Review

Drug Testing and Analysis

Received: 5 September 2011 Revised: 3 November 2011 Accepted: 21 November 2011 Published online in Wiley Online Library: 28 February 2012

(wileyonlinelibrary.com) DOI 10.1002/dta.406

Detection of EPO doping and blood doping: the haematological module of the Athlete Biological Passport

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The increase of the body's capacity to transport oxygen is a prime target for doping athletes in all endurance sports. For this pupose, blood transfusions or erythropoiesis stimulating agents (ESA), such as erythropoietin, NESP, and CERA are used. As direct detection of such manipulations is difficult, biomarkers that are connected to the haematopoietic system (haemoglobin concentration, reticulocytes) are monitored over time (Athlete Biological Passport (ABP)) and analyzed using mathematical models to identify patterns suspicious of doping. With this information, athletes can either be sanctioned directly based on their profile or targeted with conventional doping tests.

Key issues for the appropriate use of the ABP are correct targeting and use of all available information (e.g. whereabouts, cross sectional population data) in a forensic manner.

Future developments of the passport include the correction of all concentration-based variables for shifts in plasma volume, which might considerably increase sensitivity. New passport markers from the genomic, proteomic, and metabolomic level might add further information, but need to be validated before integration into the passport procedure.

A first assessment of blood data of federations that have implemented the passport show encouraging signs of a decreased blood-doping prevalence in their athletes, which adds scientific credibility to this innovative concept in the fight against ESA- and blood doping. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: erythropoietin; transfusion; performance; sports; manipulation

Introduction

The capacity of the organism to transport oxygen to the working muscle is a key factor for endurance performance.[1] For many years, one of the prime targets of manipulating athletes was therefore to improve the oxygen-carrying capacity of the blood as it offered gains in performance of 5–8%.[2] As early as 1970, athletes started to use the then legal transfusion of blood (blood doping) to enhance their endurance. This technique was banned in 1988 and is since a prohibited method listed by the World Anti-Doping Agency (WADA). The logistical requirements for blood manipulations were rather significant, which limited their use in the athletic population. With the commercial introduction of recombinant human erythropoietin (EPO) – the first erythropoiesis stimulating agent (ESA) – the increase of red cell mass to boost performance became available to everyone and was soon widespread in most endurance sports. This paper reviews the development of the fight against ESA doping and blood doping and focuses on the most recent technique, the haematological module of the Athlete Biological Passport (ABP).

Doping with erythropoiesis-stimulating agents (ESA)

The first recombinant human erythropoietin (rhEPO) was developed in the early 1980s and marketed in 1989 as a treatment for acute anaemia mainly associated with chronic kidney disease but also with cancer, AIDS, hepatitis C infection, bone marrow transplantation, autoimmune diseases, heart failure, or chronic infections. The use of rhEPO is particularly powerful in improving the physical performance of patients, reducing exertional dyspnea, and, more generally, improving their quality of life. Additionally, it avoids the need for blood transfusions and diminishes therefore the risks of incompatibility reactions, viral infections, and iron overload.[3] rhEPO is a blockbuster drug and different pharmaceutical companies have produced many different forms of rhEPOs.[4] First-Generation rhEPOs (epoietin-alpha and epoietin-beta) have a similar amino acid sequence as the endogenous EPO, but there are differences in the structure of glycans that constitute the side chains of the molecule. In early 2000 and in order to increase the half-life of rhEPO by the factor 3 to 4 (24–26 h vs. 4–8 h), two extra N-glycans were added to the second-generation rhEPO, Darbepoietin. In 2007, CERA (continuous erythropoietin receptor activator) was launched with a half-life of about one week. This epoietin contains a 30 KDa methoxy-polyethylene glycol polymer attached to a normal epoietin beta backbone. This drug is excreted in very low concentrations in

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urine due to the large size of the drug. With the expiration of the patent for epoietin molecules in Europe in 2004, many manufacturers have started to produce generics. Additionally, in some developing countries, a lot of black-market EPOs are synthesized and commercially available. In the new developments, EPO mimetic peptides such as Peginesatide (Affymax, Inc.) are in trials to treat patients suffering from pure red-cell aplasia due to the appearance of neutralizing anti-EPO auto-antibodies. Orally active compounds that stimulate endogenous EPO production and erythropoiesis are also being developed. These compounds called HIF (hypoxia-inducible factor) stabilizers induce the expression of the EPO gene and 200 other genes, which make the side-effects of the substance incalculable, as the activation of these other genes might trigger unwanted and uncontrollable processes in the body. Other drugs activating the endogenous EPO gene or EPO gene transfer are currently under development.[5–7]

