

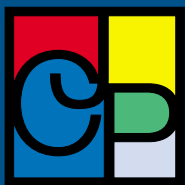
Bloodline[®]

Volume 1, Issue 3, 2001

www.bloodline.net

HEMATOLOGICAL TESTING FOR
BLOOD DOPING IN SPORTS

REVIEWS



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Bloodline Reviews is published by:
Carden Jennings Publishing Co., Ltd.
375 Greenbrier Drive, Suite 100
Charlottesville, VA 22901
Phone: (434) 817-2000 Fax: (434) 817-2020
Web: www.cjp.com

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This issue of Bloodline Reviews is supported by a grant from



Bloodline Reviews is dedicated to the publication of original research and pertinent reviews in the fields of hematology and oncology.

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The content of this issue is based on selected presentations made at the XIVth International Symposium on Technological Innovations in Laboratory Hematology, held in May 2001, in Montpellier, France.

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The EPO Epidemic in Sport

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It is interesting to note that performance-enhancing drugs have been used since the beginning of organized sports. There is evidence that such substances were used as early as the 4th century B.C., at which time there existed substances and methods to create feelings of euphoria, decrease fatigue, and increase strength.

With the advent of modern organized sporting activities – dating back to the 19th century – has come ever-increasing interest in superior performance. The ability to accurately measure time and strength contributed to this interest, and the lure of prizes and fame led to the undertaking of sports as a profession.

Notably, in recent decades there have been well publicized increases in the use of new synthetic substances, including morphine, codeine, amphetamines, anabolic steroids, and corticosteroids to increase performance. There has also been a strong trend toward increased use of dietary supplements.

Most recently we have seen the introduction of synthetic peptides such as erythropoietin (EPO) – the first substances that have quantifiable impacts on athletic performance, and which are very hard to detect.

Since the introduction of these substances, we have seen significant performance increases, as evidenced by the rate of new world records being set in endurance sports, such as swimming, cycling, and speed skating.

Prior to the introduction of synthetic peptides there was no real scientific evidence that other drugs used in the athletic community, such as amphetamines and steroids, had any measurable effect on endurance. This changed, however, with the introduction of EPO, which can enhance performance in some tests of endurance by as much as 20 percent.

This problem of doping in sports may well have something to do with the general medicalization of society. More and more pharmaceutical products are taken and have become almost a normal part of life. There is also an idealization of success – that it should come at almost any price. These pressures to perform at the highest possible levels exert a powerful stimulus on athletes to use performance-enhancing substances.

To many of us involved in sports professionally, it is a given fact that every athlete seeks to enhance their performance. Whether they do it with legal products – those not on the list of forbidden substances for their sport – or they do it with forbidden products, the goal of performance enhancement is the same.

This drive for performance has resulted in increasing use and abuse of legal drugs, particularly in those countries where doping control is very tough. The use of these products is allowed within certain limits.

Use of performance-enhancing substances has somewhat of a ‘snowball’ effect. When one athlete does it, others feel forced to do the same thing, because they feel themselves to be at a disadvantage when competing against others who use such substances. They, quite simply, don’t want to be at the mercy of others’ use of these substances, which is how they feel.

My federation – Union Cycliste Internationale (UCI) – began its fight against doping in 1965 after we had a cyclist in the Olympic Games in Rome whose death was linked to the use of performance-enhancing drugs. We established our first anti-doping rules in 1965, and have continued our anti-doping efforts to the present day.

When UCI began testing its athletes in 1965, approximately 25 percent of 250 athletes tested positive for doping. This usage level decreased significantly and rapidly following the implementation of control measures. In contrast, UCI tested approximately 5500 athletes in 2000, out of which 1.12 percent of results were positive. It should be noted that this later statistic does not necessarily mean that there is not a higher prevalence of doping – only that, perhaps, there are substances being used which are, as yet, undetectable.

Also in 2000, approximately 8000 anti-doping tests were conducted by various national and international sports federations. The majority of substances detected were light stimulants.

It should be mentioned that, in addition to purposeful doping by athletes, we are faced with the problem that more and more forbidden substances are being found in food products that are for sale in normal retail outlets. This has led to an increase in the number of athletes who test positive for doping, but who have not knowingly taken any doping agents.

In January, 1997, my organization, the UCI, introduced a health protection program to address the doping problem – specifically that of EPO.

This program consisted of two steps, the first of which involved conducting blood tests prior to races. Three hours prior to a race, small blood samples were obtained from our athletes. Any athlete who had a hematocrit level over 50 was removed from the race and given a two-week rest period. These athletes with a hematocrit result over 50 were not suspended, per se, because we could not prove that the elevated hematocrit was due to EPO use. These athletes were tested again after the two-week rest period and allowed to resume competition if their hematocrit level was below 50.

The second part of our program was a medical monitoring system introduced in January 1999, which was carefully developed with both physicians and lawyers. Under this system, our athletes go through certain medical checks four times per year. This

monitoring system is followed very carefully to ensure that our cyclists are able to perform safely in a sport that is very physically demanding.

It is important to note that this two-part health-monitoring program is conducted with the full consent of the athletes.

