Review Article

Biological passport parameters

MARIO ZORZOLI

International Cycling Union

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INTRODUCTION

The "no-start rule"

The fight against doping aims to ensure health protection to athletes and fair competitions. It is therefore the role of anti-doping authorities (International Olympic Committee in the past and now the World Anti-Doping Agency) to establish a list of prohibited substances and methods, the Prohibited List, which includes substances or methods which are either dangerous for the health of the athlete or are performance enhancers or are considered contrary to the ethic of sport.

Substances and methods enhancing oxygen transport (i.e. erythropoiesis stimulating agents, autologous or homologous blood transfusion, hemoglobin based oxygen carriers, and others) define blood doping. This is one of the most dangerous forms of doping, due to the risks for the health and because of the clear performance enhancement.

From the end of the 1980s, since the introduction on the market of recombinant human EPO (rhEPO) in 1987, and despite the fact that sports authorities had forbidden its use since 1988, athletes have been using it to increase performance, especially in endurance disciplines, or to facilitate recovery. Numerous reports or anecdotes show that particularly in the 1990s many athletes abusing these drugs had extremely high hematocrit (HCT) values, with consequent risk of thrombovascular accidents.

The challenge of detecting rhEPO has led to the proposal of different strategies: on one side scientists have tried to find methods for the direct detection of the drug in the urine (a method based on immuno-electro-focusing has been validated and introduced in 2000); on the other side, other groups have addressed the indirect detection of rhEPO and other blood doping agents through the analysis of different haematological parameters known to be influenced by these drugs.

Corresponding author. International Cycling Union
E-mail: mario.zorzoli@uci.ch
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Because rhEPO could not be yet detected, in order to dissuade athletes from using rhEPO, some International Federations (International Ski Federation [FIS], International Biathlon Union [IBU], International Skating Union, UCI) introduced the so called "no-start rule". The aim was to prevent athletes from competing when their (HCT) or hemoglobin (HGB) values were higher than the established limit, which was the same limit for all athletes (population limit). For some sporting bodies it was considered as a medical rule, while for others it was a competition rule. The UCI introduced it in March 1997, and athletes were subjected to tests at or during competitions but not out-of-competition. Tests were conducted on the field, and the samples were analyzed on site. During unannounced bloods tests, several haematological parameters were measured, which included HCT, HGB, % reticulocytes (RET%) and the calculation of the stimulation index or OFF-hr score (Hb [g/L] – 60*√Ret[%]) (OFF-score).

Indirect parameters and anti-doping

The measurement of these parameters during several years in the same conditions clearly evidenced a difference of results in regard to RET% and HGB values, namely a decreased number of samples showing reticulocytosis and increased number of samples showing reticulocytopenia associated with increased HGB. This non physiological combination led to the conclusion that a change had occurred in the way a limited number of athletes where using blood doping. The changes in the RET%/HGB results were the consequences (and other International Federations and information from the field confirmed this interpretation) of the fact the rhEPO was used differently from previous years. In fact, in order to avoid urinary EPO detection at the time of the competition, the new strategy applied consisted in decreasing the dose of intra-venous injected rhEPO (in order to reduce the detection window) and administering the drug well before the competition. In addition, it was evident that blood transfusion was making its come-back because the detection is only limited to homologous blood transfusion (since August 2004). The consequence of these manipulations was that at the time of our tests during the competition, some athletes showed the combination of high haemoglobin and reticulocytopenia, which is a characteristic of a prior increased of red blood cells through blood manipulation. Additionally, the measurement of these parameters allowed to highlight those athletes who could be suspected of blood doping, because of abnormal results, or abnormal evolution of blood parameters, and target them conducting anti-doping tests, in and out-of-competition.

The athlete's passport

While some authors were already evoking the possibility of a longitudinal follow-up of athletes biological parameters, the Australian and the Lausanne groups intensively explored the indirect marker approach and came to the same conclusions: each athlete should become his own reference, meaning that individual limits should be applied instead of population limits, and one could use the athlete's previous measurements as basal levels. A first practical application was developed by Berglund in 2005, the Swedish Blood Pass project.

