14		

Males	*Hematocrit (%)*	*Reticulocyte hematocrit (%)*	*Macrocytes (%)*	*EPO (mU/ml)*	*sTfr (mg/L)*	*ON-model score*	*OFF-model score*
Range - Modal group	38.8–49.6	0.31–1.12	0.0–1.1	4.6–18.6	0.81–1.87	1.78–2.51	1.24–2.24
Mean (SD)	44.2 (2.7)	0.81 (0.12)	0.21 (*1.97)	9.2 (*1.43)	1.23 (*1.24)	2.14 (0.18)	1.74 (0.25)
Ethnicity							
Asian	39.4–50.3				0.85–1.95	1.83–2.56	
African					0.87–2.03		
Oceanian		0.35–1.21			0.89–2.09		
Sport							
Endurance			0.0–1.4	5.1–20.8	0.84–1.94	1.84–2.56	
Altitude							
1730 to 2220 m	41.0–52.0	0.42–1.34	0.0–0.8	4.9–20.0	0.89–2.05	1.91–2.65	

*[a]Mean macrocyte percent shown is the back-transformed mean of the log-transformed value. [b]Data shown are for the square root of the value provided by the instrument. *Standard deviations are multiplicative factors that are applied to this mean (see online appendix for explanatory details).*

Many of the blood parameter values differed substantially between males and females, consequently reference ranges were estimated separately for each sex. For brevity, only data for various groups and effects that were substantially different (greater than 0.2 standard deviations)[17] from those of the modal group have been highlighted and discussed.

Interpretation of reference ranges

The reference ranges in Table 2 for groups other than the modal group are appropriate for individuals who differ only by a single category from the modal group, for example if they are a different ethnic group, or perform a different sport, or reside at altitude. For an athlete exposed to multiple effects, the appropriate reference range can be estimated using the approach illustrated in the online appendix.

Reference ranges for hematologic parameters

The span of the 95% reference range for Hct was of similar magnitude for females and males, whilst the mean Hct value for males exceeded the mean female value by 4.5 points. The largest effect on Hct values, consistent across gender, was due to altitude; residing at a moderate altitude of ~1730-2220 m was associated with an average increase of 1.8-2.3 Hct points.

Serum EPO levels were comparable between females and males (Table 2). The highest value recorded in a female was 83.3 mU/mL from an iron-deficient athlete (serum ferritin 4.4 ng/mL, Hct 0.30). The two highest serum EPO values recorded in males were 81.9 and 75.6 mU/mL.

The effect of ethnicity on sTfr levels was modest with appreciable differences between all four ethnic groups and both sexes (Table 2). Although the span of the 95% reference range was similar in all groups, the upper range for African and Asian athletes was higher than in Caucasians (for both males and females). The small sample size of our Oceanian athlete group makes extrapolation from our data tenuous for this group. Although not included in the

detection models, ferritin concentration was measured to confirm iron deficiency anemia in cases in which Hct and/or Hb were low. Nineteen female and 13 male athletes had serum ferritin values under 10 and 20 ng/mL, respectively. The highest ferritin values were 694 and 1154 ng/mL; the lower score was for a male athlete who had a previously diagnosed disorder of hereditary spherocytosis. We also collected and have reported further hematologic parameters (hemoglobin concentration, red cell count, mean cell volume), as well as reticulocyte characteristics (absolute count and percentages, mean cell volume of reticulocytes) and readers interested in these data are directed to the online appendix.

