

SPECIAL WORKSHOP

Blood Doping in Sports and Detection Strategies

I. The EPO Epidemic in Sport

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From time immemorial, throughout the world, man has always tried to find substances of mineral or vegetable origin that could give him different sensations (excitement, reducing tiredness, strength enhancing) or to heal and prevent various ailments.

With the birth of modern sport, these substances have served to enhance performance in sport. Thanks to pharmacological progress, the choice of products has widened: morphine, ephedrin, strychnin, amphetamines, etc. More recently, corticoids, anabolic steroids and autohaemotransfusion have been introduced, and in the last decade, peptide-like hormones (EPO, GH) have appeared.

This is not a characteristic that is peculiar to the world of sport, rather it reflects the trend in our society. In our individualistic and competitive life, it becomes vital to show a strong performance in order not to suffer failure, and to achieve success. One has to excel in all activities, professional, leisure or sports. All means are justified to reach the targeted goals, particularly making use of medicines, drugs, and other methods.

What is the difference between sporting practice and daily social practice? The sportsperson makes use of the same products (but is prohibited from using certain products because of his sporting status, while other individuals can freely consume them), favouring those that cannot be detected and have proven their effectiveness, like EPO. While the system can induce some to consume such products, following a logic of high performance and undetectability, there is also a 'snowball' effect which increases the spread of these products to every level. The fear of not being competitive, the conviction that it is essential to take them in order to achieve success, and above all the rewards (financial, media and social) brought about by success engender a constant quest to obtain these molecules.

Faced with substances that cannot be detected (EPO today, others in the future), the International Cycling Union has decided to use various strategies in order to preserve the runners' health. In 1996, it funded Canadian and Swiss research aimed at detection in the urine, which did not, however, have the hoped-for results. In order to limit the risks inherent in taking EPO, it introduced a haematocrit test before the competition from 1997, setting a limit above which the runner was declared unfit for 15 days.

In 2000, the Chatenay-Malabry laboratory developed a method of detecting EPO in the urine. However, this

method has a drawback since detection is only possible 2-3 days after absorption.

Since then, the UCI has been investigating the possibility of carrying out combined blood-urine tests, with the aim of subjecting those with haematological values found during the pre-race tests, which suggested a stimulation of the bone marrow, to anti-doping tests.

This is only a first step, because we are aware that new challenges will present themselves in the months and years to come, due to the arrival on the market of new forms of erythropoietin (EPO-retard, EPO-mimetics), oxygen transmitters (reticulated and synthetic haemoglobins, PFC), not to mention the threat of genetic manipulation.

2. Hematology Parameters: Usefulness and Limitations in Monitoring Red Blood Cell Production

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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. We will review here the relative value of many of them with specific reference to their possible application to the detection of hematopoietic changes induced by increased erythropoietic stimulation induced by endogenous Epo production or exogenous rhu-Epo administration.

Automated blood cell counting: quantitative evaluation. Hemoglobin concentration and packed red cell volume (hematocrit) are commonly used for the assessment of the degree of anemia or polycythemia. Although the analytical precision and accuracy of these measurements obtained by automated blood cell counters is usually acceptable for clinical decision purposes, a number of analytical variables, including the many different causes of preanalytical variability, must be taken into account. In general terms, the accuracy of hematocrit is more subject to interferences and pitfalls, since hematocrit provided by cell counters is a virtual parameter calculated from red cell count and MCV. Thus, regardless of tradition and recent sports legislation, the measurement of hemoglobin should be preferred as an indicator of anemia or polycythemia.

Automated blood cell counting: analysis of red cell properties. MCV and RDW are provided by all hematology analyzers and, together with the shape of red cell distribution histograms, permit a first level classification of anemia. Increased RDW may indicate exogenous erythropoietic stimulation,

although definitive studies are missing. Percentages of red cells with abnormal size or hemoglobin content have been successfully used by several investigators to point out the production of young hypochromic macrocytes following rHu-Epo.