After the Olympic Games of Turin in February 2006, where the no-start rule had prevented several athletes from competing, WADA decided to address the issue of the indirect markers of blood doping from the anti-doping perspective and constituted a working group with the mission to evaluate the feasibility of introducing an Athlete's Passport. This Haematological Working Group was composed of scientists and representatives of International Federations, and the conclusions drawn were that WADA could proceed with the implementation of an Athlete's Haematological Passport. It was recommended to measure HGB and OFF-score on samples collected in- and out-of-competition in order to generate profiles that would be computed with the Bayesian statistical model developed by the Lausanne Anti-Doping Laboratory.

The principles of the Biological Passport have been described in the WADA Athlete Passport Operating Guidelines which came into force in January 2010. As it is stated in the introduction of the document, the two major innovative elements that the Passport has introduced in the anti-doping world are

- It is not the substance itself which is detected but rather its effects on the body;
- A list of relevant and significant variables for a specific class of substance (e.g. substances enhancing oxygen transfer, such as rhEPO) must be identified and then monitored on a regular basis in order to constitute an individual and longitudinal profile for any given athlete with the subject becoming his/her own reference.

In order to decrease the variability due to the pre-analytical and analytical factors, standardized protocols were established and became mandatory. They define how samples should be collected, handled and transported to the laboratory, analyzed and how the result should be evaluated.

Finally, the scope of the passport is double: on one side it will help in identifying athletes for further targeted analytical testing (recombinant EPO test, CERA test, homologous blood transfusion test, etc.); on the other side to pursue possible anti-doping rule violations in accordance with Article 2.2. of the World Anti-Doping Code which concerns the use of prohibited substances or methods.

Sample collection

The main part of the programme is of course sample collection. The Athlete's Biological Passport requires that athletes be tested not only at competition sites but also out-of-competition. The WADA Guidelines and technical documents define the modalities of samples collection:

- Samples can only be collected 2 hours after a physical effort (training or competition);
- The athlete has to remain seated for at least 10 minutes prior to providing a sample;
- The athlete should be questioned about specific issues, like altitude (natural or simulated), blood losses, donations and transfusions.

For the purpose of measuring the hematological parameters of the biological passport, one A tube (3ml tube containing EDTA as anti-coagulant) is sufficient as it is not required to conduct additional analysis. Nevertheless, in order to increase the deterrent effect and the efficiency of the program, a collection of two EDTA tubes (tube A and tube B) should be obtained as this enables the potential, in case of an abnormal result, for the immediate request of an anti-doping test (in whole blood or plasma) for the detection of homologous blood transfusion (HBT), Continuous Erythropoiesis Stimulating Agent (CERA) or Hemoglobin Based Oxygen Carriers (HBOC).

Finally, at least three blood tests are necessary in order to evaluate the entire sequence in addition to the single value.

Samples analysis

Once collected, samples should be stored and transported to the closest WADA anti-doping accredited laboratory in refrigerated conditions (2°- 12°C) in order to be analyzed within 36 hours of their collection. The drawback of this requirement is that it increases the costs incurred through transportation and that there are remote part of the world where it is almost impossible to collect a sample and have it analyzed within 36h in a WADA anti-doping accredited laboratory. This is why the principle of accrediting satellite non-anti-doping laboratories has been accepted.

Laboratories have to respect some preliminary conditions in order to be accredited to perform hematological analysis:

- They all have to use the same technology, in order to decrease the variability especially associated to reticulocytes measurement;
- They must be part of a common external quality assessment;
- The same procedures must be applied when calibrating the machines (quality control checks, fresh blood samples analysis) and analyzing the samples.

Each blood sample shall be analyzed twice and the absolute differences between the results of the two analyses should be within the established limits. If this is the case, only the first results is reported. If absolute differences between the results of the two analyses are greater than those defined than the whole analysis shall be started again.

The parameters that are measured, within the hematological module, are the following:

- Red blood cells (RBC);
- Hematocrit (HCT);
- Hemoglobin (HGB);
- Mean Corpuscular Hemoglobin (MCH);
- Mean Corpuscular Hemoglobin Content (MCHC);
- Mean Corpuscular Volume (MCV);
- Off-hr score (HGH - 60√RET%);
- Absolute number of reticulocytes (RET#);
- Reticulocytes percentage (RET%).

Actually, only the HGB and OFF-score are taken into account by the Bayesian model in order to define a possible anti-doping rule violation.

