

## The effect of common hematologic abnormalities on the ability of blood models to detect erythropoietin abuse by athletes

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**Background and Objectives.** Algorithms that combine scores from multiple blood parameters are demonstrably effective in highlighting recombinant human erythropoietin (rHuEPO) administration, and have been used to deter rHuEPO use by athletes. These models are sensitive to atypical levels of blood parameters encountered during altered states of red cell production. Because hematologic abnormalities can also result in unusual blood profiles, the aim of this study was to document the incidence and magnitude of such abnormalities in an elite athlete population.

**Design and Methods.** We screened blood samples obtained from 413 female and 739 male elite athletes from 12 countries for known hematologic abnormalities, and compared the algorithm scores for these athletes with those of their healthy counterparts. We also established the magnitude of blood parameters required for model scores to exceed cut-offs associated with rHuEPO use.

**Results.** We found that 0.7% of male and 2.4% of female athletes were iron deficient either with our without anemia. An additional 1.4% of males and 1.0% of females had hemoglobinopathies. On average these athletes' model scores were at or below the score of their healthy counterparts. The greatest influence on our models was hemoglobin concentration. Values of other parameters must exceed normal ranges by a substantial margin in order for model scores to approach levels associated with rHuEPO use.

**Interpretation and Conclusions.** The hematologic disorders we encountered in elite athletes were not associated with model scores that exceeded the nominal cut-offs that we have previously recommended to delineate rHuEPO use. We did not find any abnormalities among elite endurance athletes that were associated with high model scores.

**Key words:** rHuEPO, athletes, blood tests, doping, hematologic abnormality.

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The advent of recombinant human erythropoietin (rHuEPO) was a spectacular clinical success. However rHuEPO's capacity to stimulate red blood cell production in anemic patients was quickly recognized by athletes as an illicit means to boost their own red cell production, and thereby boost the oxygen-carrying capacity of their blood and improve endurance performance.<sup>1</sup> Although subsequently banned by the International Olympic Committee and other institutions, deterring the widespread abuse of rHuEPO in elite sport has been problematic.<sup>2</sup>

Efforts to identify rHuEPO use have focused on levels of hematologic markers which clinical research had shown increase with rHuEPO administration,<sup>3,4</sup> including reticulocyte counts and markers of iron metabolism.<sup>5-9</sup> Contemporary models sensitive to current (ON) or recent (OFF) rHuEPO administration incorporate only four parameters (hemoglobin, percent reticulocytes, serum erythropoietin and soluble transferrin receptor) but demonstrate superior sensitivity to the previous models.<sup>10</sup> Elevated ON- and OFF-model scores caused by rHuEPO treatment can be distinguished from the scores of elite athletes not receiving rHuEPO injections.<sup>10,11</sup>

Since our approach is sensitive to atypical combinations of blood parameters, it is conceivable that the hematologic disturbances associated with blood disorders may give rise to elevated model scores, which could be mistakenly interpreted as evidence that an individual had used rHuEPO. The impact that such diseases could have on the ability of our models to detect rHuEPO use in sport will depend on the prevalence of hematologic anomalies among the elite athlete population, and the resulting disturbance in model scores associated with these blood disorders. Although the majority of hematologic conditions can be diagnosed on the basis of predictable changes in blood parameters, the magnitude of these changes is highly variable between individuals, thus defying any attempt to automatically stipulate the model score associated with a particular ailment. Moreover, a substantial portion of hematologic abnormalities have physiologic sequelae that would prevent a subject from attaining the level of fitness required to be internationally competitive. The aims of this research were, therefore, to report the prevalence of hematologic abnormalities we encountered during a survey of 1152 elite international athletes, to document the associated model scores for these athletes with abnormalities, and to quantify the magnitude of our component variables that are required for our models to exceed nominal cut-off scores.

## Design and Methods

### Subjects

The study population comprised 1152 (739 males, 413 females) state/national level volunteer athletes from 12 countries who were recruited to establish reference ranges for key hematologic parameters used in antidoping research.<sup>12</sup> Among these, there was a subset of 34 athletes (16 male, 18 female) whose laboratory studies revealed a hematologic abnormality. This subgroup was designated as having a hematological abnormality that might potentially confound our blood models (athletes with hematologic abnormalities were not removed from the original cohort).

Suitability for inclusion in the athlete cohort 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 having 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.

For inclusion in the subset of athletes with hematologic abnormalities, a blood sample from each athlete 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 detection and quantification of hemoglobin A, A<sub>2</sub>, F, S and E.

Athletes were classified as iron-deficient with or without anemia using the following criteria:<sup>13</sup> females: ferritin  $\leq 10$  ng/mL; transferrin saturation  $\leq 15\%$ , and Hb  $\leq 12.0$  g/dL; males: ferritin  $\leq 30$  ng/mL; % transferrin saturation  $\leq 15\%$ , and Hb  $\leq 14.0$  g/dL.