Reference ranges for the ON-model

The width of the 95% reference ranges for both males and females was comparable, whilst mean scores were higher for males than females (Table 2). The effects of ethnicity, age, sport, time since exercise or time of day were neither marked nor consistent between sexes (data reported in online appendix). The largest single effect was due to altitude, which increased ON-model scores in males by an average of 0.14. The influence of this effect was not evident in female athletes. Both male and female athletes who were categorized as endurance athletes demonstrated ON-model scores that were 0.05-0.06 higher than those of non-endurance athletes. The 95% reference range for males in the modal group was 1.78-2.51. There were nine males who recorded an ON-model score greater than 2.80, and a further three who recorded ON-model scores of 3.00 or more (3.01, 3.06 and 3.32). The (Central/Southern African) athlete who had the highest score presented with low leukocyte (2.2×10^9/L) and platelet (100×10^9/L) counts, suggesting the presence of a significant abnormality. Unfortunately our attempts to follow-up this result failed due to our inability to locate the athlete. By comparison, all 26 male recreational athletes injected with rHuEPO in trials held in Sydney and Beijing reached ON-model scores of greater than 2.88 by the end of the 4-week period of rHuEPO administration, and 72% exceeded a score of 3.00 at some time during the four weeks.[2] For females in the modal group the 95% reference range was 1.63-2.42, with a maximum score of 2.76 recorded in one athlete. By comparison, in the Sydney and Beijing rHuEPO administration trials, all 15 females injected with rHuEPO attained an ON-model score of more than 2.54 by the end of the 4-week period of rHuEPO administration, and 80% exceeded a score of 2.76 at some time during the four weeks.[2]

Reference ranges for the OFF-model

The width of the 95% reference ranges for both males and females was comparable, with mean scores higher for males than females. The influence of effects, including ethnicity, age, sport, altitude, time of day or time since exercise, was not consistent across sexes. The largest effect was due to ethnicity, with female African athletes demonstrating OFF-scores on average 0.14 lower than the modal group. This effect was not evident in male African athletes. An effect attributable to endurance sport was evident in female athletes (scores 0.07 higher) but not their male counterparts.

For males in the modal group the 95% reference range was 1.24-2.24, with a maximum score of 2.55 recorded in one athlete. In the Sydney and Beijing administration trials, 92% of the males who were injected with rHuEPO reached OFF-model scores greater than 2.24 at some stage after their last injection. The 95% reference range for females in the modal group was 1.01-2.01, with a maximum score of 2.12 recorded in one athlete. Every female injected with rHuEPO during the Sydney and Beijing trials exceeded an OFF-model score of 2.01 at some stage after rHuEPO administration ceased.

Changes in the ON- and OFF-model scores over time

The mean (±SD) changes in ON-model scores over a 7-8 day period in the modal group were 0.00±0.12 and 0.02±0.14 for males and females, respectively. The largest change in ON-model scores over this time period was –0.43 (95% reference range –0.25 to 0.24) for males and –0.52 (95% reference range –0.26 to 0.29) for females (the negative sign indicates that the change was a decrease over time).

The mean (±SD) changes in OFF-model scores over a 7-8 day period in the modal group were –0.10± 0.18 and –0.05±0.22 for males and females, respectively. The largest change in OFF-model score was 1.21 (95% reference range –0.45 to 0.26) for males and –0.93 (95% reference range –0.48 to 0.39) for females.

Incidence of hematologic abnormalities

Only one of the 21 male athletes with demonstrable hematologic abnormalities had an ON-model score that was higher than the upper 95% reference limit of 2.51. This athlete had a known abnormality (hereditary spherocytosis) and gave a score of 3.01. ON-model scores for all 19 of the female athletes with demonstrable haematological abnormalities fell within the 95% reference range. None of these 40 athletes produced OFF-model scores greater than the upper limit of the 95% reference

range, whilst 11 athletes had OFF-model scores that were below the lower limit. The effects of hematologic abnormalities on the component variables and model scores will be reported in detail in a separate manuscript.

Discussion

The major findings of this study were that in a large cohort of international elite athletes, we identified a small number of athletes with ON-model scores that were considerably in excess of the expected range. This implies that if an athlete is found to possess a high ON-model score, further evidence (such as the presence of rHuEPO in the urine) is required to establish whether or not the athlete used rHuEPO. However the observed distribution of OFF- and CHANGE-model scores suggests that these models may, by themselves, be extremely useful tools for identifying athletes who have recently ceased using rHuEPO.

Fluctuations in hematologic parameters associated with extraneous factors

With the goal of providing a framework within which to judge the merit of using our indirect blood tests to detect rHuEPO use, we estimated the variation in our component parameters caused by circumstances potentially encountered by elite athletes. Such factors may need to be considered and included when setting threshold/decision limits for individual athletes. The main factors considered in the analysis of our data were ethnicity, whether the athlete participated in an endurance sport, and residence at altitude.