Real time process

ADAMS is the web platform which allows all data of the Athlete's Biological Passport program to be centralized and shared by all of the stake-holders. In addition to whereabouts (and therapeutic uses exemptions or declaration of use), testing missions are planned using ADAMS. The testing authority, by creating the mission order, defines which athlete should be tested; where and when the test should take place; who should be the sample collection authority; what kind of sample should be collected; to which anti-doping laboratory the sample should be sent; what kind of analysis should be requested. Once the sample has been collected, the doping control form is entered by the doping control officer (DCO) and blood collection officer (BCO) and the laboratory results are also transferred from the analyzer (Figure 1). This process, which is completed within a short delay after the sample collection, allows the matching between the athlete and the result. The testing authority can therefore rapidly react when an abnormal result is received. They can immediately ask to conduct an anti-doping test on the collected sample (for CERA, HBT or synthetic hemoglobin) or decide to collect an additional target sample. The only part which is not automated is the creation of the updated profile. This is managed by the Athlete Passport Management Unit (APMU) which is in Lausanne and is financed by the Laboratoire d'Analyse du Dopage (LAD) and WADA. The APMU is responsible for the anonymous extraction of data from ADAMS and for

computing this information into the ABP software which generates an individual encoded athlete's ABP profile. This anonymous profile is what the experts receive for their evaluation and recommendation.

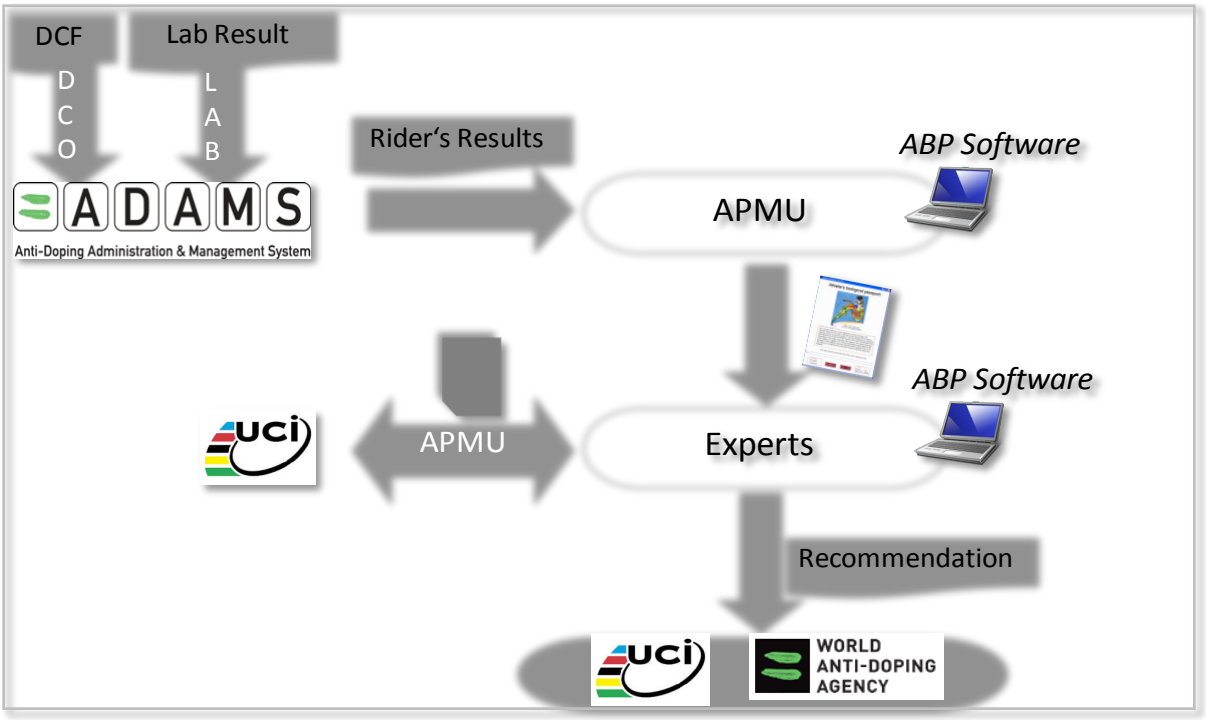

Figure 1. *The real time process.*

Profile evaluation

Once the raw data are extracted from ADAMS, the ABP software applies the statistical adaptive model. This model relies on the Bayesian theory, and its goal is to evaluate how likely the passport data are assuming a normal physiological condition. Similar models are used for the early diagnosis of cancer with biomarkers of disease and in clinical trials with biomarkers of safety and efficacy. In practical terms, new evidence or observations are used to update or to newly infer the probability that a hypothesis may be true or to discriminate between conflicting hypotheses. It has two levels: the single result of the whole sequence.