Erythropoiesis-stimulating agents – direct detection

The traditional method in the fight against doping is the direct detection of a substance in a biological matrix. This approach has the undeniable advantage to identify the drug itself and/or its metabolites, but most probably cannot detect an EPO intake dating from more than a few days. Recombiant EPO and endogenous EPO have the same amino acid sequence, but they differ from slight glycosilation differences. Indeed, glycosilation of rhEPO takes place in CHO (Chinese hamster ovary) or BHK (baby hamster kidney) cells rather than in human cells.[8] Therefore rhEPO exhibit less sialic acid residues on their surface and are consequently less negative than endogenous ones. These different sugar structures have different apparent electric charges and enable the discrimination between endogenous and exogenous EPO isoforms.[9] For the first time in 1995, Wide *et al*. managed to separate both types of EPOs in blood and urine.[10] The use of an electrophoresis in a 0.10% agarose suspension at pH 8.6 enabled to determine the median charge of EPO. Finally, the overall results were expressed in terms of electrophoretic mobility. At that time, this method was never fully validated and accepted, because the detection window was considered too small and it could not be reproduced in another laboratory. In 2000, just before the summer Olympic games in Sydney, the Paris anti-doping laboratory proposed the isoelectric separation of urinary EPO isoforms on a polyacrylamide gel followed by a double-blotting process.[11] Due to the different isoelectric points of EPO isoforms, they could be separated by using the isoelectric focusing (IEF). This method was time-consuming and expensive, but on the contrary to the technique proposed by Wide *et al*., it was reproducible and reliable.[12] It was validated and finally accredited in various anti-doping laboratories. The first adverse analytical finding (AAF) was reported by the Swiss Laboratory for Doping analyses (LAD) in 2001. The laboratory lost this case in front of the court (Court of Arbitration for Sport – CAS), because there was a lack of harmonization between anti-doping laboratories. Nowadays and thanks to WADA, technical documents define better positivity criteria necessary to return an AAF. The three positivity criteria currently in use are (1) samples positive for rhEPO must present at least three acceptable bands in the basic area which correspond to bands 1,2 and 3 of the reference standard; (2) the two most intense bands must be consecutive and be bands 1,2, or 3; and (3) the density (measured by densitometry) of these bands must be higher (double or more) of any band in the endogenous area. The urine EPO anti-doping test was initially designed to detect the first-generation rhEPOs (epoetins $-\alpha$, $-\beta$ and $-\omega$). Once the second- and third-generation EPOs were commercially available, they were analyzed with the IEF–double blotting test. This latter showed it could easily detect both rhEPOs, but the positivity criteria had to be adapted.[13]

In order to clean up the biological samples, an immunopurification tool was set up to improve the overall quality of the results. This tool also had the advantage of excluding all potential cross-reactivity of the anti-EPO antibody.[14] The distribution of CERA by Roche (a third-generation rhEPO) at the end of 2007 required the use of this immunopurification tool for confirmation purposes, because the specific ELISA set up by Roche, WADA, and LAD was not sufficient to declare an AAF.[15] Indeed, the International Standard for Laboratories (ISL) clearly stipulates that antibodies recognizing different epitopes should be used. Immunopurification is also routinely used in combination with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as an IEF orthogonal confirmation tool. This specific confirmation test was set-up, because of some atypical IEF EPO profiles observed on some degraded urines samples (bacterial degradation) or when urine samples were collected immediately after a strenuous physical effort.[16] The use of the SDS-PAGE approach to separate apparent different molecular masses avoids the misinterpretation of IEF EPO profiles and enables to identify some of the actual rhEPO copies available is some of the emerging countries such as China and India.

Two alternative approaches have been suggested for rhEPO screening in urine: the membrane-assisted isoform immunoassay (MAIIA) and the liquid chromatography coupled to mass spectrometry.[17–19] Up to now, both approaches give very promising results in laboratory conditions, but have not yet been fully validated, accredited, and accepted by WADA. The main reason is that the receiver operating characteristic (ROC) curve has not been documented with real samples coming from clinical studies and from top level athletes.