### Sample analysis

All blood from athletes was collected by trained phlebotomists and was sampled from an antecubital vein after five minutes of supine rest. To minimize inter-group variation between samples taken at international sites, 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, FRIENHAUSEN Germany) and two 2 mL K<sub>3</sub>EDTA tubes (Vacuette, Greiner Labortechnik). The analytical and collection procedures have been previously referenced.<sup>12</sup>

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 were used during the study). Each ADVIA was calibrated against appropriate reference materials, and controlled daily using *Bayer ADVIA TESTpoint* Hematology Low, Normal and High controls and *Bayer ADVIA TESTpoint* Reticulocyte Low and High controls. The average coefficient of variation (CV) for the parameters used in the models were as follows: percent reticulocytes (%Retic) 11%; 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 sera from overseas were separated and aliquoted into cryotubes, stored at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ , packed on dry-ice, then shipped to Australia for analysis. The EPO and sTfr concentrations for all samples 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.

### Statistical analysis

The models we chose for analysis of sensitivity were as follows:

$$\begin{aligned} \text{ON-he} &= \text{Hb} + 9.74\ln(\text{EPO}); \\ \text{ON-hes} &= \text{Hb} + 6.62\ln(\text{EPO}) + 19.4\ln(\text{sTfr}); \\ \text{OFF-hr} &= \text{Hb} - 60\sqrt{(\text{Ret})}; \\ \text{OFF-hre} &= \text{Hb} - 50\sqrt{(\text{Ret})} - 7\ln(\text{EPO}). \end{aligned}$$

*Abbreviations (and units):* ln, natural logarithm; Hb, hemoglobin concentration (g/L); reticulocytes (%); EPO, erythropoietin concentration (mU/mL); sTfr, serum transferrin receptor (mg/L).

Throughout this paper we have utilized 95% reference ranges and mean scores for hematologic parameters and model scores we derived from a sample of 1152 elite athletes reported previously

(including those athletes with hematologic abnormalities). Specifically, the 95% reference ranges are appropriate for athletes participating in endurance sports, with measures taken at sea level.<sup>12</sup> We chose cut-off scores that were associated with a 1 in 1000 likelihood of a false-positive result.<sup>10</sup> We evaluated the strength of the relationship between model scores and Hb by squaring the Pearson product-moment correlation coefficient and multiplying by 100. The resulting value (variation explained, or  $R^2$ ) depicts the proportion of variation in the model score explained by Hb values.

## Results

### **Frequency of blood disorders in elite athletes**

Within our cohort of 739 male and 413 female athletes, we found 34 cases of a demonstrable blood disorder. These athletes practised the following disciplines: gymnastics 3, track and field 1, combat sports 14, power sports 1, power/endurance 2, team sports 12 and unknown 1 (these categories have been defined previously).<sup>12</sup> Therefore, although approximately 30% of our 1152 athletes came from sports we designated as *endurance*, only 4 of these endurance competitors (1.2%) were found to have a blood disorder.

Nine athletes (five males, four females including one endurance athlete) were identified with iron deficiency anemia. A further three males and seven females, including three endurance athletes, were characterized as being iron deficient (Table 1). This meant that 1.1% and 2.7% of the male and female sample populations (respectively) were iron deficient with/without anemia.

Hemoglobinopathies were diagnosed in 13 male and 4 female athletes, all of whom were of Central Asian or Central/Southern African origin (1.8% and 1.0% of the male and female sample populations, respectively). Another female of Central Asian origin presented with a Hb of 118 g/L, mean cell volume of 64.5 fL, serum ferritin of 89.8 ng/mL, 35.1% microcytes and 13.6% hypochromic erythrocytes, which are findings consistent with hemoglobinopathy rather than iron deficiency. Her HbA<sub>2</sub> level of 13.3%, perhaps consistent with Hb Lepore, was deemed too high (manufacturer's reference range 4-9%) to support a diagnosis of  $\beta$ -thalassemia, and too low for a diagnosis of heterozygous HbE diagnosis (30-35%) according to the Variant manufacturers.

In addition to these abnormalities, there were two noteworthy cases of athletes with atypical model scores. One male non-endurance athlete residing at altitude produced an *ON-hes* score of 204.3 which exceeded the appropriate 1 in 10 cut-off. This athlete of Central/Southern African background had leukocyte and platelet counts which

bordered on abnormal ( $2.2 \times 10^9/L$  and  $100 \times 10^9/L$  respectively) although his Hb value of 168 g/L did not exceed the 95% reference range for non-endurance athletes at altitude. Although there was evidence of substantially accelerated erythropoiesis (5.2% reticulocytes and sTfr of 2.76 mg/L) his serum EPO was normal (12.2 IU/L). A second male athlete of Asian background was found to have extreme *ON-he* and *OFF model* scores during at least one of his multiple visits to the laboratory (the highest scores recorded in our profiling study). This was initially attributed to hereditary spherocytosis in our previous publication. However subsequent review of his medical records discovered a clerical error and this subject had no hematologic anomaly that was discernible from our blood report (our efforts to implement a medical follow-up were unsuccessful). Therefore although this athlete did not satisfy the criteria for inclusion in the current study his values have been included due to their extreme nature.