The adaptive model is capable of triggering "alerts" and self-identifying abnormal profiles that warrant further attention and review. A profile in which the Adaptive Model has identified the HGB or OFF-score abnormal with a 99.9% probability or more has to be reviewed by a panel of three experts. It is important to include experts with different backgrounds (hematology, sports medicine, exercise physiology or blood doping) so that a profile can be evaluated under different perspectives.

At any time, and in order to better evaluate the anonymous ABP profile, the experts may request additional information, such as athletes' whereabouts, competition schedule, strange journeys, laboratory documentation packages or medical information. Once evaluated, the experts may formulate a number of recommendations. The profile can be judged as normal, pathological or as suspicious for doping (Figures 2 and 3).

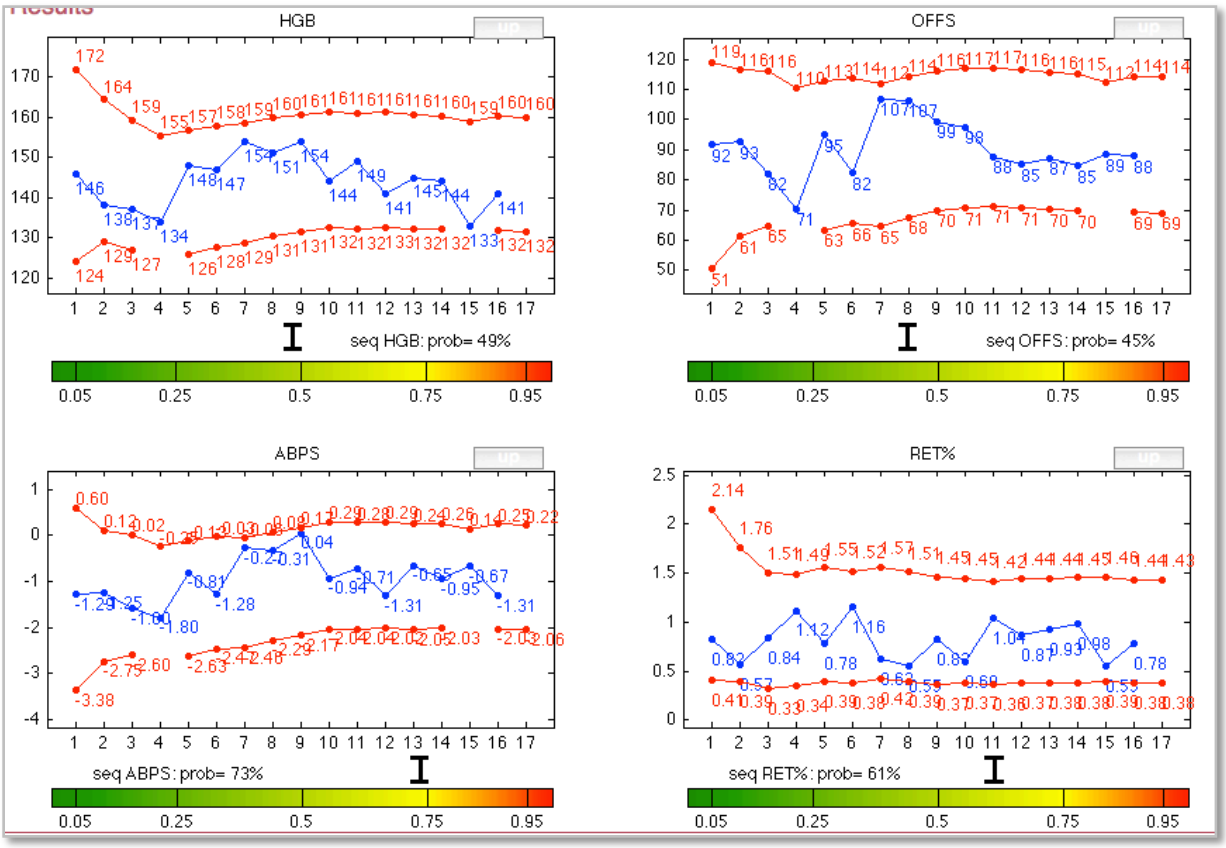

Figure 2. *A normal passport profile.*

If it is suspicious, targeted tests can be conducted to detect forbidden substances and methods or to strengthen the suspiciousness of the profile. If the experts agree that the profile is abnormal and consistent with a pattern of blood doping recommendations are made to the anti-doping authority to undertake the procedure for possible anti-doping rules violation. In fact, the 2009 World Anti-Doping Code enables the possibility to sanction an athlete for the use of doping based on an established abnormal blood or urine profile.