New/custom-made rhEPO

As mentioned earlier, rhEPO copies are actually the new threat for anti-doping laboratories, because they are cheap and easy to access via the Internet. Furthermore, it is a real analytical challenge to enable the continuous detection of these molecules; because the production process is not mastered, the EPO forms are slightly different and can change from batch to batch. Conversely, when applying the official urinary EPO anti-doping test, some EPO copies do not fulfil all three WADA positivity criteria and it is extremely difficult to find the right standard. To further tackle the hurdles of such undetectable substances, WADA recently made agreements with Interpol and the World Customs Organisation in the area of evidence gathering and sharing of information on trafficking of these black-market drugs.

Despite the advances in drug development described, the gap between cheaters and the fight against doping is narrowing more and more, notably due to the recent agreement between WADA and the pharmaceutical sector to establish a framework of collaboration to facilitate the identification and transfer of information on drugs in development. Nevertheless, the high number of different ESAs available or still under development today, the limited detection window, and the necessity to collect

different matrices (urine vs. blood) to identify one or the other ESAs, make the traditional direct anti-doping analyses fairly expensive and inefficient.

Erythropoiesis-stimulating agents (ESA) – Indirect detection

To overcome these problems and the obvious and widespread abuse of rhEPO in all endurance sports, in the mid-1990s several sporting federations such as cycling and skiing introduced so-called no-start rules, where athletes who exceeded certain values in their haematologic markers (e.g. haematocrit >50%, Hb over 17 g/dl) were precluded from racing. This approach was called the 'indirect detection' of doping, as not the abused substances, but rather their effects on the body were searched for. Although the sensitivity and the specificity regarding doping of these early tests was rather poor, they had the merit to limit excessive doping.[20] Based on this first experience, a new method using a combination algorithm of markers (haemoglobin, reticulocytes and other indicators of erythropoiesis) was developed soon after.[21] These calculation algorithms, the ON- and OFF score (the so-called second-generation tests) give an indication of the status of the erythropoietic system at the time of testing. The ON score is sensible to changes in indirect markers during EPO abuse; the OFF score increases when EPO is discontinued (and red cell mass increased) and the erythropoietic system thereby suppressed. It later appeared that some of these markers, initially developed for ESA detection, were sensitive to other types of blood manipulation that enhances red cell mass, such as blood transfusion.[22,23]

Based on these first experiences relying on a cross sectional setting (the data of an athlete was compared to the data of a reference group), a more elaborated concept with a longitudinal approach was proposed,[24] where the results obtained from an athlete were compared with his or her own previous data. This theory soon after resulted in the first algorithms for the individual, longitudinal evaluation of biomarkers.[25–27]

The process of the haematological module of the ABP

Anti-doping organizations, such as international federations or national anti-doping bodies can implement an ABP programme for their athletes based on the framework outlined in WADA's *Athlete Biological Passport Operating Guidelines*. To guarantee independence between planning, interpretation, and results management, Athlete Passport Management Units (APMU) that liaise between the anti-doping organizations and the operational side of the passport should be implemented.

The nature of the passport is to use information of biological tests as indirect evidence to detect doping. For this reason, a stringent process for sample collection, processing, and evaluation of the data has been put into place to guarantee the objective use of this tool. This is of particular importance, as blood is a living tissue that even under physiological conditions, undergoes permanent changes that need either to be limited as far as possible in the process by using standardized procedures or to be taken into account in the evaluation of the data.

Collection

The conditions of the collection, especially exercise in the hours prior to the sampling and the posture adopted in the minutes before the blood draw, can considerable alter most concentration-based blood markers due to shifts in plasma volume. For this reason, the current guidelines require that blood sample should be taken after the athlete has sat in an upright position for 10 min and only if he has not exercised in the two hours preceeding the test, in order to allow the vascular volumes to equilibrate.[28]

Transport and storage

As inadaequate storage during transport of blood samples might affect several variables in the blood, most notably hematocrit or reticulocyte percentage, blood samples must ideally be stored at cold temperature (ideally between +2 and +12°) until analysis. If such storage conditions have not been respected or are not documented, careful scrutiny of the red cell indices (e.g. MCV, to exclude temperature induced swelling of red cells) is required to determine whether the result of the test has been influenced to the disadvantage of the athlete by inadaequate storage.[29,30]

Analysis

Since 2009, all samples to be included must be analyzed in WADA-accredited laboratories, where strict quality control criteria apply in order to provide analysis at forensic level. These quality control procedures comprise strict internal and external test schemes that go beyond what is used as laboratory standards in clinical chemistry or medicine.