### **Variation in ON model scores explained by Hb**

The variation in *ON-he* model score attributable to Hb was comparable for both males and females, whether the subject was an elite athlete with or without a hematologic abnormality, or for subjects treated with rHuEPO during an administration trial (Table 2). The remaining variation was attributable to the second parameter, EPO. For a male endurance athlete with a Hb value of 151 g/L (the mean for typical endurance athletes at sea level), EPO would have to be higher than 206 mU/mL to exceed the 1 in 1000 cut-off (the corresponding EPO was 33 mU/mL for an athlete with a Hb of 169 g/L, which is the upper limit of the 95% reference range for typical endurance athletes at sea level). For female athletes, the EPO would have to exceed 212 mU/mL if Hb was equal to the mean score of 137 g/L (or 34 mU/mL if the nominal Hb was equal to the upper limit of the 95% reference range, 155 g/L).

The variation in *ON-hes* score attributable to Hb was found to be of a similar magnitude to that in the *ON-he* model, and comparable among healthy elite athletes, elite athletes with hematologic anomalies and recreational athletes injected with rHuEPO (Table 2). Quantifying the parameter values required for the *ON-hes* score to exceed a nominal cut-off is complicated by the need to specify the values of EPO and sTfr in addition to Hb. Figure 1a illustrates the combination of EPO and sTfr values required for a male athlete with Hb values equal to either the mean (151 g/L) or the upper limit of the 95% reference range (169 g/L) to exceed the 1 in 1000 cut-off: an athlete would have to concomitantly have EPO and sTfr values above and to the right of the respective lines.

**Table 1. Hematologic parameters and model scores for elite athletes identified with haematological abnormalities. Athletes were among 1152 volunteers for an international profiling study and were subsequently classified as having a hematologic abnormality on the basis of laboratory screening for hemoglobinopathies and iron deficiency criteria. Y (yes) or N (no) denotes whether athletes resided at an altitude >1730m above sea level (Alt), or participated in endurance sport (End). \*Denotes that the athlete exceeded the 1 in 10 cut-off but not the 1 in 100 cut-off. #Denotes above 1 in 1000, ##denotes above 1 in 10000. Cut-offs for individual athletes were appropriate for gender, sport and altitude above sea level.**

Abnormality	Sex	Alt/End	Hb (g/L)	Retics (%)	EPO	sTfr (mU/mL)	ON- (mg/L)	ON-hes he	OFF-hr	OFF-hre
<b>Anemias</b>										
Iron def. anemia	F	Y/N	91	1.6	72.2	4.31	132.7	147.7	15.1	-2.2
Iron def. anemia	F		109	1.4	21.7	4.54	139	158.7	38	28.3
Iron def. anemia	M		122	1.4	75.6	4.13	164.1	178.1	51	32.6
Iron def. anemia	M		127	0.9	12.6	2.06	151.7	157.8	70.1	61.8
Iron def. anemia	M	Y/N	132	2.0	15.0	1.86	158.4	162.0	47.2	42.3
Iron def. anemia	M		137	1.4	10.7	1.13	160.1	155.1	66	61.2
Iron def. anemia	M	N/Y	137	1.6	11.3	1.97	160.6	166.2	61.1	56.
Iron def. anemia	F	Y/N	116	1.3	39	2.29	151.7	156.3	47.6	33.3
Iron def. anemia	F		116	0.9	25.3	1.69	147.5	147.6	59.1	46
Iron deficiency	F		122	1.5	25.2	1.43	153.4	150.3	48.5	38.2
Iron deficiency	F	N/Y	123	1.3	12.1	1.59	147.3	148.5	54.6	48.5
Iron deficiency	F		125	1.4	10.5	1.6	147.9	149.7	54	49.4
Iron deficiency	F	Y/N	127	2.3	29.3	1.59	159.9	158.4	36	27.5
Iron deficiency	F		132	1.1	14.8	2.17	158.2	164.9	69.1	60.7
Iron deficiency	F		135	1.3	19.9	2.32	164.1	*171.1	66.6	57.1
Iron deficiency	F	Y/N	135	1.5	19	2.42	163.7	171.6	61.5	53.2
Iron deficiency	M	N/Y	142	1.5	9.4	1.36	163.8	162.8	68.5	65.1
Iron deficiency	M	N/Y	152	1.8	8.1	1.80	172.4	177.3	71.5	70.3
Iron deficiency	M	Y/N	157	0.8	16.7	1.54	184.4	184	103.3	92.6
<b>Hemoglobinopathies</b>										
Hetero Hb E/variant	t	F	118	1.1	15.4	1.62	144.6	145.5	55.1	46.4
Heterozygous HbE	F		112	0.9	15.8	1.08	138.9	131.8	55.1	45.2
Heterozygous HbE	F		133	0.6	10.6	1.52	156	156.8	*86.5	*77.7
Heterozygous HbE	M		131	0.6	11.3	1.14	154.6	149.6	84.5	75.3
Heterozygous HbE	M		136	0.6	7.1	1.01	155.1	149.2	89.5	83.5
Heterozygous HbE	M		144	0.7	7.9	1.4	164.1	164.2	93.8	87.7
Heterozygous HbE	M		155	1.3	7.1	1.13	174.1	170.3	86.6	84.3
Heterozygous HbS	F	Y/N	122	1.2	14.7	1.57	148.2	148.5	56.3	48.4
Heterozygous HbS	F	Y/N	148	2.2	13.3	1.42	173.2	171.9	59	55.7
Heterozygous HbS	M		142	1.5	18.9	1.03	170.6	162	68.5	60.2
Heterozygous HbS	M	Y/N	143	2.9	12.2	1.49	167.4	167.3	40.8	40.3
Heterozygous HbS	M	Y/N	145	1.3	10.0	1.25	167.4	164.6	79.3	74.1
Heterozygous HbS	M	Y/N	150	1.7	9.3	1.44	171.7	171.9	71.8	69.2
Heterozygous HbS	M	Y/N	153	0.8	10.1	2.07	175.5	182.4	99.3	92.1
Heterozygous HbS	M	Y/N	154	1.7	9.9	1.64	176.3	178.8	75.8	72.8
Heterozygous HbS	M	Y/N	155	1.6	8.6	1.06	176.0	170.4	79.1	76.7
Heterozygous HbS	M	Y/N	169	1.3	8.4	1.8	189.7	194.5	100.6	97.1
Heterozygous HbS	M	Y/N	176	1.4	8.5	1.92	196.8	*202.8	105	101.9
<b>Miscellaneous</b>										
Hereditary spherocytosis	M		160	2.3	19.9	2.87	189.1	*200.3	69.0	63.2
Undefined	M		185	0.8	9.4	1.36	*206.8	*205.8	**131.3	*124.6
Undefined	M	Y/N	168	5.2	12.2	2.76	192.4	*204.3	31.2	36.5