The procedure foreseen by the WADA Guidelines requires:

- To inform the athlete that the anti-doping organization (ADO) is considering bringing an anti-doping rule violation against him;
- To give him a copy of any document made available to the expert;

- To allow him to rebut the allegation by letting him provide his own explanation for the abnormal profile. He can for instance demonstrate that the results were the consequence of a pathological condition.

This explanation is further reviewed by the experts who must finally and unanimously decide if, in their opinion, there is no known reasonable explanation for the abnormal blood profile other than the use of a prohibited substance or method. In such a case, the three experts sign a statement asserting that the profile provides « convincing evidence of the use of a prohibited method », and make an official recommendation to the ADO to open a disciplinary procedure against the athlete.

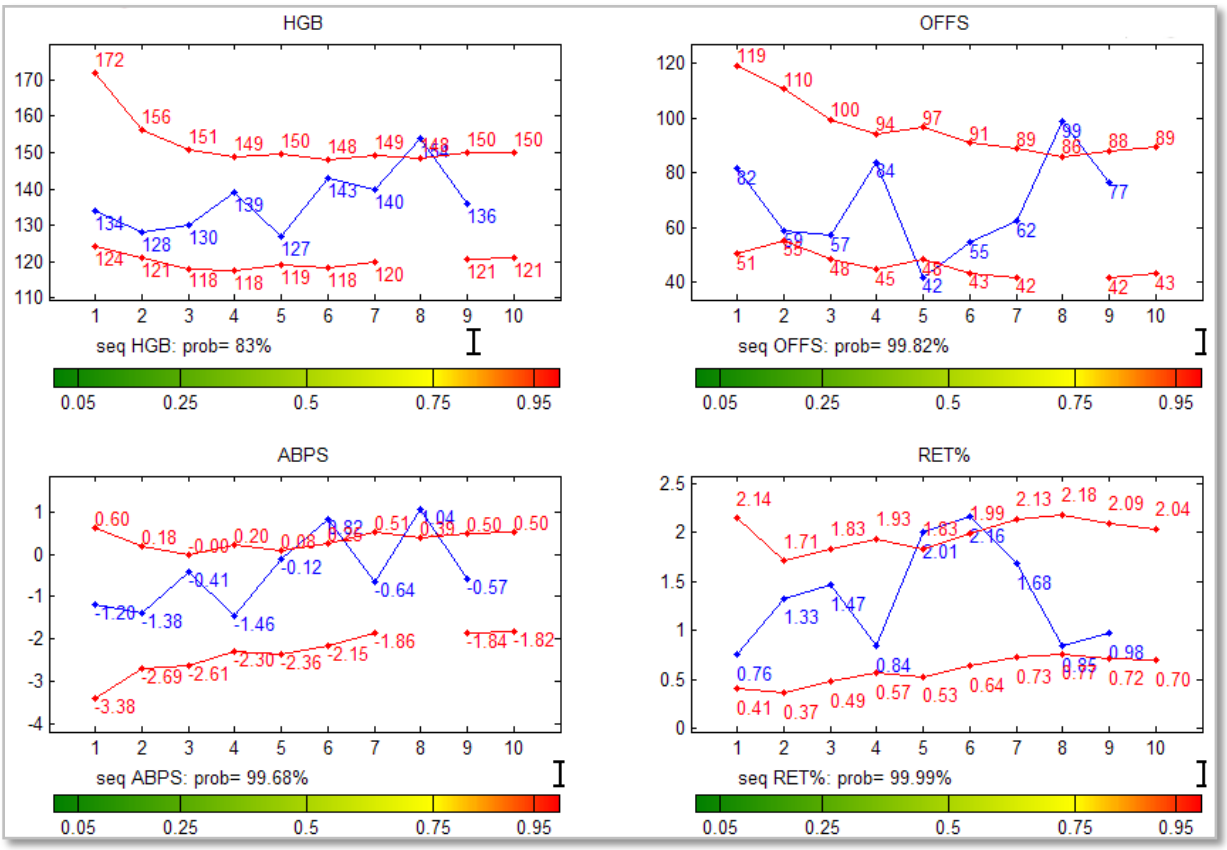

Figure 3. *A passport profile of an athlete convicted of doping.*

Forensic reasoning

It is important to understand that within the passport program, the evaluation of evidence follows the same rules as for the evaluation of forensic evidence (DNA evidence, traces, fibers, prints...). It is therefore important to act in accordance with a correct logic in the evaluation of the evidence in order to avoid any possible fallacy.

Three questions have to be answered by the expert when analyzing a profile:

1. How likely are the passport data assuming a normal physiological condition?
2. How likely are the passport data assuming a medical condition?
3. How likely are the passport data assuming doping?

and to compare the answers to determine the "weight of evidence". While the first answer is provided by the adaptive model, the experts have to answers to the two other questions. It is therefore their task to define a doping scenario which fits to the profile they evaluate.

The UCI pilot project

Following a meeting which took place in Paris in October 2007, between WADA, the French sporting authorities and the cycling family, UCI launched in 2008 a pilot project for the implementation of the hematological module of the Athlete's Passport.