Again concentrating on EPO, which increases oxygen transportation through increasing the number of red blood cells, we have cutoff hematocrit levels of 50 for men and 47 for women. Detecting increased hematocrit is done through a relatively simple and rapid blood test, but again, this cannot prove that an athlete has used EPO.

Of course, while the measurement of hematocrit levels is an indirect method of testing for blood doping, we also have the direct

urine test method currently being developed in France. The urine test, as a direct method, can prove the presence of EPO, and a positive result can, therefore, be used as the basis for sanctioning an athlete. This method also has disadvantages, namely that it has a limited detection period of 2-3 days following the injection of EPO, and it can only be performed at a few laboratories worldwide, at present.

Our anti-doping efforts have resulted in a rapid decline in the number of our athletes who test positive for various forbidden substances. These efforts have also been very expensive. We spent a total of \$2.2 million in 1999 and \$2.4 million in 2000 on these anti-doping efforts. The costs were, however, justified in that they protected the health of our athletes and helped to ensure the validity of our athletic performances. *BLR*

Hematology Parameters: Usefulness and Limitations in Monitoring Red Cell Production

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Overview:

A variety of instruments and methods are available for the evaluation of hematological parameters in the context of erythropoiesis. Among the standard parameters and methods used for such evaluation, there exists a strong need for standardization, which should take into account biological, analytical, pre-analytical variabilities, all of which can significantly affect the data being obtained through hematological analysis.

If our goal as clinical laboratory hematologists is to monitor and detect abnormal stimulation of erythropoiesis, we must use valid criteria to assess the value of testing procedures used for this purpose.

This means that we have to select appropriate tests – ones that meet the accepted performance levels in terms of sensitivity, specificity, and predictive value. We also need, most importantly, to standardize the tests used.

Standardize, in this sense, is a very general word, which covers the areas of calibration and quality control, the elimination of imprecision, assuring the accuracy of instruments and methods, and the harmonization between these factors. It also recognizes the importance of biological and analytical variability, and of the performance goals and critical differences between testing methods.

Numerous studies have shown the effects of recombinant EPO on various hematological parameters, including hematocrit, hemoglobin, and reticulocyte counts. EPO has also been shown to affect various properties of red cells themselves, such as shape and size.

Among other things, studies have shown that reticulocyte counts increase much earlier than hematocrit or hemoglobin in response to EPO administration, and that there is a decrease in the reticulocyte count following the cessation of EPO use.

The Australian research group of Robin Parisotto and Mike Ashenden introduced the use of reticulocyte parameters as fine-line indicators of changes in the production of red blood cells.

It should be understood that laboratory data have no value in themselves. They must be subjected to the transformation process known as interpretation. This can be done for our purposes through two methods: transversal evaluation and longitudinal evaluation.

In transversal evaluation, data is compared with that of a reference population or a threshold value, and adjudged to be normal or abnormal. Examples of this type of evaluation include the UCI's use of hematocrit cut-off values, and the FIS' use of hemoglobin cut-off values.

Longitudinal evaluation involves the comparison of data with earlier data from the same individual and is defined as changed or not changed. This evaluation method is probably much more

effective than transversal evaluation, and is the basis for the UCI's plans to prepare an effective method of examining athletes periodically.

As an example of the ways in which an athlete is evaluated, consider a hypothetical case study of an individual. This athlete arrives in France today, has his blood taken and analyzed on a Bayer ADVIA 120 analyzer. He then travels to the United States, and after eight days, a new blood sample is taken and analyzed on a Coulter machine. Then, after one week, the athlete flies to Japan and his blood is analyzed there on a Sysmex analyzer. Continuing his travel, blood samples are drawn over the course of several weeks on an ABX analyzer in Jerusalem, and on an unknown analyzer in Moscow. Finally, the athlete returns to France, where blood is drawn once again and analyzed on the original Bayer machine.

We would expect that any change observed over the course of all these analyses is that any change observed in hemoglobin or cell counts were the result of biological variations, and not due to the fact that the analyses were performed on different instruments, at different times, or in different places. However, the lack of testing standardization across all these analyses can be the cause of significant variation, which is the reason standardization has become an area of focus for many international sporting and hematology organizations.

Clinical interpretation of testing data depends on a number of variables, including biological, pre-analytical, and analytical variabilities. Biological variability, including such factors as actual physiological changes and training changes, can be within one individual or between different individuals. Pre-analytical variability includes such factors as food and fluid intake, exercise, and posture, prior to or during sampling.

Analytical variability is a combination of analytical imprecision and systematic errors of method. This analytical variability can be lessened through in-laboratory internal quality control methods, and through standard quality assessment schemes and harmonization processes, so that results from different methods and different instruments can be compared.

It is apparent that the issue of instrument calibration is very important. This calibration of blood cell analyzers involves adjustments to correct for many factors, including dilation and signal amplitude in the different analyzer channels. These adjustments can be made by comparing values obtained from an analysis of fresh blood with values obtained by a reference method or through the use of reference materials. This need for calibration can be clearly seen through incidents in which samples obtained from an individual athlete at the same time have been analyzed

using the same tools in different laboratories, with significantly different results being obtained.