Athlete Passport Management Unit (APMU)

The APMU plays a key role in the passport procedure. To guarantee an independent and unbiased treatment of the passport results and a qualified follow up, this unit, usually attached to anti-doping laboratories, is in charge of the operational side of the respective passport programmes. It provides expertise for targeted testing and/or appropriate test scheduling, manages the laboratory data, compiles the analytical results, performs an initial review, and liaises between the anti-doping organizations and the external experts for the in-depth evaluation of the passport data. The APMU will relate potential 'adverse passport findings' (analogous to 'adverse analytical findings' in conventional doping analysis) to the anti-doping organization. Staff at the APMU must therefore be skilled in anti-doping administration and management including legal aspects, be familiar with the pre-analytical and analytical procedures, and have knowledge in exercise physiology to ensure skilled scheduling of tests in each sporting discipline based on the expected effects of the most common doping agents.

Experts

Unlike conventional anti-doping tests, where the unequivocal presence or absence of a substance or metabolite in a sample determines whether a sample is positive or negative, the passport concept relies on scientific expertise and thus a subjective element for the evaluation of a profile. Therefore, the role of the experts who evaluate the blood profiles is crucial to decide whether the findings are indicative of doping or might originate from other causes. Aside from experience with the longitudinal evaluation of biological data, a broad range of qualifications should therefore be represented in the expert panel. Typically, experts from a laboratory medicine or analytical anti-doping background, exercise physiologists, haematologists, and sports medicine practitioners form the backbone of such expert panels.

After the first three years of experience with the haematological module of the ABP, clear tendencies for the most appropriate use and the effects of this new tool in the fight against doping became apparent:

1. It can be used to sanction athletes directly based on abnormalities in their profilesSeveral athletes have been sanctioned based on data from the ABP (see TAS 2010/A/2308, TAS 2010/A/2178). These decisions clearly underline the possibility to use the ABP as a sanctioning tool.
2. It can be used to target suspicious athletes. Information from the blood tests contained in the ABP give a clear picture on the state of the haematological system of the athlete at the time of testing. This information can be used to identify potential doping patterns and plan future anti doping tests accordingly.
3. It has a considerable deterrent effect. Population data has shown that the behaviour of the athletes has considerably changed since the introduction of the ABP. Zorzoli and Rossi[31] showed that the percentage of reticulocytes has very significantly changed and normalised since 2008, which points to a decrease in prevalence of blood manipulations in the professional cycling peloton.

However, the ABP is presently not yet used to its full capacity. The right timing of the blood tests that constitute a profile is crucial for all three points outlined. In fact, in its use as a sanctioning tool, the ABP is required to provide evidence of clear doping scenarios that can be presented and defended in front of disciplinary panels. For this reason, tests must be conducted during the periods where the haematological sytem of the athlete is most likely manipulated and, on the other hand, at times when it is normal in order to clearly illustrate the potential abnormalities in the profile.

From these facts emerges a strategy that should be used by sporting bodies implementing the ABP (Figure 1). In a first step, all athletes that are part of the programme should be submitted to a certain number of tests with the tests equally distributed during the period of competitions (thus periods where manipulation is more likely) and during periods, where the athlete is not active (and manipulation is unlikely). Profiles obtained using this approach should then be evaluated by experts in order to identify suspicious patterns. In the next step, the available passport resources are mainly to be focused on the profiles that have been found suspicious. The next tests should essentially be targeted based on information obtained from the blood values combined with the training and competition schedules (Anti-Doping Administration and Management System (ADAMS)) of the athletes, and, ideally, other sources in a forensic manner.[32,33] It seems crucial to use the expertise of the APMU or the experts already at this stage. Using this approach, testing can be scheduled to either convict the athlete with conventional, direct testing methods used at the right time (e.g. if the profile suggests the use of EPO or other drugs that can be detected using conventional methods), or to further expose doping patterns of the athlete in view of an Anti-Doping Rule Violation (ADRV) based on indirect markers. As during this process, information that might compromise the anonymity of the athlete, such as competition schedules and or training locations can considerably enhance the efficiency of the control system, it must be discussed in the future to what extend the anonymity of the athlete can be lifted at a certain stage of the process. Standards from forensic medicine should apply in this context.

In the current anti-doping structures, where the targeting is very often performed by the same structures that run the respective sporting discipline and sanction the cheating athlete, this approach cannot be implemented to its full extent, due to potential conflicts of interest. Just as in the basic principles of democracy, where a clear separation between legislative, executive, and judicative power is endorsed, the passport system should aim at separating its structures to avoid such conflicts. In the context of the passport this involves a framework with independent and skilled APMUs (e.g. attached to anti-doping laboratories) for the operational tasks. The framework outlined in WADA's *Athlete Passport Guidelines* provides such a set-up. Furthermore, sporting federations are starting to share responsibilities with independent anti-doping bodies (e.g. national anti-doping organizations) and transfer tasks such as the result management of positive cases (judicative power). This can be considered as a first step in this direction.