Since then, more than 800 athletes per year were enrolled in the program. UCI signed contracts with several sample collection providers in order to face the high demand of in- and out-of-competition tests. At the beginning samples were only analyzed in the group of laboratories that have been involved with the no-start tests for many years, because they were already participating in the same external quality control program that WADA has chosen (Swiss Center for Quality Control) and were all using the same technology. Progressively, as other WADA accredited laboratories were fulfilling the requirements to conduct hematological passport tests, UCI only relied on these laboratories for three major reasons:

- The samples could be directly analyzed for the detection of forbidden substances or methods;
- Decreased costs with sample transport when collection included both urine and blood;
- Ease of the laboratory with technical and legal aspects related to anti-doping procedures (documentation package; chain of custody; testifying in court).
-

The experts were chosen by the UCI and WADA. All qualified in the field of hematology (either clinical or laboratory), sports medicine, exercise physiology or blood doping. Each week, 10 to 15 updated profiles were sent by the APMU to the experts for review. In these profiles the Bayesian adaptive model has identified the Hb or Off-hr score abnormal with a 99% probability (either for the single measurement as a function of previous results or for the complete sequence) or with normal or lower levels of probability.

Results

From the beginning of the program more than 15'000 blood and almost 10'000 tests have been conducted on the registered athletes. More than 60% of the riders have more than 15 blood results in their profile.

The results of experts' evaluations are presented in the figure 4. Only about half of the profiles were sent for evaluation, and this included all those who had an abnormality higher than 99% plus some which were below this limit. Among the evaluated profiles, still a large majority was considered as normal by one or

more experts. At the end, only a few tens could be considered by the experts as reflecting a possible blood doping manipulation.

It was then decided to gather all the experts together and to discuss all these highly suspicious profiles on the light also of the additional information available: whereabouts, competition schedule and documentation packages. Only a unanimous consensus from the experts would have led to the recommendation to UCI to open a disciplinary procedure against the athlete.