### Variation in OFF model scores explained by Hb

The influence of Hb on OFF model scores was noticeably lower for those subjects who had recently ceased using rHuEPO, especially so for males but also for females. The variance in the OFF-hr model score explained by Hb is shown in Table 2. For a male athlete with Hb equivalent to the mean score of 151 g/L, reticulocyte percentage would have to be less than 0.24% in order to exceed the 1 in 1000 cut-off. A male with a Hb of 169 g/L (the upper limit of the 95% range) would exceed the threshold with <0.63% reticulocytes. A female athlete with Hb equal to the mean value of

137 g/L would require  $\leq 0.23\%$  reticulocytes to exceed the 1 in 1000 cut-off, while a female with a Hb equal to the 95% reference range limit of 155 g/L would exceed the same cut-off with  $\leq 0.61\%$  reticulocytes.

For the OFF-hre model, Figure 1b shows the combination of reticulocyte percentage and EPO required to exceed the nominal cut-off for a male athlete with a Hb level equal to the mean value of 151 g/L, or the upper 95% reference range limit of 169 g/L. In contrast to the situation illustrated in the ON-hre graph, athletes would have to have scores below and to the left of the respective data points to fail the OFF-hre model threshold.

**Table 2.** The component of ON- and OFF-model score variation explained by hemoglobin concentration (Hb). Values were obtained by squaring the correlation coefficient between model score and Hb, then multiplying by 100. *Abnormalities* variation was derived from the correlation of n=22 male and n=18 female athletes diagnosed with a hematologic abnormality (one sample per subject). *Athletes* variation was derived from the correlation of n=1817 male and n=1066 female samples (multiple samples per subject); *EPO* variation was derived from the correlation of n=108 male and n=75 female samples (multiple samples per subject) obtained from volunteers injected with 20-50 IU/kg rHuEPO three times weekly (ON- and OFF-model variations were derived from samples collected during those visits used to derive the original models).<sup>13</sup>

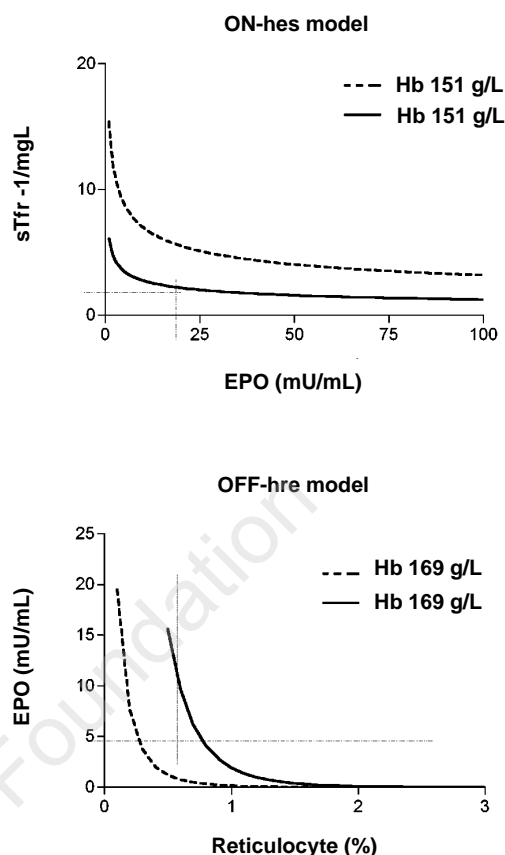
	Variation in model scores attributable to Hb concentration (%)							
	ON-he		ON-hes		OFF-hr		OFF-hre	
	M	F	M	F	M	F	M	F
Abnormalities	86	85	61	50	38	48	50	53
Athletes	90	85	83	69	41	46	49	55
EPO	81	88	64	71	16	35	18	35

**Table 3.** Means and standard deviations of ON and OFF models for healthy athletes and those who were iron deficient (either with or without anemia) or who had a hemoglobinopathy. Subjects were identified through screening of 413 female and 739 male elite athletes from 12 countries.