When we want to quantify increases in erythropoiesis, there are two hematological parameters that are of particular importance: hemoglobin concentration and hematocrit.

Hemoglobin concentration, which is the most precise, accurate, standardized and harmonized hematology parameter, is obtained by photometric measurement after conversion to cyanmethemoglobin, or with new cyanide-free methods. Measurement of this parameter is supported by a unique hemoglobincyanide reference preparation, which was proposed by the International Council for Standardization in Hematology (ICSH), and also by an established reference method, which was proposed and accepted by the ICSH in 1987.

Hematocrit values, on the other hand, can be measured using either manual methods – as a volume ratio of red blood cells to whole blood – or automated methods, in which the basic object of the direct measurement is the electrical impulse generated by the cell when it passes through impedance-based or optical-based analyzers.

There are discrepancies in manual versus automated hematocrit measurements, especially due to the plasma trapping in the corner of red blood cells, which can be as high as 5-6 percent in abnormal samples and up to 20 percent in very ill abnormal cells. This causes a false increase of the mean cellular volume in centrifuge hematocrit. What this means is that when we calculate hematocrit from pulse size and red cell count, an underestimation of the hematocrit can occur. A correction of about three percent on automated hematocrit measurements has been proposed in the past to deal with type of discrepancy, but this would not be valid, as the effect is not linear, since plasma trapping increases with microcytosis and anemia.

Different extraneous factors affect how automated hematocrit measurements are made by different instruments. The fundamental difficulty is that the electrical impulse produced by any cell is only approximately proportional to the volume of the cell

(shape effect). Also, aberrant impulses, which do not truly represent cell size, can occur on any counter (coincidence, edge effect, recirculation), occurring more frequently on aperture-impedance counters without sheat flow. Owing to their unusual characteristics, suitable electronic circuits edit these aberrant impulses, resulting in measurement variances.

This information shows that variability is increased in hematocrit measurement, suggesting that hematocrit is not the best parameter for red cell quantitative assessment. This parameter represents red cell production relative to whole blood, and is sensitive to changes in plasma volume, as well as red cell volume. It is also a virtual, or calculated, parameter, which is more difficult to standardize.

Hemoglobin, on the other hand, is the most precise, accurate, direct and standardized blood parameter. It expresses more directly the oxygen-carrying capacity of the blood, which is the target of blood doping.

Another hematological parameter useful to examine is reticulocyte measures. Both absolute reticulocyte and percent reticulocytes are very useful parameters, which, while always standardized, have some measurement problems at very low volumes. Reference values for reticulocyte measurements are very heterogenous, and change from one analyzer to the next.

Different methods of measuring the immature reticulocyte fraction have been shown to have very poor inter-method correlation. However, reticulocyte volume measurements obtained using different methods are well correlated.

In conclusion, hematology laboratories at the start of the new century are provided with a growing number of parameters and methods aimed at assessing the degree and efficiency of erythropoiesis, some of which have been shown to be more accurate or useful than others.

Finally, the importance of how our tests and methods should be selected, standardized, and controlled should be kept in mind as we continue to develop our anti-doping strategies and methods. *BLR*

Erythropoietin, Iron and Red Blood Cell Production: Laboratory Evaluation

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Several clinical settings have furthered our understanding of the relationship between erythropoietin, iron, and the erythropoietic response to anemia. These settings include hereditary hemolytic anemia, hemochromatosis, autologous blood donation, and therapy with recombinant human erythropoietin (r-HuEPO).

The knowledge acquired in these settings has allowed us to identify a "relative iron deficiency" that occurs when increased erythron iron requirements exceed the available supply of iron, even in the presence of storage iron. The identification of this relative (or functional) iron deficiency is important for the proper assessment of r-HuEPO use.

Known indicators of iron metabolism and r-HuEPO can be broken down into two main categories: biochemical indicators, which include serum iron, transferrin, transferrin saturation (Tsat), ferritin, and circulating transferrin receptor (TfR), and hematological indicators, which include erythrocyte zinc protoporphyrin (ZPP), percent hypochromic cells (%hypo), and reticulocyte hemoglobin content (CHr).

Of the biochemical indicators, one of the more recent developments has been in the area of circulating transferrin receptor (TfR), which is a truncated form of the transferrin receptor that circulates in plasma, usually attached to transferrin. Several studies have shown that the levels of TfR are elevated in iron deficiency anemia, in some people living at high altitude, and in disorders with expanded erythropoiesis. TfR levels have also been shown to be decreased slightly in the setting of iron overload and in disorders with reduced erythropoiesis.

Clinical research on the clinical values of TfR in the setting of r-HuEPO therapy has found that TfR is predictive of responses to the initiation of r-HuEPO therapy,¹ and that r-HuEPO therapy brings about an increase in TfR. This increase is of lower magnitude in patients with delayed or absent response to r-HuEPO therapy.²

The advantages of using measurements of TfR to assess r-HuEPO use are that it is a good, dynamic test that reflects changes in real time and is a good index of erythropoietic mass at a given location. The disadvantages of this method include the facts that the assays used for TfR are better suited to research, rather than clinical use, and that although there is a good correlation between different methods of testing, these methods all use different units. Additionally, the accuracy of using TfR to identify iron deficiency in the setting of r-HuEPO-driven erythropoiesis has yet to be determined.

