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One year's experiment with the blood screening test to fight against rhEPO doping.

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Introduction

Since the mid 90's, several proposals were published showing the ability of blood secondary hematological and biological parameters to prove the abuse of recombinant EPO in sport [1,2,3,4]. Gareau et al [1] were strongly supporting the idea that the ratio of serum transferrin receptor/ferritin (sTFR/Ferr) could be the best tool to diagnose this abuse. Knowing from several internal reports [5] that iron was absorbed in huge quantities in some endurance sports, especially in cycling, this single ratio strongly dependant on the iron metabolism was finally shown not to be so useful. Naturally, the scientists chose to go in a multi-factorial approach based on a combination of several hematological and biochemical measurements [3]. It became nevertheless evident that this approach could be more interesting in a longitudinal hematological survey of an athlete (hematological passport) rather than a real anti-doping test in itself. It was chosen in Sydney as a part of the EPO anti-doping test together with the urinary EPO French technique [6]. Just before the games, these authors showed that urine samples could still be employed for the detection of EPO doping based on the different IEF pattern of glycoforms of rhEPO.

The original part of their work was published in 2001 [7]. The Paris laboratory allowed several IOC accredited laboratories to acquire this technology in order to perform these delicate analyses.

Based on a long term experience on blood samples in sport (more than 7'500 blood samples since 1997) and on a controlled double blind study with volunteers treated with rhEPO, We introduced this method after having been educated in Paris. The urinary detection of rhEPO was applied on samples chosen randomly or after a screening blood test performed in the morning of the competition.

Materials and Methods

Double blind study

The study was approved by the ethics committee of our clinic and the nature of the experiment was explained to the volunteers and their written consent was obtained. Then a full medical exam and a blood test were undertaken. All subjects had a normal iron status assessed from ferritin values, a normal haematological blood count and no signs of infection. Thirty one healthy, sporty, Caucasian male took part to this randomised double blind study. The mean age was 26 years old (range 19 to 36 years), height 1.80 m (range 1.71 – 1.91 m) and weight 76.1 kg (range 59.8 – 98.6 kg) respectively. Because it was impossible to handle all men at the same time, each volunteer had his own schedule and had to come always at the same time. This was done in order to prevent biological variations due to a circadian rhythm effect for some parameters (EPO and iron for example). All volunteers were asked not to abuse of alcohol, caffeine, were not allowed to smoke and could take drugs (aspirin, paracetamol...) only after medical advice. A proper diet and enough sleep was recommended to all men. Blood parameters were analyzed directly after blood collection. Cell counting was performed with a Cell-Dyn 4000 (Abbott diagnostics division, Baar, Switzerland.) and sTFR on serum was analyzed with an EIA automate Advantage (Nichols diagnostics division, Allschwil, Switzerland.).

Blood collections on the field

The International cycling federation (UCI) is performing blood sampling on the competition sites since 1997. In 2001, in addition to the hematocrit and hemoglobin measurements (Coulter ACT, Beckman Coulter, Nyon, Switzerland), the reticulocytes were counted by a transportable machine Sysmex, R-500 (Sysmex, Digitana, Horgen, Switzerland.) both measurements were performed on the same sample, collected early in the morning. This approach allowed to standardize, as much as possible, the pre-analytic process and to provide the results before the start of the race.

Urinary EPO test

This analysis was performed on urines collected in or out of competition samples, with or without a blood screening. The method employed here was strictly the same as described by Françoise Lasne et al [6,7,8] and in the inter-laboratory study presented in this book [9]

Results

Double blind study

Only partial results are showed here, the entire set of data being published elsewhere [10]. The reticulocytes unsurprisingly rise rapidly directly after the beginning of the treatment. The mean peak is reached after 10 days to decrease to the normal level even during the treatment after four weeks (Fig. 1). The same type of curve is drawn by the sTFR with almost one week of delay. This shows clearly that the solubilization of the receptors are strongly depending on the reticulocytes maturation. The number of reticulocytes then cannot be considered as a highly specific signal of EPO doping in a spot blood sample. Nevertheless, in a longitudinal follow-up, the evolution of the relation between the 2 parameters can be of great help to eventually indicate a manipulation.