Automated blood cell counting: flow-cytometric reticulocyte parameters. The introduction of flow-cytometric methods for reticulocyte counting has increased the diagnostic power of this time-honored parameter thanks to lower imprecision and the availability of new measurements. Among these, reticulocyte indices (MCV, MCH and MCHC) and the fraction of immature stress macroreticulocytes (IRF) can be monitored to point out their sudden changes following erythropoietic stimulation or inhibition. MCV and IRF seem particularly effective to show the reticulocyte response to rHu-Epo in normal subject as well as in patients with uremia.

Automated blood cell counting: nucleated red blood cell count (NRBC). Accurate and reproducible count of NRBC is now provided by two different hematology analyzers. The presence of circulating NRBC is associated with hypoxia and severe anemia.

The evaluation of different combination of these hematologic parameters, in association with biochemical tests (serum or urine Epo, sTfR, ferritin) can certainly provide a real time effective assessment of erythropoietic function in diseases. In healthy subjects and athletes using rHu-Epo their relative utility is under evaluation.

3. Erythropoietin Pathophysiology, Clinical Uses of Recombinant Human Erythropoietin, and Medical Risks of Its Abuse in Endurance Sports

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Erythropoietin (Epo) is primarily made by a single organ, the kidney, outside the bone marrow and participates in a classic negative feedback control system. Factors other than tissue hypoxia, however, appear to be involved in the regulation of erythropoietin production. In addition, there is an inverse relationship between red cell precursor mass and serum Epo level: the higher the number of red cell precursors, the faster the erythropoietin clearance. Erythropoietin is mainly a survival factor for human erythroid progenitor cells, in particular CFU-Es and proerythroblasts. These cells require continual presence of erythropoietin to survive although they vary widely in their erythropoietin sensitivity. This physiological control of erythropoiesis is relevant to the clinical use of recombinant human Epo (rHuEpo). In fact, when erythropoietin levels are inappropriately low, administration of rHuEpo can be effective in allowing survival of more CFU-Es and generation of erythroid precursors that subsequently mature to red cells. The definition of defective erythropoietin production relies on a low serum erythropoietin in compar-

ison with reference patients with similar Hct (or Hb). Practicing physicians should assess the adequacy of endogenous erythropoietin production in an individual patient through the observed/predicted log (serum erythropoietin) ratio (O/P ratio). The O/P ratio is below 1 if the observed value is lower than the predicted one; in reference subjects, the 95% confidence interval ranged from 0.80 to 1.20.

Over 500,000 patients throughout the world are now receiving rHuEpo for the treatment of anemia of renal failure and deriving great benefit from such treatment in terms of both quality of life and prolongation of survival. In the last years, in addition, rHuEpo has been approved for other indications, including prevention of anemia in surgical patients or in patients undergoing platinum-based chemotherapy, treatment of anemia of prematurity, of anemia induced by zidovudine therapy in HIV-infected patients, and of anemia induced by chemotherapy. Erythropoietin should routinely be given subcutaneously to maximize its effects. Most patients under rHuEpo treatment develop functional iron deficiency, a situation where iron supply to the erythroid marrow is inadequate for the red cell precursor demand. Iron supplementation should therefore be given to all individuals receiving rHuEpo but those with increased serum iron and transferrin saturation. rHuEpo is remarkably well tolerated when employed for the above indications. Adverse reactions have been described mostly in chronic renal failure, with development of hypertension and seizures, as a consequence of a rapid increase in red cell mass.

Several observations indicate that blood doping with rHuEpo is particularly common in endurance sports, and that this is no longer a problem restricted to professional athletes, since it now involves also amateurs and young athletes. Consequently, this vitally important drug – that can prolong survival of thousands of patients – is nowadays reported by the media as a doping drug. Prospects for the next years are discouraging. In fact, the major pharmaceutical companies are currently developing long-acting, modified Epo molecules. One weekly injection of a long-acting stimulator of erythropoiesis would be the ideal procedure for dishonest athletes.