Erythrocyte zinc protoporphyrin (ZPP), a red cell parameter long used to detect lead poisoning in children, has been the subject of numerous studies in the context of EPO use. It has been shown that patients taking EPO in a clinical setting showed modest but significant increases in ZPP.

The use of ZPP to follow patients on EPO is, however, limited. A paper in *Clinical Chemistry* in 1992 showed that a large number of

substances interfere with the measurement of ZPP and that very different measurements are obtained if red cells are washed and resuspended in saline. For these reasons, measurement of ZPP is not suited to following patients on EPO or to identify iron deficiency.

Another parameter that has been studied is the percentage of hypochromic red cells (%hypo). This is the percentage of cells that have a hemoglobin concentration below an arbitrary cut-off point of 28 g/dL.

One of the first papers to examine the concept of functional iron deficiency showed that patients on dialysis treated with EPO tended to develop functional iron deficiency, as identified by the increased appearance of hypochromic red cells.³ These cells had abnormally low hemoglobin concentrations, very similar to those seen in cases of iron deficiency.

A similar concept was shown in a study that used flow cytometric assessment of volume and volume concentration. In this study, it was found that the newly formed cells associated with EPO administration had increased ZPP, decreased volume concentration, and decreased red cell volume.³ This suggests that, in normal subjects, use of EPO can result in the production of red cells that are indistinguishable from those seen in patients with iron deficiency anemia.

Several studies have suggested that %hypo measurement can be useful in studying iron deficiency and EPO use.^{4,5} A number of other studies, however, have shown no value for this parameter in the setting of r-HuEPO.^{6,7}

%Hypo has the advantages of already being used in Europe and of being part of the regular CBC. This parameter does, however, have apparent disadvantages, such as incorporating both hypochromic red cells and normal reticulocytes and increasing with storage at room temperature. Also, %hypo can only be measured using Bayer instruments.

Numerous studies have shown various effects of EPO on reticulocytes. These effects include a significant release of immature reticulocytes within 1.5 days of, and peaking 3.5 days after, EPO administration. EPO has also been shown to bring about an increase in the overall number of reticulocytes, peaking at 5 days after EPO administration, as well as a decrease in serum ferritin, with the nadir at 4.0 days after EPO administration.⁷

Several recent publications have addressed the use of reticulocyte indices and reticulocyte hemoglobin content (CHr) in dialysis patients. In the initial publications, measurements of CHr were obtained with the H*3 Bayer hematology analyzer. CHr has been shown to be a reliable indicator of iron status in patients undergoing chronic hemodialysis.⁷ Patients with CHr values lower than red cell MCH had lower transferrin saturation and lower hematocrit, and they all had a recent increase in r-HuEPO dose. With iron deficiency being defined as a state that shows an increase in reticulocyte counts following intravenous (IV) iron, CHr showed 100% sensitivity and 80% specificity. CHr was a much more accurate pre-

dicator of iron deficiency than serum ferritin, transferrin saturation, or percentage of hypochromic erythrocytes.⁷

In another study, a baseline CHr < 28 pg had 78% sensitivity and 71% specificity to diagnose functional iron deficiency, compared with 50% and 39% for traditional biochemical measures of iron deficiency.⁸

In patients treated with subcutaneous (SC) r-HuEPO and IV iron, CHr was shown to increase during IV iron therapy, indicating response to treatment and potential value as an early indicator of functional iron deficiency.⁹

More recent studies have used the ADVIA 120 Bayer hematology analyzer, which provides CHr data that are on the average 3 pg greater than the H*3 analyzer. This difference is important when cut-off values are used as decision points for anemia management in chronic dialysis patients. In a study by Fishbane et al., which was presented at the ASN 2000 meeting, management of iron therapy in dialysis patients based exclusively on CHr (< 29 pg) resulted in similar Hct and EPO dose and greatly reduced iron exposure compared with management based on biochemical parameters (ferritin < 100 ng/ml or transferrin saturation < 20%).

In another report, a combination of %hypo > 6% and CHr < 29 pg provided the greatest diagnostic efficiency (90.4%), with 86.3% sensitivity and 93.2% specificity to identify hemodialysis patients who will respond to IV iron therapy.¹⁰

The advantages of the reticulocyte hemoglobin content parameter are that it provides a real-time estimate of iron availability and that it is provided as part of an automated reticulocyte count. Additionally, it is insensitive to storage-induced changes. It does, however, have disadvantages, namely that the measurement is available on only one class of instruments, it is not widely used in the United States or Europe, and it is not informative in patients with thalassemias or macrocytosis.

Looking at numerous studies, we can see that there are several indicators of r-HuEPO-driven erythropoiesis. These indicators, which were used in the indirect EPO-use detection method for the 2000 Olympic games, include: hemoglobin, transferrin receptors,

percentage of hypochromic red cells, reticulocyte hematocrit (absolute retic count x MCVr), reticulocyte hemoglobin (absolute retic count x CHr), and serum EPO.