	Males		Females	
	Mean	SD	Mean	SD
<b>ON-he</b>				
Iron deficient	164.8	12.1	153.3	8.2
Hemoglobinopathy	172.4	13.5	152.2	13.3
Normal	175.1	10.8	159.2	9.9
<b>ON-hes</b>				
Iron deficient	167.6	12.8	157.7	9.0
Hemoglobinopathy	172.1	17.7	150.9	14.8
Normal	173.5	12.0	156.8	11.1
<b>OFF-hr</b>				
Iron deficient	71.8	19.2	53.5	11.1
Hemoglobinopathy	84.4	19.0	62.4	13.6
Normal	83.0	13.8	65.6	14.2
<b>OFF-hre</b>				
Iron deficient	44.2	11.8	44.2	11.8
Hemoglobinopathy	79.5	18.4	54.7	13.5
Normal	78.9	13.5	60.8	14.4

**Comparison of model scores for healthy athletes and subjects with hematologic or clinical anomalies**

A comparison of the average model scores for healthy athletes and those with hematologic abnormalities is shown in Table 3. Graphs depicting the relative position of model scores for athletes with hematologic abnormalities are shown in Figure 2. There was a considerable overlap for the model scores of subjects with a hematologic anomaly and those of healthy athletes. A substantial proportion of the athletes with a hematologic