There is speculation that blood doping with rHuEpo may be involved in the death of professional cyclists from the Netherlands in the early '90s. At that time, rHuEpo abuse was largely uncontrolled and Hct values in excess of 60% were presumably achieved. These polycythemic conditions compounded by dehydration during exercise readily predisposed athletes to thromboembolic complications. Nowadays rHuEpo abuse is undoubtedly more finely tuned. However, the medical risks associated with blood doping are still considerable.

Erythropoietin markedly enhances endothelial activation and platelet reactivity in humans, and these may substantially increase the risk of thromboembolic complications especially in individuals with a genetic predisposition to thrombophilia. Although a minority of athletes abusing rHuEpo will eventually develop a thromboembolic disease, the

unlucky ones might die because of this, or experience serious handicaps for the rest of their life. Administration of rHuEpo also involves an increase in the systolic blood pressure during submaximal exercise. Recent observations in animals are particularly worrying with respect to erythropoietin abuse. In a rat model, cessation of intensive rHuEpo therapy was followed by a strong inhibition of erythropoietic activity with secondary anemia. This was not due to antibodies or other soluble inhibitory factors, a defect in endogenous Epo production, or a loss of sensitivity to Epo. The above effect rather represented intrinsic erythroid marrow exhaustion, mostly at the level of erythroid progenitors. In addition, chronic inborn erythrocytosis leads to cardiac dysfunction and premature death in mice overexpressing erythropoietin. These findings suggest that erythrocytosis per se may result in cardiac dysfunction.

A large portion of the professional cyclists whose data have been examined in recent investigations by Italian magistrates show a degree of iron overload comparable to that of patients with genetic hemochromatosis, with ferritin levels often in excess of 1,000 ng/mL. These individuals were clearly given intravenous iron together with rHuEpo. Although intravenous iron is primarily taken up by the reticuloendothelial cells, it is later redistributed to parenchymal cells. Therefore, this type of iron overload will eventually produce organ damage comparable to that occurring in genetic hemochromatosis, including the risk of developing hepatic carcinoma.

As physicians, one of our major duties is to prevent diseases, and we have sworn this with the Hippocratic oath. Since abuse of rHuEpo exposes athletes to several medical risks, we must do every effort to prevent it.

4. 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. They include hereditary hemolytic anemia, hemochromatosis, autologous blood donation, and therapy with recombinant human erythropoietin (r-HuEPO). The knowledge acquired in these setting has allowed us to identify a "relative iron deficiency" which 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 utilization of r-HuEPO. Several studies have assessed the value of biochemical and laboratory parameters in identifying this condition. The in-depth knowledge on the behavior of these parameters in normal subject and patients treated with r-HuEPO has led to the indirect method for detection of r-HuEPO-induced

blood doping used in the Sydney Olympic Games. Biochemical parameters such as iron saturation, ferritin, and circulating transferrin receptor (TfR) have not been proven to be useful in this settings: they lack the sensitivity, and specificity required to reliably identify a transient state of iron deficiency. Erythrocyte parameters such as erythrocyte ferritin and erythrocyte zinc protoporphyrin (ZPP) have also not proven useful. Macdougall IC et al (*Br. Med. J.* 1992; 304:6821) provided the first evidence for functional iron deficiency in the setting of r-HuEPO therapy with the identification of increased production of hypochromic red cells. Similar findings were obtained in normal subjects treated with r-HuEPO in the setting of autologous donation (Brugnara C et al. *Blood* 1993; 81:956). Since instruments based on electrical impedance are known to provide inaccurately higher values of MCHC in samples with hypochromic erythrocytes, most of the published studies in this area have used flow cytometric-based methods. Reticulocyte parameters such as absolute reticulocyte count and reticulocyte Hb content (CHr) have also proven to be useful in the identification of functionally iron deficient states. The advantage of CHr is that it provides a real-time assessment of the balance between iron availability and Hb synthesis. Studies have shown the value of this parameter in a simulated perisurgical setting and in the anemia of chronic renal failure (Brugnara C et al., *J. Lab. Clin. Med.* 123:660; Fishbane S et al., *Kidney Inter.* 1997; 52:217). The challenge is to now to integrate these laboratory parameters into reliable and simple algorithms for physicians to use in patients being treated with r-HuEPO or with any novel stimulator of erythropoiesis.