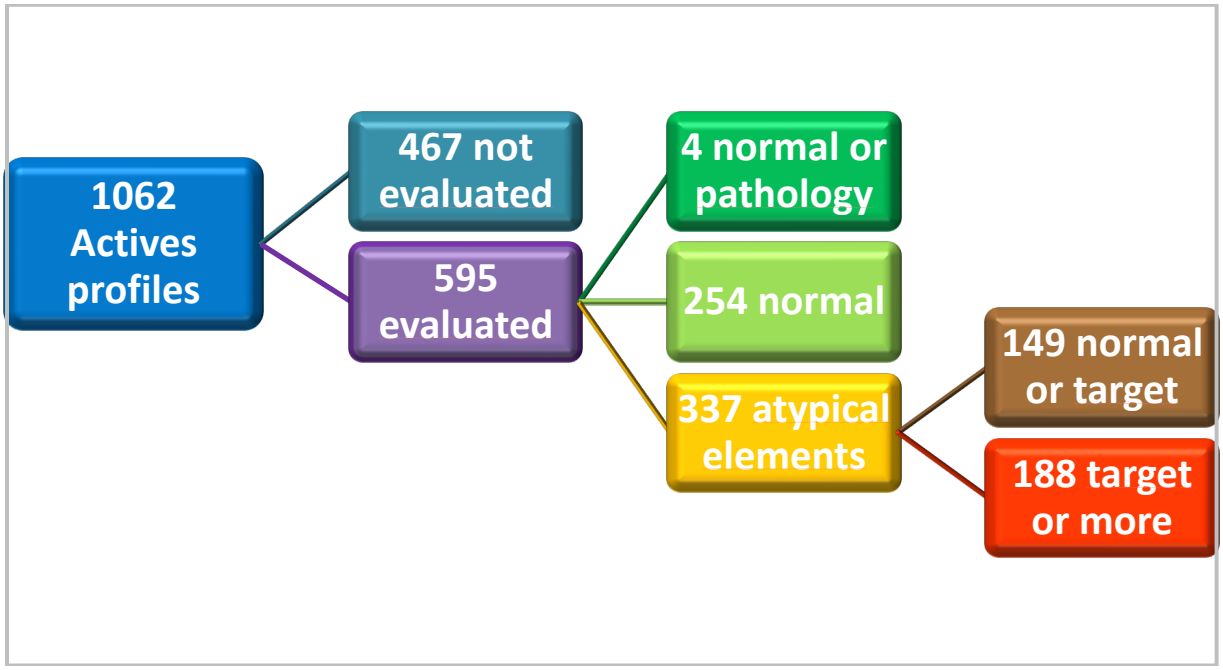

Figure 4. *The experts' evaluation of the profiles.*

Outcome

Up to the end of 2010 nine procedures had been opened for a possible anti-doping rules violation for use of blood doping based on passport profiles. It is also worth mentioning that, for some of these athletes, additional evidence was available: in one case, UCI reviewed all of the EPO tests conducted in previous years and requested the laboratory to reanalyze a urine sample, which was finally considered positive for Dynepo; in another case, a single biological passport sample revealed the presence of CERA. Of these cases four were judged and sanctioned at the national level (as per UCI rules) and the athlete didn't appeal the decision. Four other cases were brought in front of the Court of Arbitration of Sport after that the national disciplinary body had acquitted two athletes and suspended two others. The defenses of the athletes were mainly arguing that:

- Some results within the profile had to be considered unreliable because of the lack of some pre-analytical, analytical or post-analytical elements (i.e the athlete was not in a seating position for 10 minutes; the sample was not analyzed twice; there was no temperature logger; the poor quality of some documentation packages);
- Some changes of the hematological parameters had to be attributed to physiological conditions, like hypoxic stimulation or variations dues to competitive season;
- Some results were explained by medical conditions such as blood loss (from dental extraction to gastro-intestinal bleeding) or dehydration from gastro-intestinal infection and heat.

The hearings have taken place and two recent decision have led to the same conclusion which is that the athlete passport is "a reliable mean for the indirect detection of doping acts" and the athletes have been sanctioned (in one case reversing the decision of the first instance body).

In addition to this, as it was stated from the beginning, the passport is an excellent and reliable tool to target athlete with conventional anti-doping tests. As a result of this, more than 20 athletes involved in the passport program have been found positives for erythropoiesis stimulating agents (ESA).

Finally, the introduction of the passport has had a highly deterrent effect which is demonstrated by at least two aspects. The first is the evolution of the values of RET% measured from 2001 to 2010 (Figure 5). It is well known that RET% is one of the most reliable markers of blood doping. After an ESA administration (boosting period – ON phase), RET% show a marked increase (green values in figure 5) up more than 2%. Contrary to this, during the maintenance and wash-out period (OFF-phase), they decrease to even lower values than the level measured prior to the ESA or transfusion therapy (blue values in figure 5) because of the natural negative feed-back on red cell production. Figure 5 indicates that from 2001 to 2007 an almost constant percentage of samples (around 10%) had RET% results in the extreme ranges (<0.4% or > 2.0%). Since the introduction of the Biological Passport this number has dramatically decreased to 2-3% notwithstanding the fact that samples were also collected out-of-competition when doping products are more likely to be taken. Additionally, very extreme values (<0.2% or >2.4%) have completely disappeared since 2009. This data suggests that the behaviour within the peloton has changed in relation to the use of blood doping agents and confirms the feed-back we get when discussing the topic within the "field".