Examining all of the hematologic parameters discussed, one can ask the question of how they can be used together to differentiate EPO-driven erythropoiesis and EPO-associated iron deficiency. The bottom line is that several parameters are available, some of which can be used clinically in patients, and some of which may be useful in the detection of EPO use for blood doping.

Table 1 shows the effects of both EPO-driven erythropoiesis and EPO-associated iron deficiency on various hematological parameters.

In conclusion, we can see that hematological parameters can be reliable indicators of iron status and that these parameters may play an important role in the management of r-HuEPO therapy and the diagnosis of iron deficient states. In particular, %hypo and CHr may provide both long-term and real-time assessment of the balance between available iron and erythropoiesis.

Taken together, these parameters may play an important role in the management of r-HuEPO therapy and diagnosis of iron deficient states, although more studies are needed to validate their clinical use. *BLR*

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Table 1: r-HuEPO-Driven Erythropoiesis and Functional Iron Deficiency

Parameter	EPO-driven erythropoiesis	EPO-associated iron deficiency
%Hypo	↑	Further ↑
Reticulocyte count	↑	No additional change, or blunted increase
MCVr	↑	No additional change, or blunted increase
Retic Hct (MCVR x count)	↑	No additional change, or blunted increase
CHr	=	↓
Retic Hb (CHr x count)	↑	↓
TfR	↑	Further ↑

Red Cell Parameters in Winter Endurance Sports

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Overview

Blood doping has been the most significant doping problem in endurance sports over the last 20 years. Blood doping has afforded the greatest performance benefit among doping agents and has been the most difficult to detect. Initially, blood doping took the form of homologous and autologous transfusions; this was followed by the use of r-HuEPO, and most recently by the use of HBOC's and other oxygen transport molecules.

The International Ski Federation (FIS) was first to introduce hemoglobin limits to allow participation. The concept was to limit both the degree of the health risk and the degree of performance enhancement provided by blood doping. These efforts were followed by implementing hematocrit limits in the International Cycling Union (UCI) and the International Biathlon Union (IBU). Researchers at the same time have attempted to work on both direct and indirect tests for r-huEPO.

Due to the problem of false positives and false negatives associated with measuring hemoglobin or hematocrit alone, in conjunction with the International Skating Union (ISU) medical committee, we have developed the SAFE (Safe And Fair Events) program for the ISU. The SAFE program is a refinement of measuring hemoglobin or hematocrit concentrations to allow starts and further serves to focus anti-doping efforts where they are most likely to be efficacious.

In 1989, Warren and Cureton came up with a very nice equation that essentially says that for every hundred-gram increase in total hemoglobin mass, there is an increase in maximum oxygen intake of about 600 mL. What this means is that if you have a one gram increase in hemoglobin concentration, the resulting increase in athletic performance, as measured by maximum oxygen intake, will be about 1-4 percent.

At the elite level of sport, contests are won or lost by a fraction of a percent, so performance increases resulting from a small increase in hemoglobin can take an athlete from, for example, 38th place, up to one of the best.

The problem is that in case of the recombinant EPO, or even growth hormone, a paradigm shift is needed in the way we approach doping control. Since the half-life of these drugs is very short, and the half-life of the effect is much longer, there is a large open window where urine tests have almost no ability to detect the EPO. Therefore, traditional direct testing, at this time, cannot work in the second and third weeks after cessation of EPO administration.

With the availability of the ADVIA 120 analyzer, however, it is possible to measure red cells and reticulocytes. The ADVIA 120 is

a unique machine, and is the only one that can measure, essentially, the hemoglobin concentration, and, thus, hemoglobin content, of each red cell.

EPO 2000

With the help of the ADVIA 120 technology, in combination with the direct urine test, the EPO 2000 project was developed. It was sponsored by the International Olympic Committee, and is a collaboration between researchers in Australia, China, Canada, France and Norway, with data included from a profiling study of 1100 athletes in thirteen different countries.

The hematological parameters used for this project model were hematocrit reticulocyte, hematocrit, and percent macrocytes – all measured using the ADVIA 120 – in addition to immunoassays for erythropoietin and soluble transferrin receptor.

Basically, what was found in this project was that, using a five-parameter on-model equation and a three-parameter off-model equation to detect when an athlete is on or off EPO, there was a complete separation in the results of athletes receiving EPO and those athletes receiving placebo. This means that we have a very effective change model that can be used in conjunction with a hematological passport to detect the use of EPO.

Later efforts in this project included calculating on-model scores based on a four-week altitude camp, which showed that the mean values for men and women almost never go above the cutoff scores for the regular EPO on-model. In the very few instances where athletes had values that exceeded the cutoff stage in response to altitude, urine tests were able to differentiate between the effects of altitude and those of EPO.

The SAFE Solution

In response to questions concerning the accuracy of hemoglobin and hematocrit limits implemented by sporting organizations, we have come up with the SAFE solution, which represents a significant paradigm shift from standard doping control protocols.