Residence at a moderate altitude (~1730–2220 m) was by far the most consistent effect, with the largest influence on blood parameters. There was an increase in Hct, RetHct, EPO and sTfr in the altitude sub-group (males and females). Numerous publications have described an increase in red blood cell count (RBC), mean cell volume (MCV), Hct, Hb and %Retic in people living at altitude compared with those at sea level: among the most comprehensive was the classic study of Hurtado *et al*.[18] The greater change in Hb (9.1%, *data reported in the online appendix*) than Hct (3.9%) is consistent with dehydration at altitude compared with sea level, and an acute decrease in plasma volume of ~7-10% is a well recognized response of lowlanders traversing to ~2000m.[19] Our Hct versus Hb data suggest that this acute effect might also be a chronic effect for altitude residents. When the plasma volume shift was estimated from changes in the Hct and Hb,[20] plasma volume was reduced for the altitude sub-group by 8.0% in males and 8.8% in females. Therefore, the confounding effect of plasma volume shifts needs to be taken into account for blood testing conducted at altitude, or after a training block conducted at altitude. The time course for parameters to *normalize* following exposure to altitudes of ~2000m therefore needs to be addressed for the detection models. Our group has investigated groups of athletes following acute and chronic exposure to various altitudes, and these data will be presented in a separate manuscript.

In our sample cohort, the effect of endurance on Hct was negligible for males but was 1.1 Hct points higher for females. Further, Hb was higher for both males and females than for their non-endurance counterparts (*complete data set reported in online appendix*). The difference noted in our study associated with endurance athletes was of a comparable magnitude, but opposite, to that found by Schumacher *et al*.,[21] who reported that in a cohort of 426 endurance and 321 mixed and power male athletes, endurance athletes tended to have Hb 5 g/L-1 lower than their non-endurance counterparts. Schumacher *et al*. noted that their protocol was unable to discern the acute effects of exercise (since their samples were taken after two days of reduced sporting activity). However, their finding that endurance training does not substantially influence Hct/Hb compared with that in sedentary subjects, together with our data showing no further influence whether or not samples were drawn within four hours of exercise, suggests that any hemodilution associated with endurance exercise is unlikely to affect Hct/Hb markedly.

When the concomitant influence of altitude was removed, male and female African athletes in our cohort possessed marginally lower Hb than their Caucasian counterparts. This is consistent with previous research that has reported that *Blacks* possess Hb levels ~5 g/L lower than *Whites*.[22] This effect was discernible in lower Hct scores for the female athletes but not males, which underlined the relatively minor magnitude of this specific influence.

Ethnicity was also found to consistently influence sTfr levels. Both male and female athletes in the Asian and African groups possessed higher sTfr values than their Caucasian counterparts. Allen *et al*. reported previously that in a cohort of 225 healthy adults, subjects they classified as *Blacks* possessed sTfr levels ~9% higher than subjects classified as either *Caucasian*, *Asian* or *Hispanic*.[23] In contrast to our study, they reported no statistical difference between sTfr levels in Asians and Caucasians. It was difficult to extrapolate from the increased (males) and decreased (females) sTfr levels encountered in

our Oceanian cohort due to the limited sample size. All serum measurements were performed in the same laboratory, and it is difficult to find an explanation for observed differences in sTfr levels between ethnic groups.

The potential for hematologic abnormalities to affect blood parameters and therefore confound the detection models was also considered. Although there were 40 athletes in which hematologic abnormalities could be demonstrated, only one subject (male) with hereditary spherocytosis had blood parameters sufficiently disturbed to result in an ON-model score (3.01) far above the relevant upper 95% reference limit. None of the 40 athletes registered OFF-model scores outside the 95% reference limit. Nevertheless, we recommend that an athlete with a model score outside the reference range should be investigated for the presence of a clinical/medical abnormality. We conclude that the overall robustness of our hematologic parameters supports their utility as potential tools to identify athletes abusing rHuEPO.

Detection of rHuEPO users

Various approaches have been considered to identify athletes abusing rHuEPO, the simplest of these using a single blood parameter such as Hct or %Retic. Given the proximity of the upper limit of our Hct and %Retic reference ranges to the cut-off thresholds, we estimate about 1 in 40 male athletes (or 1 in 10 if residing at ~2000 m) risk failing the *50% Hct rule*, and about 1 in 100 male athletes residing at moderate altitude risk failing the 2.4% reticulocyte threshold. For females the chance of exceeding the 47% Hct cut-off is less, but still around 1 in 40 for female athletes who live/train at moderate altitude. Additional detail is provided in Table 3.