Another important deterrent effect of the passport is that more and more athletes are required to provide the results of their profile when negotiating a contract with a new team or when they are selected to compete in the National Team. We are aware of situations where new teams or National Federations have refused to hire or select athletes because of their abnormal profile.

CONCLUSIONS

The introduction of the Biological Passport is clearly a major step forward in the fight against doping. It allows the efficient combination of two different but complementary strategies. The classical method based on toxicological science for detecting a forbidden substance in a human sample can now be coupled with a more subtle detection of the biological consequences induced by these drugs. The downside is the increase of costs due to the particularity of these tests where blood samples have to be handled differently from normal urine samples. Furthermore, the result's management is more complex than in traditional anti-doping tests and the legal challenges are more important. In fact not only the validity of the numerous results can be questioned, but also the interpretation of the expert is subject to debate. Nevertheless, the Court of Arbitration of Sport has recently stated that the athlete passport is a valid and reliable mean of proving doping.

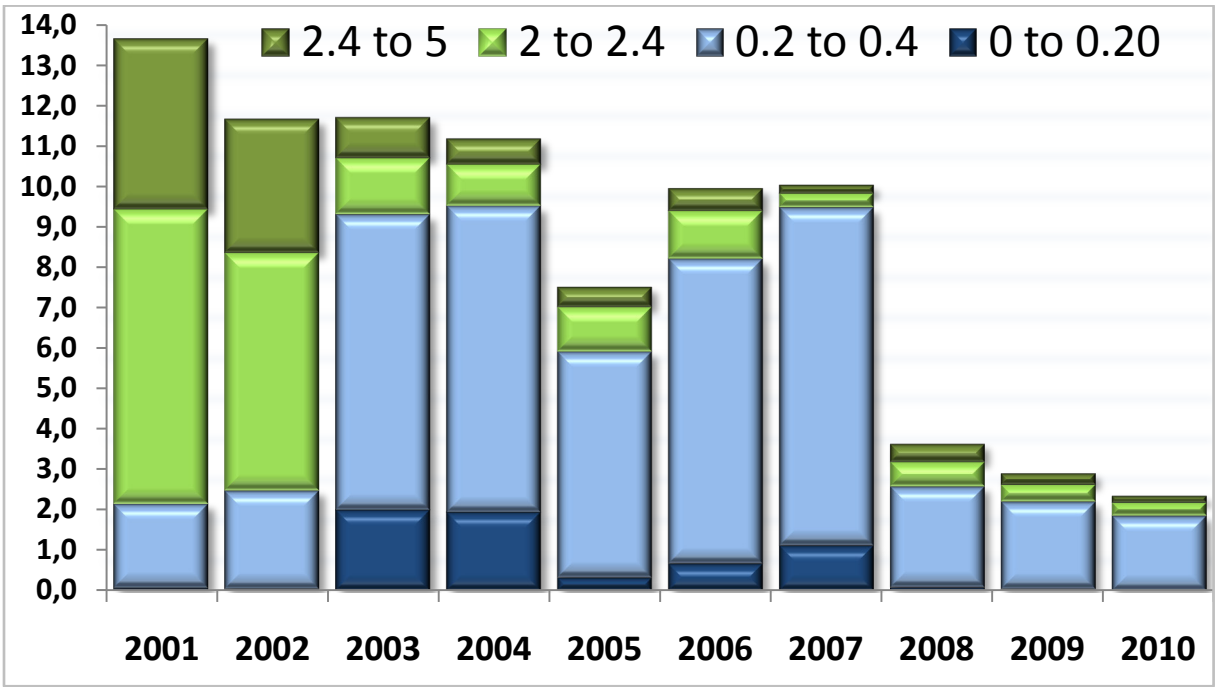

Figure 5. *Changes in RET% from 2001 to 2010 (x= years; y= percentage of samples analyzed).*

In order to increase efficiency at equal costs, anti-doping organizations should opt for the collection of a double sample (A+B) of whole blood. This will allow to directly requesting an anti-doping test to detect a forbidden substance or method when the profile is suspicious.

In the future, additional modules should be added in order to improve the indirect detection of numerous forbidden drugs (urinary steroid profile for exogenous steroids; IGF1 and P-III-P as indirect markers of human growth hormone).

We are just at the beginning of a new promising era in the world of anti-doping.

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