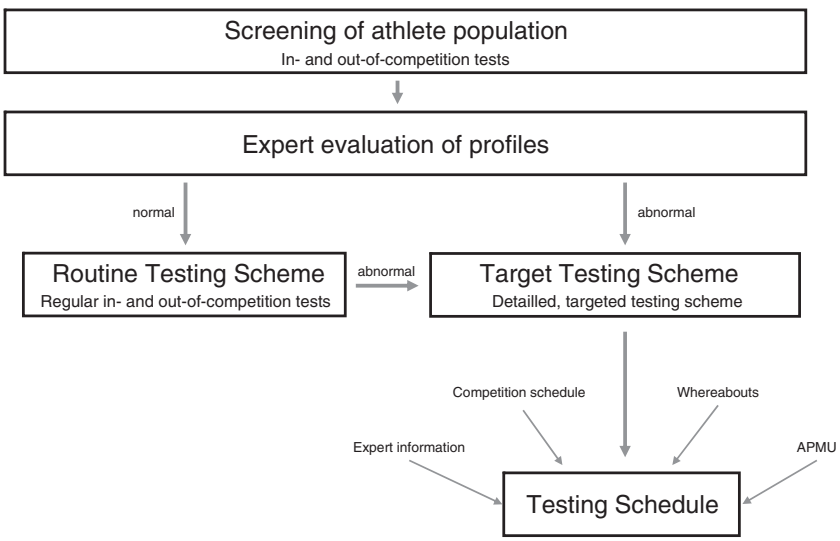

Figure 1. Suggested Testing Scheme for organisations implementing the Athlete Biological Passport System.

Passports of typical manipulation techniques

In the following figures, several exemplary suspicious passport patterns are displayed. The first passport (Figure 2) shows a typical profile observed when EPO or other erythropoietic stimulants are used. It is important to interpret a profile in connection with the competition schedule taken from the whereabouts in ADAMS for each athlete. In the present example, the athlete had a competition period when samples 8–10 were taken. He uses EPO in the lead up to a race (ON phase, illustrated by relatively high Ret% in the weeks before racing (samples 3–7). He then discontinues his use before/during the race to avoid detection in the direct test (samples 8–10). The abrupt cessation of erythropoietic stimulation leads to a significant and prolonged suppression of retics, paired with an elevated red cell mass (slightly increase in Hb) (OFF phase).

Figure 3 depicts a classic transfusion scenario: There is evidence of recent blood withdrawal approx. 4–6 weeks prior to a major race with a sharp drop in haemoglobin concentration. Several days after this occasion, the reticulocytes start to rise. Before the start of the race, blood is re-infused, illustrated by a sudden increase in Hb concentration. Retic formation is more and more suppressed due to the artificially increased red cell mass.

Figure 4 shows evidence of an athlete having re-infused blood during competition. The physiological reaction to heavy endurance exercise is usually haemodilution, therefore, one can expect a drop in haemoglobin values after the first days of a multi-day cycling stage race. This is the reason why during such multi-day events, the individual lower limit for haemoglobin in the passport is removed (the racing period is therefore indicated by the missing lower limit in the haemoglobin and OFF score panel). If such drop in haemoglobin during racing is not visible, further scrutiny of the athlete in question is advised. In Figure 4, an athlete, after an initial drop in the first in competition blood test (sample 6), shows an increase of his Hb values with ongoing racing and a drop in reticulocytes. This is a characteristic feature of blood re-infusion during racing with a gradual reduction in reticulocytes due to the supraphysiological red cell mass.

It has to be pointed out that in real passports, the described patterns are only rarely seen in the clarity displayed here, as tests are not always carried out at the right moment. Furthermore, athletes use a combination of different doping techniques or microdoses to blurr their passport picture. Recently, 'masking doping' to confuse the passport (e.g. use of infusions to dilute and lower high Hb values, well-imed EPO injections to increase low retic values) is becoming more and more common. Other athletes provide rather inventive remarks on the doping control forms to 'pre-explain' potentially suspicious values, such as fictional surgery with blood loss (to hide blood withdrawal in view of future re-infusion) or altitude sojourns or the use of hypoxic tents (to cover variations in reticulocytes caused by EPo or transfusions). Such strategies should be kept in mind whileevaluating ABP profiles. In the same context, it has to be acknowledged that information from this or similar papers might be used by cheating athletes to adapt their doping strategy.