Under the SAFE paradigm, all athletes are screened on a non-competition day, thus allaying athletes' concerns about testing interfering with their performance.

To implement this screening we have protocols that take up about ten minutes of an athlete's time, and we can process two hundred athletes in a period of about five hours. Further, these protocols are relatively inexpensive, meaning that we are better able to focus anti-doping efforts where they are more likely to be successful, and thereby make better use of funds spent on anti-doping efforts.

The SAFE program is an extension of the EPO 2000 project, and is a refinement of hemoglobin or hematocrit measurement. It forms a basis for an individual athlete's hematologic passport.

Essentially, what happens under this model is that the athlete comes in on a non-competition day, and 3 mL of blood is drawn in a standard position after they have been seated for five minutes. This five-minute period is used to allow for the stabilization of plasma volume. After the blood sample is taken, it is analyzed on the ADVIA 120.

The first decision in the SAFE paradigm is whether or not an athlete's hematocrit level is within the limits for their particular sport. For those who have levels exceeding the limit, we look at the other hematologic parameters of erythropoiesis. If these other parameters show signs of normal erythropoiesis, the athlete is approved for participation in the sporting event.

On the other hand, if the hematocrit level is over the limit and the hematological parameters show signs of accelerated or decelerated erythropoiesis, there is an increased degree of confidence that by preventing this athlete from starting you are doing the right thing. This is not necessarily considered a positive doping test, it is just saying that a significant accelerated or decelerated erythropoietic picture here justifies not allowing the athlete to take part in the sporting event.

It is also worth noting that, for those athletes who have hematocrit levels below the cutoff points, these procedures aid in the establishment of a 'normal' picture from an erythropoietic standpoint. These athletes are, of course, allowed to compete.

When an athlete shows signs of abnormal erythropoiesis, but is allowed to compete, a system of focused investigation comes into play, under which the athlete undergoes more comprehensive testing.

One potential problem with these assessments is that when humans are involved in making decisions about which athletes will compete or which athletes will undergo further doping-control tests, a certain amount of bias is unavoidable. Therefore, as part of the program we have designed a computer macro that takes the export file off the ADVIA 120 and analyzes it. This analysis decides if a given athlete starts or not and whether follow-up testing is indicated or not. This computerized categorization, however, is supervised by a medical official from the sport's governing body to ensure correct results.

Using the SAFE paradigm, we have obtained more than 1500 samples from about 600 world-class endurance athletes in ISU, IBU and FIS over the course of the last two winter World Cup seasons. Samples were obtained during World Cup or World Championship competitions in Europe, North America and Japan. Approximately 1100 samples were obtained at sea level, 200 samples at 1400m and 200 at 1750m. The specimens were analyzed on six different ADVIA 120 hematology analyzers, which were carefully calibrated prior to use.

If this testing paradigm had been in place as an official function, the hematological parameter measurements would have resulted in only 1 percent of the athletes being excluded from competition, while 22 percent would have been required to undergo further testing.

Our testing using the ADVIA 120, it should be noted, can indicate accelerated erythropoiesis by detecting high reticulocyte counts, or high percent reticulocyte counts, in addition to the size and hemoglobin concentration of reticulocytes. Decelerated erythropoiesis is detected using essentially the same measurements.

It is worth considering the possibility that through efficient detection methods for EPO, we may push athletes into using other forms of doping. However, with the ADVIA 120 testing procedures we use in the SAFE paradigm, we can also detect indicators of the use of hemoglobin-based oxygen carriers, as well as transfusions.

Overall, what we can do in the case of accelerated erythropoiesis as evidenced by hematological parameters, is go ahead and measure EPO and transferrin receptors and calculate an on-model score. In addition, urine samples are collected and tested for recombinant EPO. If these additional tests are positive, it is fairly certain that an incidence of doping will be detected.

In the case of decelerated erythropoiesis you can have an extremely high degree of certainty that the deceleration is due to stoppage of a banned substance, as opposed to altitude changes or any other normal factor that may affect erythropoiesis. This can be verified through additional blood testing after 5-10 days, which will show either another decelerated model or a normal model. A normal or higher measurement is indicative of a positive change model, which is a very strong indication of the use of doping.

In summary, the anti-doping methods described include the measurement of a series of parameters on the ADVIA 120 analyzer that give an indirect indication of accelerated or decelerated erythropoiesis, the use of hemoglobin-based oxygen carrying molecules, or transfusions. Also in place are additional confirmation tests for doping, some of which are still under development.

With current doping methods, it is theoretically possible for an athlete to use such low doses of prohibited substances that their parameters remain within normal levels. However, the performance benefits and repeated testing over time is likely to detect very substantial changes in their hematological parameters if they cease doping.