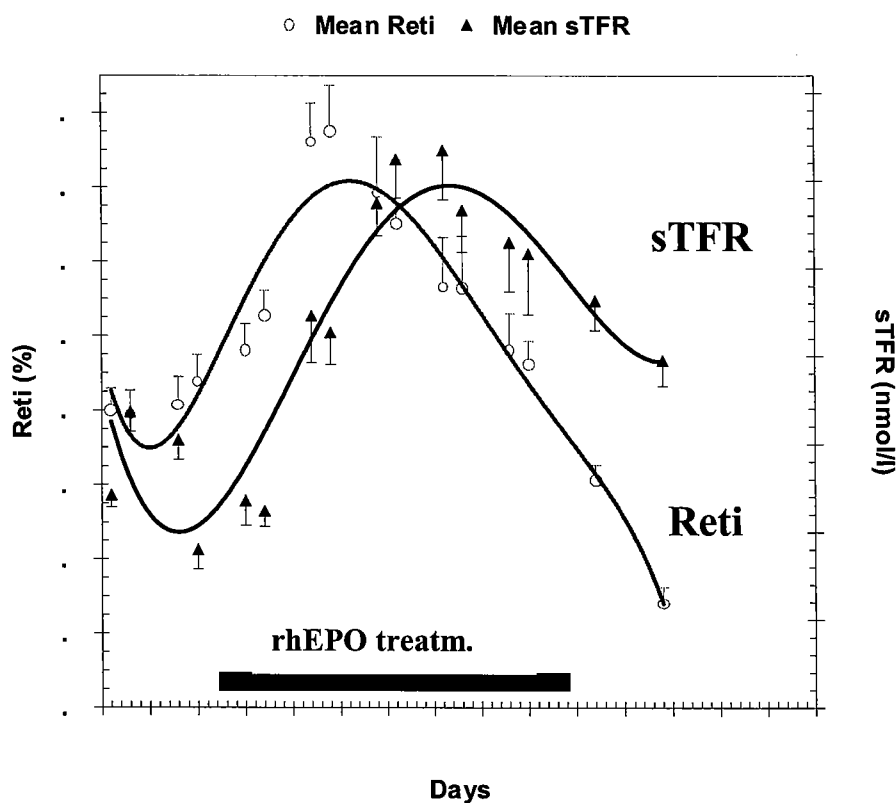


Figure 1: Mean reticulocytes and sTFR for the 80 IU/kg (3 times/week) treated subjects

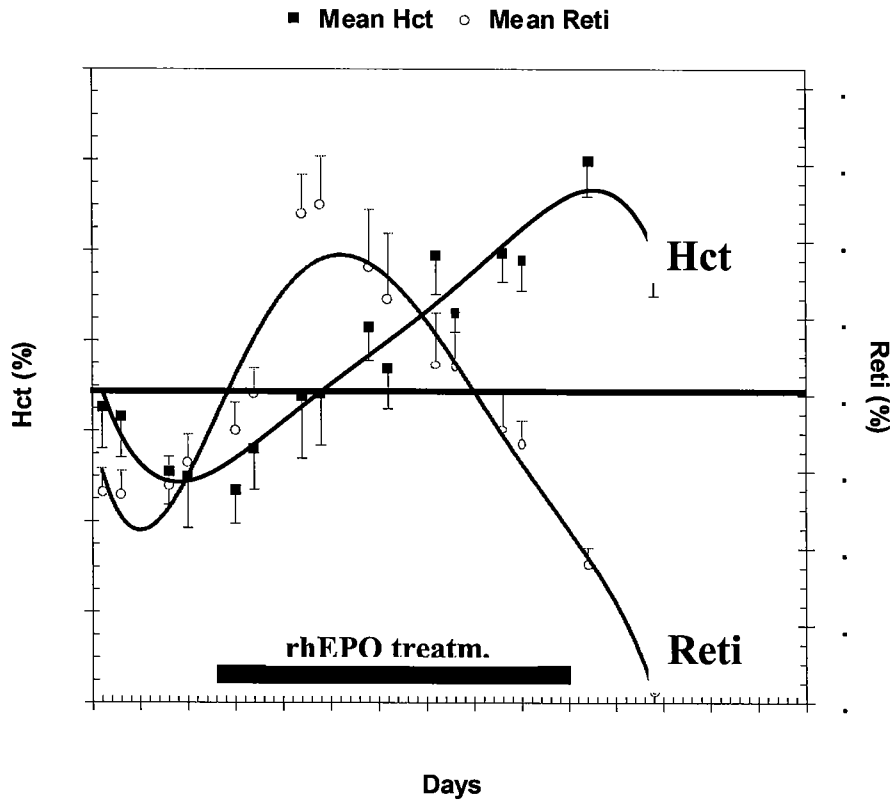


Figure 2: Mean reticulocytes and hematocrit for the 80 IU/kg (3 times/week) treated subjects