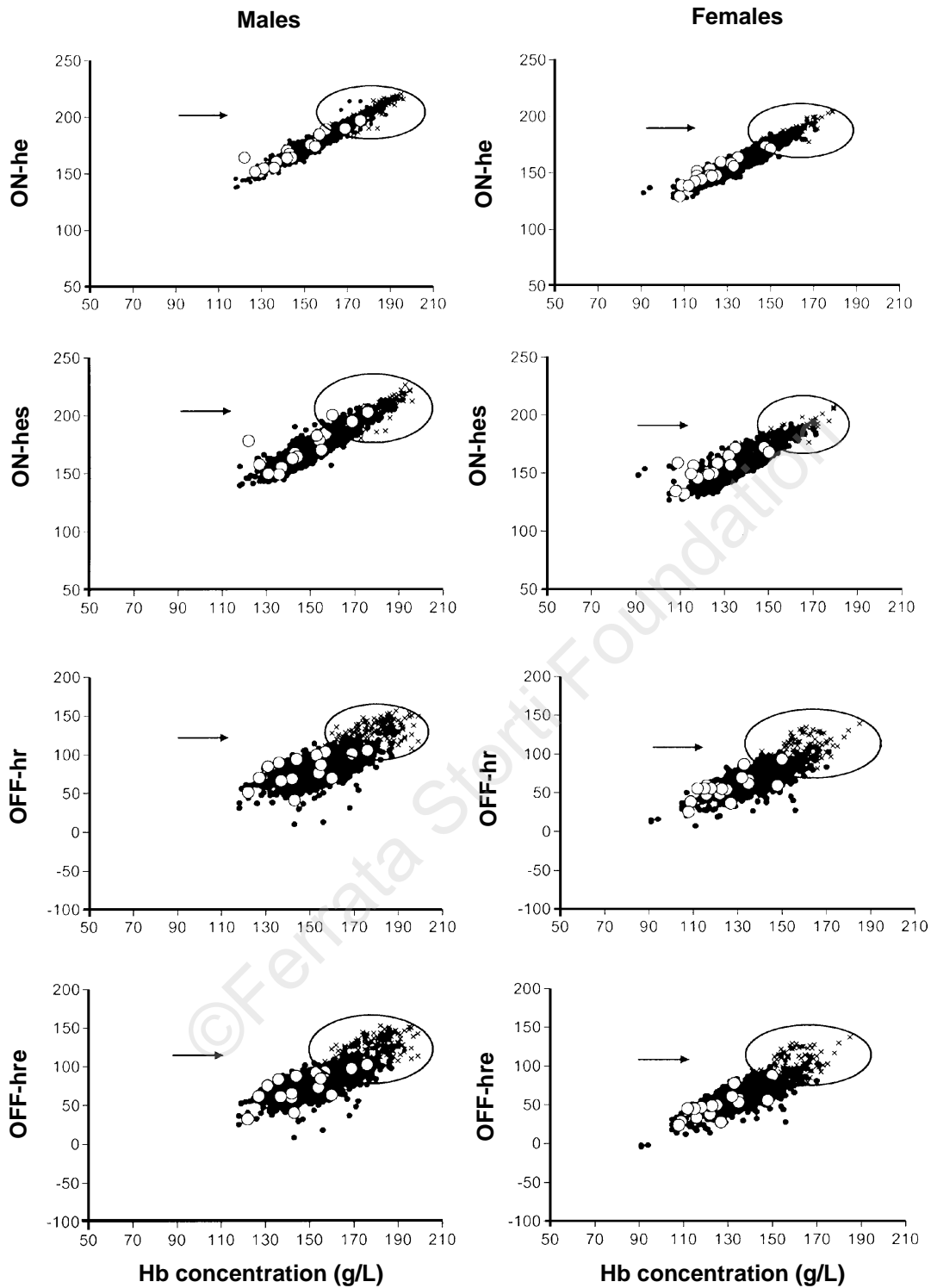


**Figure 1.** Serum erythropoietin, serum transferrin receptor and/or percent reticulocyte values required for a male athlete to exceed a nominal cut-off of 1 in 1000 for the ON-hes model (Figure 1a) and OFF-hre model (Figure 1b) if Hb concentration is equivalent to the average (151 g/L) or the upper limit of the 95% reference range (169 g/L). The dashed straight lines depict the upper (ON-hes) and lower (OFF-hre) limits of the 95% reference range for the respective parameters. We applied the 95% reference ranges that we had established for elite endurance athletes measured at sea level.<sup>12</sup> To exceed the cut-off, athletes would need to have a combination of sTfr and EPO that was above and to the right of the lines in Figure 1A, and EPO and reticulocytes that was below and to the left of lines in 1B.

disorder fell within the lower left corner of the graphs, since these athletes had lower Hb and model scores. Subjects who received rHuEPO treatment, or who had ceased injections, were typically in the upper right corner of the ON and OFF model graphs, respectively.

**Discussion**

Screening the data from our international profiling of elite athletes revealed a very low percentage of athletes with hematologic abnormalities, the



**Figure 2.** Comparison of model scores (ON-hes, ON-he, OFF-hre and OFF-hr) for males and females in the cohort of healthy athletes (●), athletes identified with hematologic abnormalities (○), and volunteer subjects injected with recombinant human erythropoietin (x). One data point was included for every visit for those athletes providing multiple samples. Multiple blood samples from volunteers injected with rHuEPO were included, using data from Visits 15 to 18 (the phase of low-dose rHuEPO administration) for comparison of the ON models, and data from Visits 19-24 (approximately 14 days after rHuEPO injections ceased) for comparison with the OFF models.<sup>11</sup> The horizontal arrows depict the respective cut-off associated with a 1 in 1000 rate of false-positive, the oval denotes the region within which values fall for volunteers injected with rHuEPO.

majority of which were associated with either iron store depletion or hemoglobinopathy. Although our limited sample size precludes us drawing definitive conclusions, the tendency for athletes who were iron deficient or had iron deficiency anemia to have low model scores, and the tendency for athletes with hemoglobinopathies to have normal model scores, are consistent with the relative importance of Hb in the ON and OFF model scores.

#### ***Incidence and model scores of hematologic anomalies in athletes***

The international profiling study from which we gleaned the current data included endurance-trained athletes, since it is this group that has historically been found to utilize blood doping. During the screening of blood samples derived from our 1152 elite athletes, we found a very small percentage of subjects with detectable hematologic anomalies, and the majority of these came from non-endurance-oriented sports. It is prescient to recognize that many hematologic abnormalities are associated with a reduction in oxygen-carrying capacity, and this characteristic is not conducive to elite-level endurance sport. Our data support the contention that a process of self-selection will result in a lower frequency of blood disorders in elite endurance athletes than in the normal population.

The ON model scores for iron-deficient athletes with or without anemia in our current study tended to be lower than scores for healthy athletes, as reflected by the difference in mean scores (Table 3). It was notable that despite a markedly reduced Hb value, athletes with iron-deficiency anemia did not automatically have a lower model score than iron-deficient athletes who were not anemic. Moreover, one of the female athlete's with iron-deficiency anemia had an ON-hes score that was slightly higher than the average value for healthy females. This can be attributed to the positive influence of elevated EPO and/or sTfr values (which are sometimes noted in iron-deficiency anemia) on the ON model scores, which tends to counteract the decrease associated with a reduced Hb value.

Since iron deficiency is associated not with a depression of reticulocyte production but with a reduction in the amount of hemoglobin contained within these immature red cells,<sup>14</sup> it was not surprising that the normal percentage of reticulocytes encountered in these athletes with iron deficiency and/or anemia resulted in reduced OFF-hr model scores. Similarly, because EPO levels are typically normal or even elevated in iron-deficiency anemia, and because both reticulocytes and EPO have a negative coefficient in OFF models, the average OFF-hre model score for iron-deficient athletes of both sexes was well below the (sex-dependent) average for OFF-hr and OFF-hre models.

The second category of hematologic anomalies that we encountered in our profiling study were the hemoglobinopathies. All 17 athletes we detected with these diseases were of Central Asian or Central/Southern African origin, which is consistent with the increased prevalence of these disorders among people of Asian, African, and Mediterranean ancestry. The incidence of hemoglobinopathies in our athletes from these ethnic backgrounds (3.7%) was somewhat lower than the 8.7% prevalence of heterozygosity reported among athletes from the Ivory Coast<sup>15</sup> or the 12.4% reported among Cameroonian runners.<sup>16</sup> Hemoglobinopathies are autosomal recessive so that heterozygous subjects can be carriers and show no clinical features, whilst homozygous subjects will show severe clinical symptoms which would inevitably preclude participation in endurance sport at an elite level. The average model scores for our elite athletes with heterozygous hemoglobinopathies was comparable to the mean score for healthy athletes, for both sexes and across all models.