Finally, the SAFE paradigm offers an effective strategy for detecting and deterring blood doping. It also reduces inconveniences to the athlete, is welcomed by the athletes because it provides them with accurate data on their own hematological parameters and health, and can be used as the basis for a hematological passport to track doping status and health changes over time. *BLR*

The Development of a Blood Test for EPO Abuse

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Overview:

Anecdotal reports have suggested that endurance athletes have been blood doping with impunity for several decades. The original practice of autologous and/or homologous blood doping was superseded with the commercial availability of recombinant human erythropoietin (r-HuEPO), highlighted by the infamous 1998 Tour de France “drug busts.” However, efforts to detect r-HuEPO abuse remained elusive primarily due to the similar chemical structure of both the synthetic and natural forms of EPO. Subsequently much of the research was directed towards “indirect” detection in blood, accompanied by the introduction of the hematocrit (Hct) test by the UCI in the late 1990’s. Many studies suggested an upper limit of a single hemopoietic marker as possible evidence of r-HuEPO abuse. The aim of our 1999 pilot study was to investigate whether multiple indirect markers of erythropoiesis were more effective in detecting use of r-HuEPO than a single marker.

The concept of using hemoglobin parameters to detect doping was first developed during the evaluation of the blood profiles of athletes at the Australian Institute of Sport. It was recognized at that time that hematological parameters were relatively stable over time, and that because these parameters were disturbed as the result of erythropoietin administration, they may be useful as indicators of doping.

Previous research in this area had usually suggested single indirect markers of r-HuEPO use. Findings had included that the serum concentrations of transferrin receptors increased with the administration of EPO,¹ and that the hemoglobin content of reticulocytes decreased with EPO use.² What emerged from looking at this previous research was the question of whether multiple markers of accelerated red blood cell production could provide greater discriminative power to detect r-HuEPO use than single markers.

The initial stage of research into this question was conducted in 1999, and involved three groups: placebo, EPO injection plus intramuscular iron injection, and EPO injection plus oral iron tablets. EPO injections were given three times per week during the administration phase, and blood samples were collected regularly. Additionally, hemoglobin mass was measured before, afterwards, and four weeks after administration ceased.

During the administration phase, participants’ hematocrit increased significantly – to such a level that would correspond to about a 4 percent increase in athletic performance terms. Percentage of macrocytic cells, reticulocyte hematocrit, and soluble transferrin receptor also increased during the administration phase, as did, of course, serum EPO levels.

It was also found that the group receiving oral iron tablets seemed to respond to EPO more than those given intramuscular

iron injections, though the reason for this is not known – possibly being attributable simply to individual responses.

To form the original experimental model, the five tested parameters (reticulocyte hematocrit, serum EPO, soluble transferrin receptor, hematocrit, and percent macrocytes) were each assigned a score. Data for the parameters from days 22 and 24 of the EPO administration phase were averaged for each group, and differences between the r-HuEPO and placebo groups were quantified. This resulted in a program in which, for example, an athlete who had elevated scores for only one parameter would have a relatively lower overall score than an athlete in whom four or five of the parameters were elevated.

Logistic Regression (logit) analysis used to evaluate all 31 possible combinations of parameters, with the valuation based on ability of logit to correctly distinguish between r-HuEPO recipients and placebo subjects at end of period of drug administration.

These models were then taken and applied to each of the blood samples collected during the study in order to identify the different levels of sensitivity and specificity of the different combinations of models we could derive. The models were also tested by application to data from 556 blood samples from athletes who had participated in studies involving natural altitude, simulated altitude, a six-day cycling race, or normal training.

This on-model score – reflecting the combination of all five parameters – correctly identified 94-100 percent of r-HuEPO recipients during the final two weeks of drug administration. There was one false-positive result in the placebo group, and one false-positive in the reference group (after the first night at simulated altitude).

Off-model analysis in this study, which was conducted using the hematocrit, reticulocyte hematocrit, and EPO parameters, was conducted 12-21 days after the cessations of EPO administration. This off-model correctly identified up to 72 percent of participants who had received EPO. There were no false-positive results in the off-model analysis in either the reference or placebo groups.

The overall conclusion, then, of this 1999 study was that multiple indirect hematological and biochemical markers used in combination are potentially effective for identifying current and recent users of r-HuEPO.

EPO 2000

During late August 1999, the International Olympic Committee (IOC) called for research to explore ways to detect EPO use. We at the Australian Institute of Sport submitted a proposal to the IOC, for which a research grant was approved in December 1999.

A major aspect of the EPO 2000 effort was to access international profiling study data. To this end, many blood centers which

had studied the population we would be testing were contacted, and cooperative arrangements were made.

The international profiling portion of EPO 2000, which included data from North and South America, Africa, Asia, Southeast Asia, and Australia, had the goal of collecting blood samples from many countries to determine whether there were factors that could confound the test. These factors included gender, injury, sport, ethnic background, exercise and altitude/hypoxia. Blood profile signatures for this phase of the study were scored during a training period, during which three samples were taken from athletes over a two-week period.

Testing was continued with a repeat of the 1999 trial protocol in Australia (using more subjects than the original trial), and in Beijing, China, using members of a different ethnic group.

Two additional trials were undertaken, in Australia and Norway, to explore whether use of low-dose EPO could affect the study methodology.

Preliminary results from the EPO 2000 study at this point showed that responses were similar for all parameters across the 1999, Sydney 2000, and Beijing trials.

Next, to develop the test that was eventually submitted to the IOC, data from the Sydney trial were used to refine the models that were first developed in the 1999 study. Data from international profiling study were used to establish typical on- and off-model scores for the athlete population, and the models were integrated into a practical r-HuEPO testing procedure.