An alternative approach is to use scores obtained from a combination of blood parameters, with cut-offs chosen to achieve an acceptable balance between sensitivity and specificity. This approach has the advantage of requiring that there be a consistent profile of altered erythropoeisis before an athlete is suspected of drug use, rather than relying on an unusual value for a single parameter. The ability of the multiple-parameter algorithms to predict rHuEPO use in athletes is illustrated by a simple comparison between the upper limits of the reference ranges obtained in our current study, with the scores elicted by subjects injected with rHuEPO in our previous research.[2] The maximum upper limit of the reference range for ON-model scores (for males, living/training < 610 m) was calculated to be 2.62 (the peak score associated with Asian, endurance athletes). In our previous administration trials conducted in Sydney and Beijing, all of the males given rHuEPO injections recorded ON-model scores in excess of 2.88 towards the end of the 25-day administration period. The corresponding maximum upper reference range limit for the ON-model in female athletes is 2.47. All of the females injected with rHuEPO in our Sydney and Beijing trials recorded scores in excess of 2.54, and 14 of the 15 recorded scores in excess of 2.65.

Table 3. Percentage of 1,152 athletes (739 males, 413 females) from 12 countries in the profiling study with blood parameters equal to or greater than the specified value. Samples were collected on the first visit to the laboratory and measured on an ADVIA analyzer.

	Females %	*Males* %
Hematocrit (%)		
44.0	15	73
47.0	1.2	32
50.0	0.0	5.4
53.0	0.0	0.3
Hemoglobin (g/L^{-1})		
155	5.1	48
165	0.7	16
175	0.0	2.9
185	0.0	0.4
Reticulocyte percentage (%)		
2.0	10	9.4
2.4	1.7	3.4
2.8	0.7	1.4

Whilst this discrimination confirms the usefulness of the model for predicting rHuEPO use in athletes, our current study showed that some hematologic conditions, such as hereditary spherocytosis, can also lead to elevated ON-model scores. Further, there were a number of outliers within the elite athlete population with ON-model scores well in excess of the 95% reference range. Since the athletes declared that they were not taking rHuEPO and they could not be excluded from the study on medical grounds, the concomitant presence of outliers and the possibility of hematologic abnormalities makes using a single ON-model score by itself tenuous. Authorities may elect to use a higher reference range to achieve 100% specificity based on our current results, but only at the cost of an almost complete loss of sensitivity in the ON-model.

For OFF-model scores the maximum upper limit of the 95% reference ranges was calculated to be 2.24 for males and 2.08 for females (Table 2). In the Sydney and Beijing administration trials, 21 of the 26

males injected with rHuEPO recorded OFF-model scores in excess of 2.50, whilst 14 of the 15 females recorded scores in excess of 2.10. Consistent with our discovery of athletes with unusual ON-model scores, there were also a number of outliers within our elite athlete sample who demonstrated unusual OFF-model scores. However, whereas the unusual ON-model scores depicted accelerated erythropoiesis and therefore could have been confused with an individual using rHuEPO, the unusual OFF-model scores were *lower* than the 95% reference range and therefore would not have been confused with an athlete who had recently ceased using rHuEPO.

Furthermore, none of the hematologic abnormalities led to elevated OFF-model scores. The absence of outliers with very high OFF-model scores suggests that the OFF-model, by itself, is an extremely useful tool to identify athletes who have recently ceased using rHuEPO. It is pertinent to note that using an appropriate cut-off score based on population data, we have never observed a false positive result using the OFF-model. Hence this approach provides a level of assurance not evident with single parameter models. We believe that the benefits of the OFF-model include its robustness in identifying athletes who should be followed-up as part of a targeted testing program to detect rHuEPO users.