#### ***The influence of abnormal blood parameter values on model scores***

The blood parameter which we have found to be the single most effective in discriminating subjects injected with either rHuEPO or placebo is Hb. It is intuitive that on average, subjects treated with rHuEPO will have higher Hb values than their non-treated counterparts. Subsequently Hb has been the foundation parameter upon which second-generation algorithms have been devised to highlight rHuEPO use by athletes.

A substantial fraction of an individual's model score will be dependent on their Hb level. As illustrated in Table 3, the variation attributable to Hb was similar whether or not the athlete had a hematologic abnormality, and of similar magnitude for both males and females. This also held true for subjects while they were being treated with rHuEPO. However after treatment had ceased, the component of the OFF model score that was explained only by Hb concentration was notably lower for both males and females when compared with that for athletes with or without an anomaly. Presuming that an athlete had average values for each of the parameters we use to index the rate of red cell production, a male athlete would need a Hb level that was more than 3.3 standard deviations (SDs) above average to exceed a nominal cut-off of 1 in 1000 for the ON models (180.2 g/dL or 183.8 g/dL, ON-he and ON-hes, respectively). For a female athlete, the Hb would also have to exceed the average by more than 3.3 SDs (166.7 g/dL or 171.2 g/dL, ON-he and ON-hes, respectively). With regard to the OFF models, a male athlete with average values for the remaining parameters would require a

Hb level that was greater than 3.9 SDs above the mean (188.1 g/dL or 185.9 g/dL for OFF-hr and OFF-hre, respectively) to exceed the 1 in 1000 cut-off, while the corresponding Hb levels for females would need to be more than 4.1 SDs above average (177.7 g/dL and 173.8 g/dL for OFF-hr and OFF-hre, respectively).

The statistical approach used in deriving our blood models revealed that the models would have enhanced sensitivity and specificity by using, in addition to Hb, parameters that reflected the rate of red blood cell production. It is instructive to quantify the required levels of these erythropoietic components necessary to exceed cut-offs for our models. For example, a male endurance athlete whose Hb was equal to the mean value noted in our profiling study of 151 g/dL would have to have an EPO of 206 mU/mL (equal to more than 8 SDs above the mean) or higher to exceed the 1 in 1000 cut-off for the ON-he model. For an athlete with a Hb of 169 g/dL, the upper limit of the 95% reference range, EPO would have to be 33 mU/mL or higher (more than 4.3 SDs above the mean) if their ON-he score was to exceed the same threshold.

Although our OFF models were derived using the same mathematical principles and also incorporate both Hb, as the foundation parameter, and an erythropoietic component to enhance sensitivity/specificity, the coefficients for percent reticulocytes and EPO are negative in our algorithms. Therefore for a given Hb level, OFF model scores increase as the erythropoietic component – reflecting lower levels of EPO and reticulocytes in the bloodstream – is reduced. A milieu of normal Hb in combination with a subnormal level of red cell production defies logic, since individuals must replace approximately 1% of their red cell population daily for erythrocyte homeostasis. This process is mediated primarily through EPO, which stimulates the bone marrow to produce and release reticulocytes. However after EPO injections cease, the body seeks to regain homeostasis by suppressing EPO production and therefore reticulocyte release (ie the erythropoietic component of the OFF models). The resulting reduction in circulating reticulocytes is present in combination with elevated Hb values for several weeks after injections cease.

In order for an athlete with a Hb of 151 g/dL to exceed our 1 in 1000 cut-off for the OFF-hr model, they would need to have less than 0.24% circulating reticulocytes, which is more than 3.8 SDs below the mean value of 1.23% we noted in our profiling study. As for the ON models, an elevated Hb value will, all other things being equal, predispose this athlete to a higher OFF model score. For example, an athlete with a Hb of 169 g/dL (the upper 95% reference limit) would need to have less than 0.63% reticulocytes, which is 2 SDs below the mean value encountered in elite athletes.

### **Potential for clinical abnormalities to influence model scores**

A convenient approach to address the plethora of known hematologic abnormalities is to deal with classes of diseases according to whether they are associated with subnormal, normal or elevated levels of Hb. This approach also dovetails in a straightforward manner with the progressive impact of Hb on our model scores.

As noted previously, anemias are associated with a reduction in oxygen transport capacity which would seem to preclude competition in endurance-oriented sport at an elite level. By definition, anemias are characterized by a Hb level well below the sex-dependent mean value. Therefore, in order to exceed nominal cut-offs, values for the erythropoietic components would need to be even more extreme than those noted earlier for subjects with normal Hb. For example, an anemic male with a Hb of 135 g/L would need to have EPO  $\geq 1065$  mU/mL to exceed the ON-he cut-off of 1 in 1000.

