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Fighting for fair competition: blood doping – a persistent challenge and smart approaches to detect it

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Since 1996, the Swiss Laboratory for Doping Analyses (LAD) has invested a lot of time and energy to fight blood doping for fair competition. Different and smart approaches have been developed since then to detect rhEPO (recombinant erythropoietin) and HBOC (haemoglobin-based oxygen carrier) abuse as well as homologous blood transfusion. The use of modern blood analysers has helped us to reach our objectives, but until now, none of the blood parameters can be used alone for anti-doping purposes. Nevertheless, haematocrit, haemoglobin and reticulocyte count are very useful to diagnose any manipulation of the blood formula. Odd data or abnormal changes over time are used by sports federations to focus their energy on some specific athletes.

To obtain optimal results, a unique type of transportable equipment (XT-2000i) is used and rigorous procedures are followed. The multitude of parameters allowed in 2003 to foresee the return of homologous blood transfusions as a doping practice, which led to the LAD launching the first anti-doping test to detect such manipulations. The constant development of new drugs forces anti-doping laboratories to find new strategies to detect cheaters, leading to the introduction of blood profiling, blood passports and indirect proof of doping based on abnormal data, without knowing necessarily the origin of the abnormality.

There are many ways for athletes to artificially increase their total red blood cell mass. Twenty years ago blood transfusion was used and it was a very efficient method but with the availability of erythropoietin (EPO) most athletes changed to this method due to its convenience, rather than a bulky unit of blood. EPO comes in ready to use injections and has a very long shelf life.

Since 1996, during the Tour de Suisse, the Swiss Laboratory for Doping Analyses has invested a lot of time and energy into the attempt to detect blood doping and so make the competitions fair. We compared data from the cyclists to that from sport's students at the University. Surprisingly there was not a great deal of difference between the two groups for the results of haematocrit (HCT) or soluble transferrin receptor. The main difference between the two groups was for EPO. Unfortunately, as the cyclists refused to give blood before the event it is not known if this difference was due to hypoxia (due to altitude, since the event took place in the Alps), due to the effects of excretion, or due to illegal EPO injections. The other parameter that demonstrated a large difference was ferritin levels. Levels in the cyclists were much higher than in the control group, with values as high as 1400 ng/mL; this may have been caused by intravenous iron injections. On the basis of these results the International Cycling Union decided to introduce a 'no start rule' designed to ban athletes with a HCT above 52.5% from participating in a race. However, the cyclist's team physicians involved decided that this value was too high and

set the level to a HCT of 50 %. In order to take into account athletes with a normal HCT of 50 % or more (such as those living at high altitudes) a certificate could be issued that this is their normal value.

Since 1996 many changes have been made in the way blood doping is detected.

- 1997, the 'no start rule' was introduced.
- 1998, the sad event of the Tour de France, the Festina team's illegal use of EPO.
- 1999, the implementation of medical follow up of the cyclists, this was mainly to detect athletes with very high ferritin levels. After this strategy was implemented it was then found that the ferritin levels of the cyclists did fall but mostly because they switched to the use of oral iron supplements rather than intravenous iron injections.
- 2000, all cyclists participating in all major tours were tested at the departure of the races
- 2001, the screening and target test for the detection of EPO was introduced.
- 2002, a strategy to detect haemoglobin based oxygen carriers was developed.
- 2004, our laboratory was the first to develop a method for the direct detection of the abuse of homologous blood transfusion.
- 2008, introduction and implementation of the blood passport. The 'no start rule' is abandoned in cycling.

Earlier methods of detecting EPO abuse using haematological parameters

An Australian group had developed a method utilising markers of altered erythropoiesis for the detection of EPO abuse in athletes[1]. This used a combination of haematological parameters to identify subjects while administering EPO, the ON-model, and another group of parameters to identify EPO users after treatment had stopped the OFF-model. However we did not want to use these models as some of the parameters are affected by effort alone, HCT will increase due to water loss, and hypoxia, will increase EPO, even at rest[2]. All previous models have been developed following continuous EPO treatment, where the measured EPO goes up as well as the reticulocyte count, HCT and soluble transferrin receptor. When treatment stops the EPO and reticulocyte count fall to below normal levels, however in athletes treatment is not continuous, they may take EPO until their HCT reaches 50 % then stop. After a period of time their HCT will fall and they will re-start treatment. At what point is it best to apply the 'ON or OFF model'?