5. Red Cell Parameters in Winter Endurance Sports

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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 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 rhEPO 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 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 rhEPO. Due to the problem of false positives and false negatives associated with measuring hemoglobin or hematocrit alone, in conjunction

with the ISU medical committee, we have developed the S.A.F.E. program for the International Skating Union (ISU). The S.A.F.E. program (Safe And Fair Events) 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. Over the course of the last two winter World Cup seasons, we have obtained over 1500 samples from ~600 world-class endurance athletes in ISU, IBU and FIS. 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 1300m and 200 at 1750m. Specimens have been analyzed on 6 different Bayer Advia 120 hematology analyzers. Analyzers were carefully calibrated to control material prior to use. We will present the range of red cell parameters measured in that material including data from the spectrophotometric channel, as well as, the flow cytometry channel. Data were examined for evidence of accelerated or decelerated erythropoiesis, free hemoglobin or transfusions. Variables of interest include; hemoglobin, hematocrit, % macrocytes, number and percentage of reticulocytes, content of reticulocytes (CHr) and mature red cells (CHm), cell volume of reticulocytes (MCVr) and mature cells (MCVm), extracellular hemoglobin, distribution characteristics of the red cell population and calculated variables including reticulocyte hematocrit and reticulocyte hemoglobin. Results will be presented.

6. Cross-linked Hemoglobin Solutions

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Although "blood substitutes" include both the hemoglobin based oxygen carriers (HBOCs) and the perfluorocarbons, the HBOCs offer the potential to enhance oxygen transport and athletic performance. The HBOCs are made from hemoglobin from various sources that are stabilized by cross-linking to reduce much of the toxicity of cell-free, unmodified hemoglobin. A number of pharmacological and physiological properties of the HBOCs affect oxygen delivery to tissue. These properties can affect blood rheology, unloading and offloading of oxygen and tissue blood flow. The interaction of these properties with the physiological modifications that enhance performance in elite athletes may lead to improved or diminished oxygen delivery and utilization in tissues during exercise. The HBOCs, being made from hemoglobin are deep red in colour and will therefore not present a problem for identification in serum/plasma samples. However, their differentiation from hemoglobin from hemolysed red cells must be made. These products have a characteristic "finger print" on chromatography such that not only their presence can be verified, but their source may also be determined. Since excretion in the urine is inconsistent, urine samples alone will not be adequate for detection.

Although the HBOCs offer the potential for performance enhancement in elite athletes, their detection in blood samples should be a relatively simple matter for qualified laboratories.

7. Hemoglobin Modifiers: Is RSR13 the Next Aerobic Enhancer?

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RSR13 (2-[4-[2-[(3,5-dimethylphenyl)amino]-2-oxoethyl]phenoxy]-2-methylpropanoic acid sodium salt [CAS #170787-99-2]) is a small molecule that facilitates oxygen unloading from blood by reducing hemoglobin-oxygen binding affinity through allosteric binding to the hemoglobin tetramer. Intravenous administration of RSR13 results in an immediate and sustained reduction of hemoglobin-oxygen affinity that emulates and amplifies the effects of natural allosteric modifiers of hemoglobin such as H⁺, CO₂, and 2,3-diphosphoglycerate. This approach has broad clinical applicability in conditions characterized by tissue hypoxia due to reduced blood flow (regional or global), reduced oxygen carrying capacity, and/or increased tissue oxygen demand. Numerous nonclinical pharmacology studies have shown that RSR13 can increase oxygenation of a variety of hypoxic tissues including myocardium, skeletal muscle, tumors, and cerebral tissue. RSR13 has never been tested in humans for athletic performance enhancement. However, Allos collaborators have demonstrated that RSR13 increased oxygen extraction in dog skeletal muscle during exercise by 15% and muscle VO_{2max} by 25%. These animals had an increase in p50 (pO₂ that results in 50% saturation of hemoglobin) of 21 mmHg and received supplemental oxygen to maintain arterial oxygen saturation.