Change in model scores over time

When we examined the stability of CHANGE scores in the multiple blood samples we collected over the three-week period, ON-model scores appeared to be more stable than OFF-model scores. The average change in the ON-model was close to zero whereas OFF-model scores showed a minor decrease over the three visits; these changes reflect the relative contributions of Hct and RetHct to the two model scores [*see the online appendix (http://www.haematologica.org/2002_12/1248.htm #appendix for a comprehensive evaluation of this trend*]. However the stability of the model scores (particularly ON-model) for an individual over time has sufficient potential for highlighting rHuEPO use to warrant further research. Specifically, when applied in unison with the iso-electric focusing technique for detecting rHuEPO in urine, this could leverage the efficacy of *out-of-competition* testing for rHuEPO where both blood and urine samples are collected. We reached this conclusion because several studies have shown that athletes who stop taking rHuEPO more than four weeks prior to competition lose any competitive advantage,[2,24] which implies that athletes must continue to use rHuEPO in the weeks immediately before competition. Athletes taking rHuEPO who are tested in this time window will tend to have a relatively high ON-model score, but may not fail the urine test if their previous injection was more than 3 days earlier. If the athlete continued taking rHuEPO up until the time of competition it would heighten the likelihood of a subsequent urine sample revealing the presence of rHuEPO.

Alternatively, if the athlete elected to minimize the risk of failing a second doping control test by discontinuing rHuEPO use, our previous data suggests there will be a significant drop in ON-model score for the second blood sample.[2] This would produce a large change in ON-model score, and if the magnitude of the CHANGE score was suspicious it could perhaps offer antidoping authorities justification to pursue subsequent monitoring of the athlete. Further, it should be noted that the ON- and OFF-models considered here were developed to discriminate between rHuEPO users and placebo subjects, but (as yet unrealised) alternate models may be more appropriate for detecting intra-athlete changes in erythropoiesis.

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KS, WH, KRE, CH, GJT, RK, MJA, CJG, RP, AGH participated in the conception/design, data collection, analysis and interpretation and drafting of the manuscript, and read and approved the final version of the manuscript. KS is responsible for Tables 1 and 3; WH for Table 2. The authors wish to recognise the outstanding contributions of the National Olympic Committees, National Sports Institutes, National Sporting organizations and numerous pathology laboratories from the following countries: Australia, China, France, Hong Kong, Italy, Kenya, Malaysia, Mexico, New Zealand, Norway, Singapore, South Africa and the USA. Arturo Terres, Carpermor Laboratories, Mexico City, merits a special thank you. We are grateful to Dr. Peter Davis, John Boultbee, Dr. David Pyne, Dr. David Martin, John Williams, Dr. Ross Smith, Robyn Power, and the staff of AIS Travel, for management, scientific and administrative support, as well as to Graeme Allbon, Christopher Alma, Zez Bobosevic, Julianne Brettargh, Helen Bull, Zophia Czarnota, John de Vrey, Gaenor Edwards, Janelle Grainger, Ann Gould, Claire-Marie Guillemot, Nicole Horvath, Maggie Lavercombe, Jenny Lenihan, Ting Lovie, Dorothy Manocha, Kylie Nelson, Annie O'Neill, Simone Ransley, Donna Ridley, Susan Soo, Katina Urlich and Sally Wright for technical and paramedical support. We wish to thank Dade Behring for donating the nephelometer and the consumables for the sTfr measurements, and Bayer Diagnostics for their technical and engineering backing of the ADVIA

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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References