There is also a lack of standardisation between different haematology instruments for some of these parameters; for example the normal range for the reticulocyte count is often instrument specific.

The development of the new approaches to detect blood doping

In our laboratory we looked at the effect of EPO treatment on volunteers. They were divided into four groups:

1. Treated with 40 IU/kg three times a week plus intravenous iron and B12 and folate
2. Treated with 80 IU/kg three times a week plus intravenous iron and B12 and folate
3. No treatment
4. Placebo group, treated with isotonic saline three times a week and intravenous iron B12 and folate

All groups were followed from baseline, during the treatment period and for some weeks after treatment had finished.

The idea was to increase the HCT to 50 % in the treated subjects, maintain it at 50 % with further administration of EPO and then observe what happens when treatment is stopped, the wash out period.

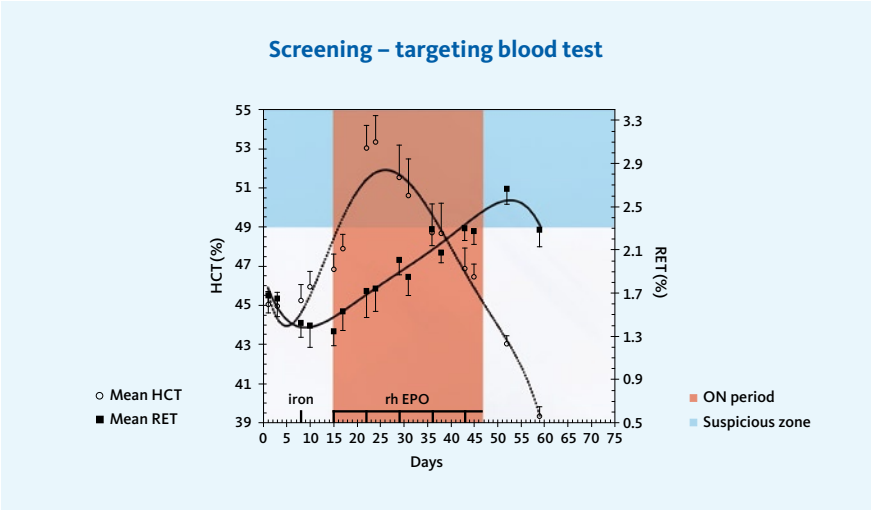

Figure 1 *Results for HCT and reticulocyte count during the ON period are in the pink area, the OFF period is after the 47th day, results that would cause suspicion fall in the blue/purple area. Adapted from Robinson et al.*[4]

We developed a primary screening test using haematological parameters commonly available[3,4]. During the ON period the reticulocyte count is high and the HCT is normal (figure 1), on the basis of these two parameters it is possible to suspect abuse and then the athlete would undergo a full anti doping test. When treatment stops, the OFF period, the haemoglobin and HCT is high and the reticulocyte count is low, again this is suspicious and a full anti doping test would be performed. An OFF-score has been created which is calculation involving haemoglobin and reticulocyte count (OFF-score = HGB [g/dL] x 10 – 60 x √RET %), a normal OFF-score in males is of < 133.

Urinary electrophoresis for EPO is expensive and time consuming so the idea of a screening test is to reduce the need to perform this test unless there is already a suspicion of EPO abuse.

Current approach for detecting blood doping

Currently, we either take our own equipment to the different sports events around the World or set up a temporary laboratory. This mobile unit solution is part of our score of accreditation (ISO 17025). The major advantage of this solution is that we can settle a facility wherever we want notably in countries where no anti-doping laboratory is present. Furthermore, blood samples are taken and analysed within 5 hours and all results can be reported immediately. The second approach is the setting up and implementation of a network of anti-doping laboratories all around the World. All laboratories have to be ISO 17025 as well as WADA (World Anti-Doping Agency) accredited. Because we are often legally challenged when reporting a positive abuse result we have to be completely sure of the quality of our results, which means a very comprehensive quality control procedure. It is mandatory that we run the blood samples on the same instrumentation, that is to say the Sysmex XT-2000i analyser. Furthermore, the analytical procedure must be the same in all laboratories:

Internal Quality controls:

- All three levels of Sysmex quality control (low, normal and high) must be analysed twice prior to any blood analyses
- At least one internal QC must be done after every thirty – fifty samples
- At the end of the batch of samples, at least one internal QC must be done

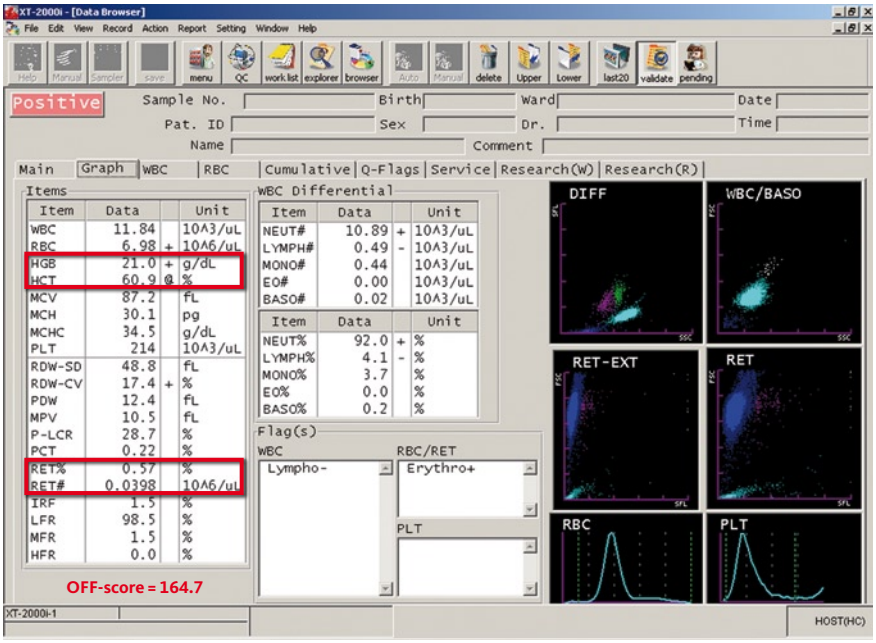

Figure 2 *Screenshot from the Sysmex XT-2000i analyser demonstrating the blood results from the case one athlete.*

External Quality controls:

- Once a month, all anti-doping laboratories must analyze an external QC material provided by the CSCQ (Quality Control Center Switzerland). In that way, we can check that all are achieving the correct target values and coefficients of variation for all parameters

In addition to these internal and external quality controls, we run a fresh blood control, taken from one of us, seven times consecutively to check for precision on the instrument. A spun HCT is also performed on this blood to check that there is good agreement between this method and the automated method. We also recommend analyzing a hand full of blood samples on our analyzer and on other analyzers (XT-2000i or XE-2100 models) located in the neighbourhood. This enables to perform some kind of ring test and to evaluate precision and bias.

In order to guarantee optimal results, all blood samples are withdrawn according to very conservative procedures and must be transported under refrigerated conditions (2 °C < T 12 °C; a datalogger is required) and analyzed within 36 hours after collection. The analytical protocol is summarized below:

- All blood samples shall be homogenized for a minimum period of 15 minutes prior to analysis.
- Each blood sample shall be analyzed twice. Absolute differences between the results of the two analyses shall be equal or less than the following for the relevant analyses to be accepted:
  - 0.1 g/dL for HGB analysis
  - 0.15 absolute difference for RET% analysis (if first measurement lower or equal to 1.00%)
  - 0.25 absolute difference for RET% analysis (if first measurement higher than 1.00%)

If absolute differences between the results of the two analyses are greater than those defined above for a specific sample, the analysis shall be started again.