A therapeutic dose of 75 – 100 mg/kg increases p50 by approximately 10 mmHg, a shift that optimally facilitates oxygen unloading to tissues. However, it is possible that a lower dose of RSR13 could enhance athletic performance. A minimally effective therapeutic dose of RSR13 is approximately 50 mg/kg. This dose would increase the p50 by approximately 2-4 mmHg for approximately 2 hours and would require an infusion volume of 350 mL administered IV over approximately 45 minutes. Peripheral venous infusion can cause pain and irritation, which limit the rate and concentration of RSR13 solution administered by this route.

The peak pharmacodynamic effect (p50) occurs immediately at the end of infusion. The half-life in healthy subjects is approximately 3-6 hours. Administration of RSR13 in excess of the therapeutic dose without supplemental oxygen administration can result in significant arterial desaturation and hypoxemia, which could lead to impairment of athletic performance. Side effects of administration can include nausea, vomiting, and headache, effects that are obviously not

conducive to enhanced athletic performance. RSR13 is bioavailable by oral administration, although the required dose, surfactant nature of RSR13, and potential for mucosal irritation would make oral ingestion problematic.

Renal elimination is the primary route of RSR13 excretion, and validated sensitive methods for the detection of RSR13 in human blood and urine exist. Even a minimally therapeutic dose of RSR13 could be easily detected in urine from the time of infusion to at least 24 hours after administration.

Phase II and III RSR13 clinical trials are ongoing in Canada and the US for enhancement of radiation therapy in patients with brain metastases, glioblastoma multiforme, or non-small cell lung cancer. More than 300 cancer patients have received daily RSR13 for up to 32 days. Allos plans to submit a New Drug Application for RSR13 in 2002 and market the drug in 2003.

8. Blood Substitutes: Detection Methods and Limits

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The intravenous use of cross-linked (XL) hemoglobin (Hgb) solutions is currently investigated as a potential replacement for red blood cell transfusions. Its main, clinical, side effect appears to be a moderate increase in mean systemic arterial pressure, while a rise in pulmonary arterial pressure has also been reported. Clinical studies in humans are still in Phase I and only a modest number of studies have been published. Use of XL-Hgb solutions in some sports is said to be widespread, but for obvious reasons no official data are available. One of the sports in which its use is well documented, at least in the lay press, is professional cycling. Since common screening procedures in cycling include measurement of the red cell packed volume or hematocrit (Hct) by hematology analyzers, the expectation is that use of XL-Hgb solutions will go undetected by that method. However, the XL-Hgb is readily detectable by spectrophotometry or regular hemoglobinometry of the supernatant plasma, after the blood is spun by centrifugation. It can also be demonstrated that this XL-Hgb is different from an individual's native Hgb, for instance by electrophoretic behavior.

We have carried out an *in vitro* study with normal blood to which XL-Hgb (Hemolink(r)) was added at various concentrations and were able to show the presence of low concentrations of XL-Hgb, equivalent to the lowest dose of 25 mg/kg as used in clinical trials with humans. We were able to detect XL-Hgb by conventional hemoglobinometry of the supernatant plasma of spun blood samples, but not by red blood cell indices. Furthermore, we could show that XL-Hgb behaves differently from native Hgb by using

reversed-phase high performance liquid chromatography and other techniques.

It is thus possible to demonstrate in the plasma of blood specimens small amounts of XL-Hgb, using regular laboratory techniques, and to prove conclusively that this hemoglobin could not have been derived from lysed autologous red blood cells.