1. Lasne F, de Ceaurriz J. Recombinant erythropoietin in urine. Nature 2000; 405:635.
2. Parisotto R, Wu M, Ashenden MJ, Emslie KR, Gore CJ, Howe C, et al. Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. Haematologica 2001; 86:128-37.
3. McKenzie S. Textbook of Hematology. 2nd edition. Baltimore: Williams & Wilkins; 1996.
4. Rifkind R, Bank A, Marks P, Nossel H, Ellison R, Lindenbaum J. Fundamentals of Hematology. 2nd edition. Chicago: Year Book Medical Publishers, Inc.; 1980.
5. Kerle KK, Nishimura KD. Exertional and sudden death associated with sickle cell trait. Mil Med 1996; 161:766-7.
6. Le Gallais D, Prefaut C, Mercier J, Bile A, Bogui P, Lonsdorfer J. Sickle cell trait as a limiting factor for high-level performance in a semi-marathon. Int J Sports Med 1994; 15: 399-402.
7. Schmidt W, Biermann B, Winchenbach P, Lison S, Boning D. How valid is the determination of hematocrit values to detect blood manipulations? Int J Sports Med 2000; 21: 133-8.
8. Reynafarje R, Lozano R, Valdivieso J. The polycythemia of high altitudes: iron metabolism and related aspects. Blood 1959; 14:433-55.
9. Jones A R, Twedt D, Swaim W, Gottfried E. Diurnal change of blood count analytes in normal subjects. Am J Clin Pathol 1996; 106:723-7.
10. Pocock SJ, Ashby D, Shaper AG, Walker M, Broughton PM. Diurnal variations in serum biochemical and haematological measurements. J Clin Pathol 1989; 42:172-9.
11. Casoni I, Ricci G, Ballarin E, Borsetto C, Grazzi G, Guglielmini C, et al. Hematological indices of erythropoietin administration in athletes. Int J Sports Med 1993; 14:307-11.
12. Birkeland K, Donike M, Ljungqvist A, Fagerhol M, Jensen J, Hemmersbach P, et al. Blood sampling in doping control. First experiences from regular testing in athletics. Int J Sports Med 1997; 18:8-12.
13. Vergouwen P, Collee T, Marx J. Haematocrit in elite athletes. Int J Sports Med 1999; 20:538-41.
14. Parisotto R, Gore C, Hahn A, Ashenden M, Olds T, Martin D, et al. Reticulocyte parameters as potential discriminators of recombinant human erythropoietin abuse in elite athletes. Int J Sports Med 2000; 21:471-9.
15. Vergouwen P, Collee T, Marx J. Hemoglobin and packed cell volume in endurance athletes prior to rhEPO. Int J Sports Med 2000; 21:228.
16. Satterthwaite FW. An approximate distribution of estimates of variance components. Biometrics Bulletin 1946; 2:110-4.
17. Cohen J. Statistical power analysis for the behavioral sciences, Lawrence Erlbaum, editor. 2nd edition.New Jersey. 1988.
18. Hurtado A, Merino C, Delgado E. Influence of anoxemia on the hemopoietic activity. Arch Intern Med 1945; 75:284-323.
19. Sawka MN, Convertino VA, Eichner ER, Schnieder SM, Young AJ. Blood volume: importance and adaptations to exercise training, environmental stresses, and trauma/sickness. Med Sci Sports Exerc 2000; 32:332-48.
20. Dill DB, Costill DL. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. J Appl Physiol 1974; 37:247-8.
21. Schumacher YO, Schmid A, Grathwöhl D, Bultermann D, Berg A. Hematological indices and iron status in athletes of various sports and performances. Med Sci Sports Exer 2002; 34:869-75.
22. Perry GS, Byers T, Yip R, Margen S. Iron nutrition does not account for the hemoglobin differences between blacks and whites. J Nutr 1992; 122:1417-24.
23. Allen J, Backstrom KR, Cooper JA, Cooper MC, Detwiler TC, Essex DW, et al. Measurement of soluble transferrin receptor in serum of healthy adults. Clin Chem 1998; 44:35-9.
24. Ekblom B, Berglund B. Effect of erythropoietin administration on maximal aerobic power. Scand J Med Sci Sports 1991; 1:88-93.
25. Cazzola M. A global strategy for prevention and detection of blood doping with erythropoietin and related drugs. Haematologica 2000; 85:561-3.

Peer Review Outcomes

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What is already known on this topic

A blood-based method to detect surreptitious use of recombinant human erythropoietin (r-HuEPO) was approved and implemented for the Sydney 2000 Olympic games. It is based on hematologic and biochemical parameters that, in combination, allow recent abuse of r-HuEPO to be detected.

What this study adds

To assess cases of potential abuse of r-HuEPO in competitive athletes, it is essential to rely on robust reference ranges for the hematologic and biochemical parameters. In addition, the effect of altitude training needs to be taken into account. This report provides reference ranges for several hematologic and biochemical parameters that are used to assess r-HuEPO abuse. It provides additional validation of the approach used for the Sydney Olympic games

Potential implications for clinical practice

This study reinforces the notion that a combination of hematologic and biochemical parameters is effective in identifying recent or past abuse of r-HuEPO in competitive athletes.

Carlo Brugnara, Deputy Editor
(Boston, USA)