Future of the haematological module of the biological passport

Despite all efforts and the obvious success of the ABP, the enhancement of the oxygen transport to the tissue will remain a prime doping technique for cheating athletes in all endurance sport disciplines. Therefore, the ABP needs to be constantly refined.

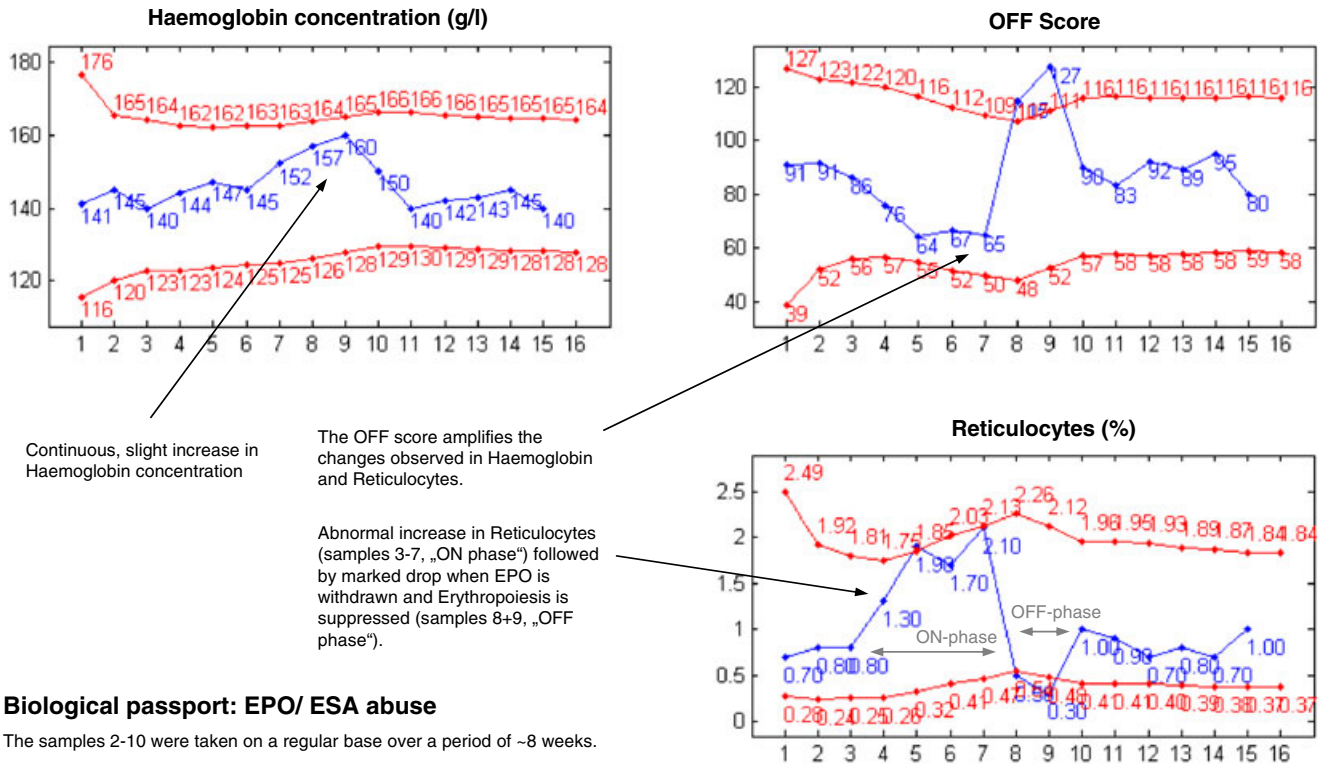

Figure 2. Haematological Module of the Athlete Biological Passport: Examples of typical blood manipulation techniques.