9. Biochemical and Hematological Parameters in Athletes

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Since 1985, our team has been studied hematological and biochemical parameters for a lot of sportmen and sportwomen, particularly in exclusive aerobic sports like running, cycling, cross country skiing or triathlon, but also combined aerobic-anaerobic like football or rugby. Excepted for some cases of subjects in situation of constitutional erythrocytosis, we always found standard levels of red cells parameters. After biological follow-up through a sport season, we often noticed a drop of red cells parameters. The monitoring of the iron status was suggestive of the negative impact of training on erythropoiesis and iron metabolism. In this context, a treatment by iron brought was a necessity with a posology and an oral administration. These situations are well known in international publications on this matter.

At the end of the nineties, we began to observe some modifications of this previous model!

In fact, on a few subjects the red cells decrease was not constant and the increase of iron parameters seemed surprising. A relation with an excessive use of iron (by injections) was noted. We also noted frequently a lot of curious disorders on indirect parameters of erythropoiesis like sTfR, EPO and ferritin.

From to 1997, we documented studies (after confession of EPO use) with significant hematological and biochemical anomalies.

From all these data, it was easy to define an hematological and biochemical profile to detect on medical bases (and not toxicological) the both use of EPO and injectable iron.

This profile was systematically applied to the biological follow-up which was obligatory undergo by the best French cyclists since 1998 [Table I].

The first results before the beginning of the season shown big anomalies of ferritin, without any inflammatory process [Table II]. On this initial population, we present figures issues of 53 subjects who admitted (under confidentiality) the uptake of EPO during the previous season, many weeks before the blood sampling.

The obligation for the cyclists to have 4 blood analysis per year permitted to study parameters a short time after EPO use (1week after). The discovery of iron overloadings, which interfere with sTfR rate, entails to realize the profile that we have specify and publish in 1999.

To understand the kinetics of many parameters of this profile, we analysed the behaviour of 6 patients which suffer of myelodysplastic syndrome and hemochromatosis [Figure].

The relation between these different parameters and the exogenous disturbances of iron metabolism in EPO users, let us think that the indirect approach is efficient to detect an EPO consumption but makes difficult its absolute use as anti-doping test. Nevertheless, it authorizes to order a sick leave.

For 3 years, this policy has been introduced by French Cycling Federation (F.F.C.) for its 700 best cyclists.

This year 2001, U.C.I. (Union Cycliste Internationale) recommends a biological surveillance on these same parameters.

The inter-technological study organized in Plouay (World Cycling Championship – 2000) under the aegis of U.C.I., shows a good correlation between different methods and machines used to analyse discriminant parameters (Hb, Ht, VGM, RDW, reticulocytes, RC and ret parameters, ferritin, sTfR).

This work has been realized with the support of 8 biomedical companies: Abbott, ABX, Bayer, Beckman Coulter, Dade Behring, Eurogénétics, Nichols and Roche Diagnostics.

10. Evaluation of Methods to Assess Erythropoietic Stimulation

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In 1987, EPO was genetically engineered for therapeutic use. Shortly thereafter, athletes began to use it to increase performance, particularly in endurance sports: It may have been used as early as 1988 in the Calgary Winter Olympics skiing events. Seventeen deaths among cyclists were attributed to EPO between 1989 and 1990, although no formal proof was ever produced. r-Hu EPO, a glycoprotein of 30400 Da, has a short half-life, although its effects in the blood only become evident 3-5 days after administration. These effects subsist for one to several weeks in cases of prolonged treatment. If treatment is stopped a few days before competition, the athlete thus benefits from its effects without risk of it being detected. We therefore oriented our antidoping research toward finding biological markers of EPO that would remain in the body after the injected molecule had itself disappeared.

Two methods were investigated. The first was in urine and was based on the work of Gareau, who noted urinary secretion of TDP (total fibrolytic factor) after EPO administration. The second was in blood by tracking sTfR (soluble transferrin receptor) and was based on the initial work of Beguin and Gareau. The first blinded experiment in 1995, with 20 subjects and 10 controls, demonstrated the interest of measuring serum sTfR and the serum sTfR/ transferrin ratio. Unfortunately, because the ferritin level can be increased by iron supplementation, this parameter was not adapted to anti-doping testing.