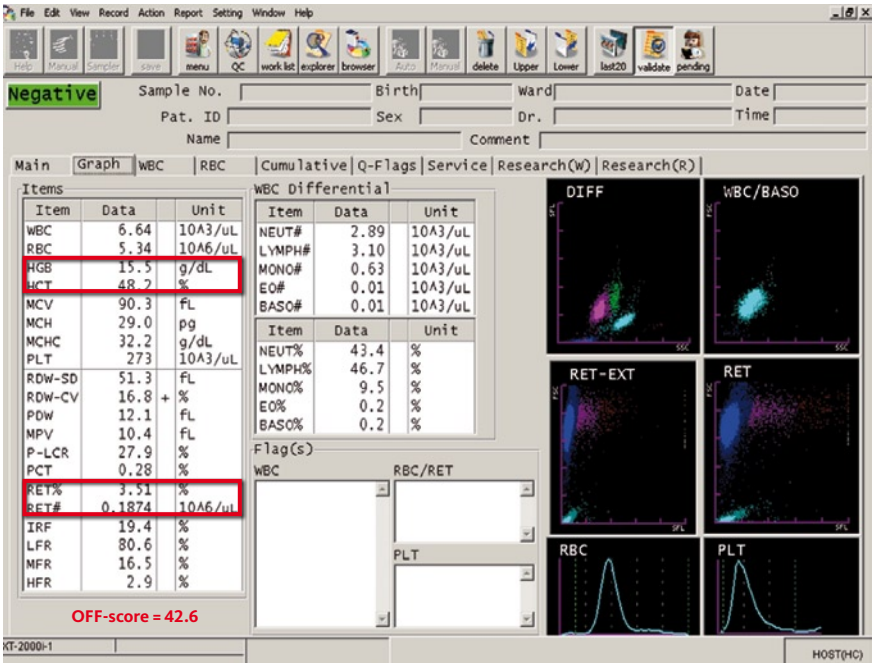

Figure 3 *Screenshot from the Sysmex XT-2000i analyser demonstrating the blood results of the case two athlete.*

Case studies

Sometimes the data is very unusual.

Case 1

This athlete had a high haemoglobin and haematocrit with a low reticulocyte count, which calculates to a high OFF-score of 164.7 (figure 2).

The suspicious results meant that a full urine test for EPO was carried out but no EPO was detected. This was not unsurprising as the high OFF-score indicates that the subject had doped but had stopped taking EPO. However due to the high OFF-score the athlete was not allowed to participate in the event.

Case 2

This athlete has a HCT just under 50 % so he decided to increase it a little with some EPO. The erythropoietic stimulation can be seen by the high reticulocyte count (a typical ON period picture). Of course the OFF period score is low (figure 3). The athlete was targeted and recombinant EPO was found in his urine.

Detection of homologous blood transfusions

Between the years 2001 to 2003 we noticed that more athletes had a low reticulocyte count, even though all the samples were analysed on the same haematology analyser. We went back to these athletes' medical records and analysed their ferritin levels. During EPO usage, or recent past EPO usage, the ferritin level decreases, but these athletes had high ferritin levels, which indicated it was likely they had been using blood transfusions, homologous or autologous, to increase their red cell mass. We launched a major project to detect the use of homologous transfusions using laboratory data.

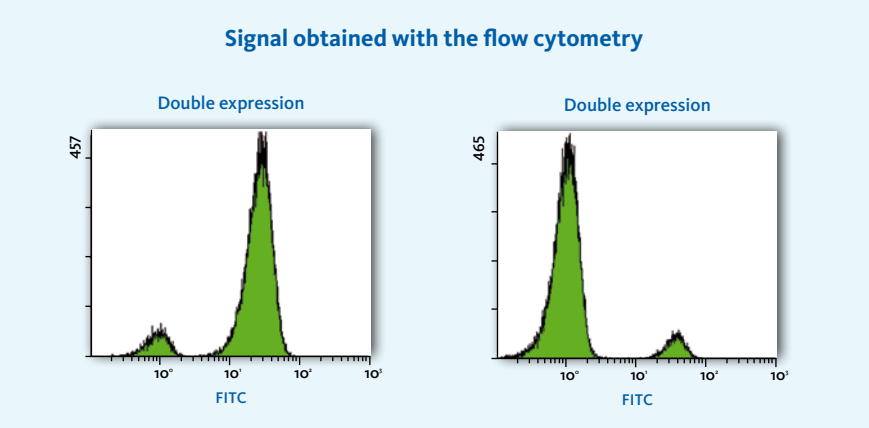

Figure 4 *The first histogram demonstrates a large population of the red cells are positive for the antigen but there is also a small population of cells which are negative (from the blood donor). The second histogram shows that the athlete is negative for the antigen but the small peak of positive cells (indicating antigen positive blood from a donor).*

The method depends on detecting specific proteins (antigens) on the red cell membrane using specific monoclonal antibodies to these proteins attached to a fluorochrome. Using a flow cytometer and specific gating strategies it is possible to detect a purely negative expression for a protein or a purely positive expression, both situations produce a single peak in a different location on the flow cytometer histogram. However a person who has received a homologous blood transfusion will have dual expression for some red cell antigens; those of their own and those of the blood donor and producing a double peak on the flow cytometer histogram (figure 4).