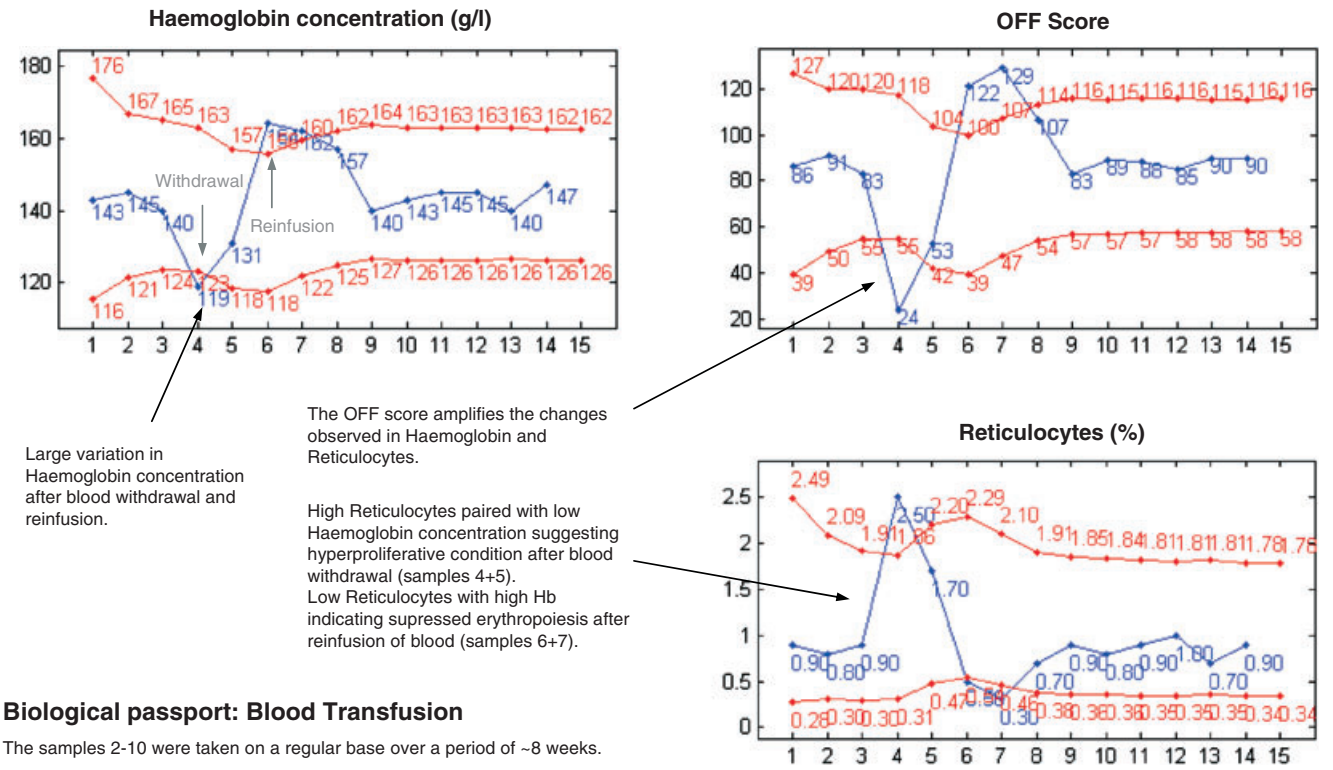

Figure 3. Haematological Module of the Athlete Biological Passport: Examples of typical blood manipulation techniques.