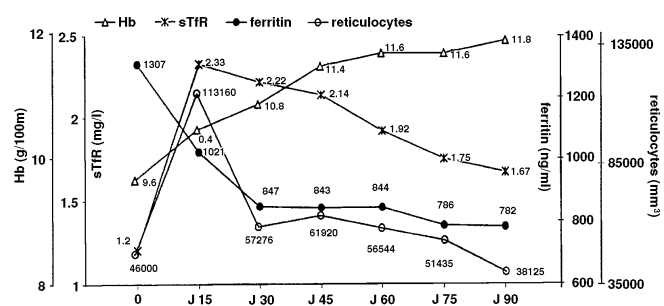
A second experiment in 1997 with high-level athletes both confirmed the interest of measuring serum sTfR and demonstrated the need to take hematological parameters into account, including hematocrit and the number of reticulocytes, as well as the serum level of EPO. Since then, complementary studies have been done with healthy volunteer athletes, athletes before and after exercise, and subjects staying at high altitude in order to fix the serum sTfR threshold above that which would allow us to confirm an illicit acceleration in erythropoiesis. Yet these studies have not resulted in an undisputed test of EPO administration. They do, however, indicate the potential of indirect methods of detection, which are the only methods that can detect the illegal use of hormones on the day of athletic competition. Such an approach was, in fact, tested during the Olympic Games in Sydney.

Table I. Biological profile in relation with a suspect stimulated erythropoiesis (EHA4, 1999)

	Male	Female
Relative polycytemia		
RBC (106 / ml)	> 5,5	> 5,2
Hb (g/100 ml)	> 16,5	> 16
Ht (%)	> 47	> 45
RDW	> 15	> 15
Reticulocytes	> 150 000	> 150 000
Ferritin (ng/ml)	> 500	> 500
EPO (mU/ml)	< 6 and >16	< 6 and >16
sTfR (UI/ml or mg/l)	>800 or 1,80	>800 or 1,80

Table II. Hyperferritinemia (Blood, 1999)

FERRITIN	Cyclists with ferritin > 500 ng/ml (n=39 / 93)	Blood donors (n=30)	Triathlon group (n=14)
Level (mean)	806 ng/ml	125 ng/ml	107 ng/ml
Ranges			
Min :	534 ng/ml	42 ng/ml	42 ng/ml
Max :	1997 ng/ml	280 ng/ml	345 ng/ml



Biological parameters in myelodysplastic and hemochromatosis patients with RhEPO

11. Recombinant Erythropoietin in Urine

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Though the use of recombinant erythropoietin (rHuEPO) in sport is officially banned since 1989, some athletes resort to this hormone in order to improve their aerobic power as a consequence of stimulated erythropoiesis.

We have developed a procedure to detect the presence of rHuEPO in urine in an anti-doping control purpose. Since some EPO is physiologically present in urine, the test must be able to differentiate between the natural and the recombinant forms of this hormone. Such a differentiation has been obtained by analysis of the EPO isoelectric patterns after ultra-filtration (MWCO:30,000 Daltons) of urine, isoelectric focusing of the retentate and a special immuno-blotting process of EPO called "double-blotting". The results indicate that the natural urinary hormone is composed of more acidic isoforms than the eliminated rHuEPO.

Though it has been developed in an anti-doping context, this new technique is a tool to study some aspects of the microheterogeneity of EPO and therefore may be useful for biological investigation of some diseases related to this hormone.

12. The Development of a Blood Test for EPO Abuse

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Background and Objectives: 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 haematocrit (Hct) test by the UCI in the late 1990's. Many studies suggested an upper limit of a single haemopoietic 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.