Detecting abuse of artificial haemoglobin, haemoglobin based oxygen carriers (HBOC)

In Switzerland there is only one HBOC compound available, Oxyglobin, which was originally developed to treat anaemia in dogs, but elsewhere another compound called Hemopure is available. Other compounds are about to be launched but they all have different chemical structures. It is, in fact, quite easy to detect the presence of these compounds in the blood; the plasma will be pink due to the free haemoglobin. The free haemoglobin can be quantitated and the plasma directly examined for the compounds using electrophoresis or high performance liquid chromatography.

Future threats for blood doping

Gene doping! An ex vivo gene therapy for the regulated delivery of EPO based on the implantation of encapsulated, genetically engineered myeoblasts, this has already been achieved in a mouse model using doxycycline-based regulation of gene expression to modulate the secretion of EPO[5,6].

The solution to detect erythropoietin abuse in athletes

An Australian group found that by comparing an athlete's haematological parameters against his or her own historical baseline (rather than population-derived reference ranges) enhances the ability to detect blood doping. Removing within-subject variability by comparing new results against a historical baseline heightens sensitivity and specificity to detection of blood doping. It is possible to delineate the longitudinal changes in either haemoglobin or the OFF-score caused by EPO treatment from the natural biological fluctuations found in subjects treated with placebo[7]. We also use a similar strategy, but with different mathematical Bayesian algorithms[8].

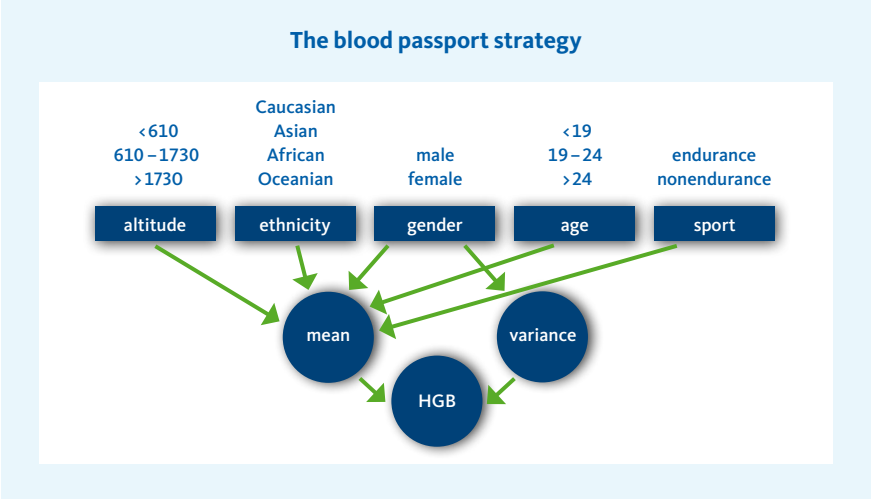

Figure 5 *The blood passport strategy: allowing for the individual's different circumstances that may affect the blood to be taken into account, and producing an algorithm for an individual's cut-off values for suspicion of blood doping.*

Our philosophy is the use of a blood passport, which takes into account heterogeneous factors which affect the blood such as altitude, gender, age and ethnicity. Theoretically, it would even be possible to include the type of instrument and type of laboratory used to measure the blood. With this type of Bayesian network it is possible to adapt the individual cut-off limits that cause suspicion in the screening tests (figure 5).

In the future genomics, proteomics and metabalomics may play a role in the detection of blood doping, but so will economics! So the blood passport is the best way to go forward, the parameters are inexpensive and easy to measure and in the future, the rules and algorithms used may well further evolve.

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Diagnostic Perspectives – The Sysmex Europe eJournal for Clinical Laboratory Medicine · Published 2010 by Sysmex