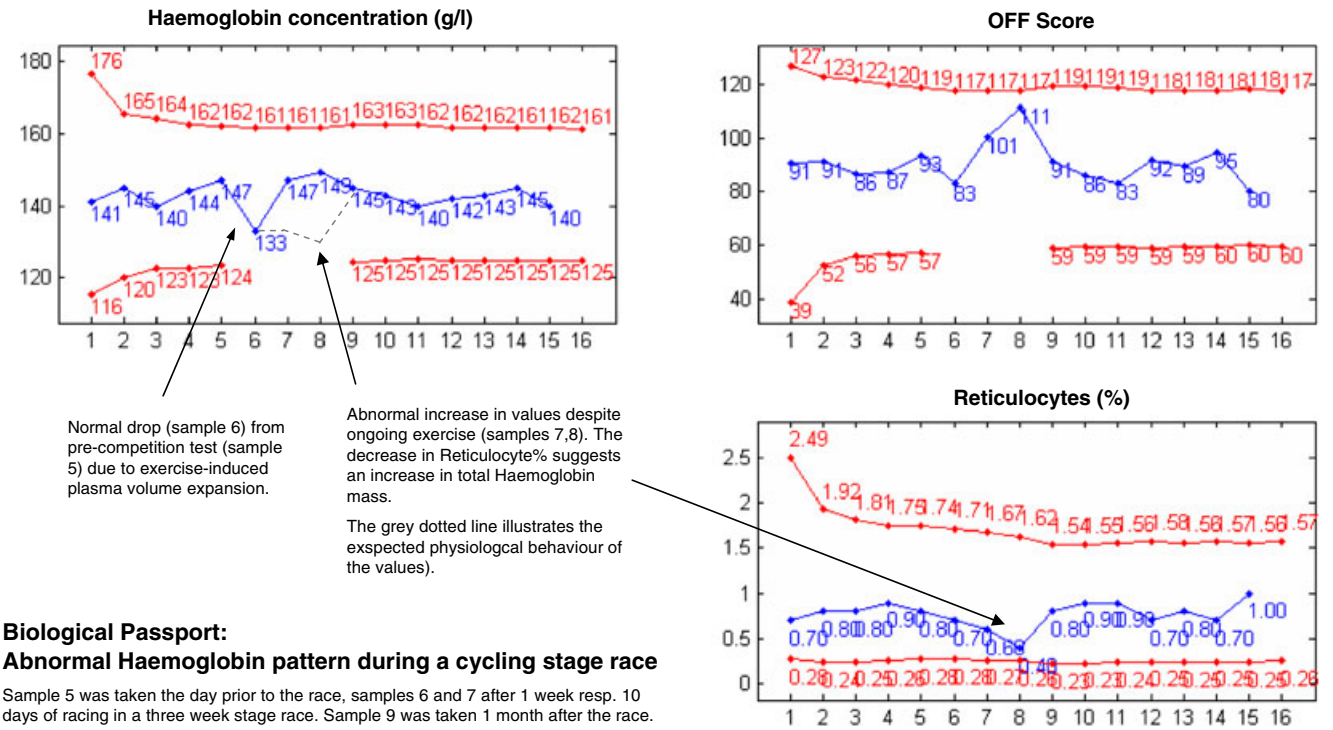

Figure 4. Haematological Module of the Athlete Biological Passport: Examples of typical blood manipulation techniques.

A key point is to reduce the variability of certain concentration-based passport markers such as haemoglobin concentration. In fact it is well known that the major causes of variation in most biomarkers are shifts in plasma volume, which are particulary prominent in athletes and might mask significant, doping-induced changes in red cell mass.[22] Therefore, techniques to estimate the magnitude of such plasma volume variations could significantly improve the sensitivity of the passport approach. New markers that allow further

insight in the oxygen carrying system of the organism must be evaluated in view of their potential use in the ABP. Until now, only very basic markers of the red blood cell system are included in the passport. Total haemoglobin mass[37] (which bears the advantage of being independent of plasma volume variations), markers of the iron metabolism or cellular subfractions of certain red cell populations might bear potential for future inclusion. The main difficulties to overcome in this context are issues of standardisation and quality control.

Recent studies have shown that variables from other analytical levels, such as genomics, proteomics, and metabolomics display distinct changes after blood manipulations which might prove suitable to be included in the passport in the future.[34] Very recently, preliminary results of a very promising strategy have been published showing that circulating microRNAs could help in the detection of ESA doping. Once again, additional data is required before the test can be used in an anti-doping context.[35]

Although the haematological passport uses a longitudinal approach, the high number of samples obtained from a limited population of athletes allows an assessment of additional information through cross-sectional analysis of population data that might improve the fight against doping. In fact, from large sampling campaigns ahead of major competitions, such as the *Tour de France* in cycling or the Athletic World Championships, doping prevalences can be estimated from deviations in biological markers in comparison to reference collectives[36] and changes in doping behaviour might be documented for certain countries or teams.[31] Such information should be taken into consideration when designing new doping control programmes and planning targeted tests.

From an organizational point of view, precise targeting will become a key issue in doping testing in the future and, as explained, it is crucial to use the passport to its full extent. Therefore, systems that improve targeting and identify suspicious athletes by taking into account information from blood profiles, cross-sectional analysis of certain groups of athletes, competition results, and other sources such as whereabouts must be developed. In this context, software algorithms that identify specific, suspicious patterns should be developed as such systems might considerably help to deal with a large numbers of profiles and use all available data in a timely and cost efficient manner.

Last, and aside from analytical and scientific questions, there is a need for more APMU or similary adapted and qualified structures that can apply the passport to its full capacities.

Conclusion

Manipulations of the haematological system using blood transfusions or ESA are a long standing problem in sports. In recent years, the introduction of the ABP has considerably improved the fight against this type of manipulation. In the future, main goals should be to refine the use of this powerful tool by improving the operative framework (target testing, expert evaluation of profiles) and by investigating and introducing new markers from different analytical fields.

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