Design and methods: The 1999 study involved 27 recreational athletes assigned to three groups with the following protocols: EPO + Intramuscular iron group (n=10), receiving 50 IU/kg r-HuEPO at 3/wk and 100 mg of intramuscular iron at 1/wk and a sham iron tablet daily; EPO + Oral

iron group (n=8), receiving 50 IU/kg r-HuEPO at 3/wk, a sham iron injection at 1/wk and 105 mg of oral elemental iron; placebo group (n=9) receiving sham r-HuEPO and iron injections and sham iron tablets. In the 2000 study recreational athletes in Sydney (n=49; 16 females and 33 males) or Beijing (n=24; 12 females and 12 males) were randomly assigned to r-HuEPO or placebo groups. Injections of r-HuEPO (or saline) were administered double blind at a dose of 50 IU/kg, 3/wk, with oral iron (105 mg) or placebo supplements taken daily by all subjects. In both studies the administration period was 25 days where blood profiles were monitored during, and for 4 weeks after the administration phase. Logit analysis (1999) and Fisher's discriminant

Results: During the period of r-HuEPO administration, our algorithm repeatedly identified 94-100% of r-HuEPO group members during the final two weeks of administration (one false positive). After ceasing r-HuEPO administration, a separate equation (including RetHct, EPO and Hct) repeatedly identified up to 72% of recent EPO users up to 21 days after the last dose (no false positives). The results obtained during the 2000 study were qualitatively similar in athletes from Sydney, and the same haematological response was also demonstrated in Beijing athletes.

Interpretation and Conclusion: The 1999 study demonstrated that multiple indirect markers used simultaneously were potentially effective for identifying current or recent users of r-HuEPO. The 2000 study confirmed that r-HuEPO administration causes a predictable and reproducible haematological response both during and for several weeks following administration. Ethnicity did not influence these markers.

13. Testing for Erythropoietin at the Next Olympic Games

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The ability to perform sustained aerobic exercise depends on both oxygen delivery to the muscles and the ability to utilize it. Rumors that various types of blood doping were being used by some athletes to enhance performance began thirty years ago. The first documented episode of doping at the Olympics occurred in 1984. When erythropoietin (EPO) became available in the late eighties, blood doping became virtually obsolete. The International Olympic Committee added erythropoietin to the list of prohibited substances in 1992 due to rumors that it was being abused by sport-persons involved in aerobic events. These rumors were confirmed when it was discovered that many cyclists competing in the 1998 Tour de France used erythropoietin. By increasing the production of red cells, EPO delivers more oxygen to muscles and therefore increases exercise capacity. If excess EPO is administered blood viscosity increases and performances declines. Until recently it has not been possible to detect syn-

thetic EPO in body fluids, thus EPO use has been discouraged by monitoring packed red cell volume. The 2000 Games of Sydney marked a breakthrough in EPO detection when an indirect screening method based on various red cell indices, and the concentration of transferrin receptor and EPO was employed (Parisotto et al. *Haematologica*, 2000;85:564). More importantly, a confirmatory method for detecting exogenous erythropoietin in urine was introduced. (Lasne and de Ceaurriz, *Nature*:2000;405:635) This test requires considerable sample preparation, separation of EPO idiotypes by isoelectric focussing, immunoblotting, and detection (CCD camera) of IgG labeled with streptiverdin-peroxidase. Since Sydney several IOC laboratories have been working to further validate the test and to render it more efficient and robust. Our plans for the Games of Salt Lake are to replicate the work of Lasne and de Ceaurriz and perhaps to improve it. At the present moment we plan to screen blood samples using the method of Parisotta et al. or a variant thereof. However, the indirect blood screen could be deleted, if progress on the direct urine test for EPO is satisfactory.

14. The U.S. Anti-Doping Agency and Future Priorities

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It is critical to separate the Olympic movement from the doping issues and benefit the health, integrity, and ethics of all athletic sports. The United States Anti-Doping Agency (USADA) was formed to bring accountability and transparency to the Olympic doping issues. The USADA way is taking drug testing to a new level. The USADA agenda requires testing, research, and education. In October 2000 USADA convened a research summit to identify and prioritize future research needs. Scientific issues included androgens/anabolic steroids, dietary supplements erythropoietin (EPO), Growth Hormones (GH) and related peptides as well as the important ethical issues related to the any doping research. This is a summary report of the priorities identified by the participants.