Also assessed were the effects of altitude, simulated altitude, repeated days of intense exercise, and various hematological abnormalities. There were no false positives associated with these factors.

The findings of the EPO 2000 validation study validated the trial results of the 1999 study, and also found that ethnicity, altitude exposure, intense exercise and/or competition did not significantly affect blood parameters.

Analysis of the on-model, which tested for current use of EPO, found that out of about 3500 subjects, only two false-positive results were returned. So, while this model was highly sensitive, it could not be used by itself for the detection of r-HuEPO abuse.

Analysis of the off-model, which tested for use of EPO up to four weeks after drug cessation, showed the test to be highly specific, with no false-positive results from the testing of about 3500 subjects.

Overall, this validation study showed that an 'indirect' approach was a viable approach for the detection of EPO use.

In July 2000, these results were taken to the IOC, which had called a special meeting of the doping commission. The researchers involved and the findings were subjected to quite intense questioning over the course of the two-day meeting, with the outcome being that the on-model protocol, in combination with the direct urine test for EPO, were accepted as a sanctionable test.

The EPO test was implemented in the Sydney Olympic Games, with approximately 300 blood samples taken in the two weeks prior to the opening ceremony. It is believed that this implementation had a deterrent effect. The testing resulted in one high on-model score among the tested athletes, but the urine test was not positive in this case, so it was not sanctioned. Of the 300 tests performed, there were also seven high off-model results.

Since the Sydney Games implementation, more research has resulted in the refinement of the models used. This research has resulted in a simplification of the models under which the hematocrit parameter was replaced by hemoglobin, reticulocyte hematocrit was replaced by percent reticulocytes, and percent macrocytes and serum transferrin were excluded, with serum EPO remaining. These changes have resulted in more sensitive models that are able to detect EPO abusers in low-dose maintenance phases. *BLR*

References

1. Gareau et al, *Horm Metab Res.* 26:311-312, 1994.
2. Brugnara et al, *J Lab Clin Med.* 123:660-667, 1994.

Laboratories Accredited by the International Olympic Committee for Doping Control Analyses

Source: IOC Medical Commission listing dated January 2001

Athens, Greece	Olympic Athletic Center of Athens	(30.1) 686 85 49
Bangkok, Thailand	National Doping Control Center	(662) 245 6701/03
Barcelona, Spain	Institut Municipal D'Investigacio Medica	(34.93) 221 10 09
Beijing, People's Republic of China	China Doping Control Center	(86.10) 64 98 05 25
Bloemfontein, Republic of South Africa	University of the Orange Free State	(27.51) 401 31 82
Cologne, Germany	German Sports University	(49.221) 497 13 13
Ghent, Belgium	Department of Doping, Ghent University	(32.09) 264 73 47
Helsinki, Finland	United Laboratories Ltd.	(358.9) 50 60 51
Huddinge, Sweden	Huddinge University Hospital, Doping Control Laboratory	(46.8) 58 58 10 75
Kreischa, Germany	Institute for Doping Analysis	(49.352) 06 20 60
Lausanne, Switzerland	Laboratoire Suisse D'Analyse du Dopage	(41.21) 314 73 30
Lisbon, Portugal	Instituto Nacional do Pesporto	(351.21) 795 40 00
London, England	Drug Control Centre	(44.20) 7848 4848
Los Angeles, California	UCLA Olympic Analytical Laboratory	(1.310) 825 2635
Madrid, Spain	Laboratorio de Control del Dopaje	(34.91) 589 68 89/88
Montreal, Canada	INRS-Institut Armand-Frappier-Santé	(1.514) 630 88 06
Moscow, Russian Federation	Antidoping Centre, Moscow Dope Control Laboratory	(70.95) 261 92 22
Oslo, Norway	Hormone Laboratory	(47.22) 89 43 68/89 40 07
Paris, France	Laboratoire National de Depistage du Dopage	(33.1) 46 60 28 69
Penang, Malaysia	Doping Control Centre	(60.4) 659 56 05
Prague, Czech Republic	General Faculty Hospital	(420.2) 81862332
Rome, Italy	Federazione Medico Sportiva Italiana	(39.06) 808 30 11
Seoul, Korea	Doping Control Center, KIST	(82.2) 958 50 65
Sydney, Australia	Australian Sports Drug Testing Laboratory	(61.2) 94 49 01 11
Tokyo, Japan	Mitsubishi Kagaku Bio-Clinical Laboratories, Inc.	(81.3) 5994 2351

Additional Resources:

World Anti-Doping Agency (WADA)

As part of the Lausanne Declaration on Doping in Sport, the World Anti-Doping Agency was established in 1999 to promote and coordinate the fight against doping in sport internationally.

<http://www.wada-ama.org>

Anti-Doping Code of the International Olympic Committee

This IOC resource includes lists of prohibited substances and methods, as well as the full text of the Olympic Movement Anti-Doping Code.

http://www.olympic.org/ioc/e/org/medcom/medcom_antidopage_e.html

Notes:

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