Hemolytic disorders can be characterized by very high reticulocyte counts and substantially increased serum EPO concentration. The direction of erythropoietic compensation is in the same direction as that induced by rHuEPO injection: therefore, although elevated reticulocyte and EPO values would reduce the OFF model scores, they might also contribute to an elevated ON model score. However, as we have demonstrated earlier, in spite of very high levels of EPO and sTfr, it is anticipated that ON model scores would be tempered by the low Hb value and therefore would be less likely to exceed nominal cut-off scores than would those in a healthy subject with typical Hb levels. However one hemolytic disorder often compensated without anemia, and therefore with near-normal Hb levels, is hereditary spherocytosis. The likelihood of an athlete with this disease exceeding a cut-off would therefore be dependent on the presence of EPO and sTfr values that were of the magnitude noted previously for subjects with near-normal Hb levels. One athlete in our profiling study with this disorder had normal Hb values in concert with mild elevations in reticulocytes, EPO and sTfr, resulting in ON model scores that were above average but did not exceed the 1 in 1000 cut-off. OFF model scores for this individual were well below the average for elite athletes.

One category of hematologic abnormalities that could conceivably confound our blood models is polycythemia, which are characterized by an increased red cell mass and therefore higher Hb values. Because of the primacy of Hb in both ON and (to a lesser extent) OFF model scores, it is clear that such conditions will tend to be associated with above-average model scores. Various types of secondary polycythemia are associated with higher than normal EPO levels in an attempt to alleviate



tissue hypoxia, although there is also an inappropriate response that can be caused by localized renal hypoxia or tumor generation of a substance that mimics the action of EPO. These scenarios would suggest that polycythemic subjects would be at greater risk of exceeding ON model rather than OFF model cut-offs. However the clinical sequelae associated with many polycythemias lessens the likelihood that they would be encountered in elite endurance athletes. Polycythemias are mostly associated with tissue hypoxia, chronic obstructive pulmonary disease, cyanotic heart disease, high-affinity hemoglobinopathy and smoking.<sup>17</sup>

It is noteworthy that given the associated medical risks of thromboembolic events, and the perception that these risks are compounded by the dehydration and/or hemoconcentration usually associated with long hard exercise, such endurance athletes are typically required to undergo careful medical examination before they are authorized to compete. The examination provides an opportunity whereby, in the unlikely event that an athlete was able to compete at an elite level in spite of a polycythemic condition, the aberrant model scores could be justified by qualified medical personnel.

### Conclusion

Given the plethora of known hematologic disorders, and the broad gamut of hematologic values that have been encountered both within and between general classes of disease, it is impractical to address every possible permutation of our component variables that might be encountered in a disease state. However the concept of using elevated blood model scores to identify rHuEPO users, and the robustness of the scores in the face of anticipated hematologic anomalies, are fortified by several important characteristics.

First, the majority of candidate hematologic disorders would not be conducive to endurance performance, and therefore it is unlikely that this scenario would be encountered when testing elite endurance athletes. Second, these disorders tend to reduce, rather than increase, model scores – primarily because most disorders are associated with a decrease in Hb. Finally, because the parameters included in our models are not a sufficient foundation for establishing the presence of a hematologic abnormality, in the event of a blood profile exceeding a cut-off score, medical evaluation and subsequent classification of a hematologic disorder by a hematologist should constitute persuasive evidence that the unusual blood profile was not caused by rHuEPO use. This adds another dimension to the validation of our current blood models for the detection of rHuEPO use in elite sport.

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## **Pre-publication Report & Outcomes of Peer Review**

### *Contributions*

RP: conception and design, analysis and interpretation, drafting and review, final approval; MJA: conception and design, analysis and interpretation, drafting and review, final approval; CJG: conception and design, analysis and interpretation, drafting and review, final approval; KS: design, analysis and interpretation, critical revision, final approval; WH: design, analysis and interpretation, critical revision, final approval; AGH: conception and design, analysis and interpretation, critical revision, final approval.

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### *Disclosures*

Conflict of interest: none.

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### *Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Dr. Carlo Brugnara, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Dr. Brugnara and the Editors. Manuscript received May 20, 2003; accepted June 11, 2003.

In the following paragraphs, Dr. Brugnara summarizes the peer-review process and its outcomes.

### *What is already known on this topic*

A model based on hematologic and biochemical parameters has been validated and used in the Sydney Olympics to identify endurance athletes who use recombinant human erythropoietin (r-HuEPO) for blood doping purposes. Athletes failing this initial testing then undergo additional testing to identify the presence of r-HuEPO in urine. However, the effect of mild hematologic disease or abnormalities on this model has not been evaluated in a systematic fashion.

### *What this study adds*

A systematic study in 1,152 athletes shows that mild hematologic abnormalities do not cause false positivity in the models developed to identify current (ON model) or recent (OFF model) r-HuEPO abuse. Variations in Hb concentration had the largest impact on these models and offset potentially falsely positive changes in the other parameters.

### *Caveats*

Careful standardization of the laboratory parameters used in this study across different reagents and instrument platforms is necessary to promote wider use of these models. In addition, establishing individual hematologic passports for competitive athletes in endurance sports will greatly facilitate the identification of blood doping due to r-HuEPO abuse or other illicit practices.