By superposing hematocrit and reticulocytes counts, the first and the last biological signal of the hematopoiesis process are used. (Fig.2)

In the group of treated volunteers (in which the treatment was stopped when Hct > 50%), elevated Retic. (>2.4%) or elevated Hct (> 47%) can be used to cover the period of time (in this experiment 6-7 weeks) during which the suspicion of doping of EPO can be stated. This scheme is of course not taking into account any discontinuous treatment.

Hematological values in a cycling population

During the 2001 season, 1510 cyclists were tested for Hct and Retic. The results are presented in the Table 1 and the Figure 3 . This shows the relation in the population between the reticulocytes and the hematocrit. The zones drawn on this Figure are defined by the cut-off values used by UCI in the screening test. The limit for reticulocyte was placed at 2.4 %, because it is the highest limit for normal values indicated by the counter's provider. The limit at 47% rather than 50% for hematocrit enlarged the population screened. Nevertheless, the

experience will show that the criteria of hematocrit was finally less diagnostic than the reticulocyte count.

	Hematocrit in %	Reticulocytes in %
mean	44.2	1.3
min	35.5	0.1
max	52.6	4.6
n \geq cut off	n \geq 47% = 255	n \geq 2.4 = 74

Table 1: hematological values of 1510 cyclists controlled for Hct and Reti in 2001 season.

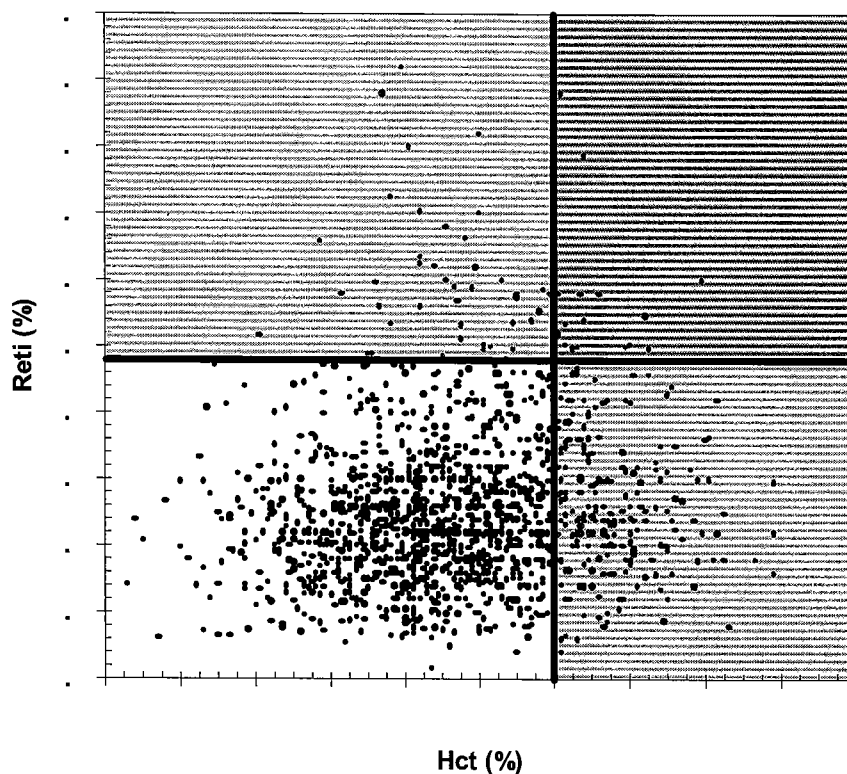


Figure 3: Relation between the Hct and Reti of 1510 cyclists in 2001 season with the limits.

EPO urine analyses

Graphic representation of the results

The first criteria for positivity adopted in our lab in 2001 was that one announced for the Sydney games and based on the negative subjects data base elaborated by the Paris laboratory [9]. Then, to announce an A sample positive to the federation, the basic bands should represent 80% of the chemi-luminescence intensity relatively to that of all the bands on the IEF lane. It became obvious during the season that this lone quantitative ratio was not sufficient to express the quite clear and evident image of a positive urine. This definition was also quite dependant on the quality of the gel and the variability due to the quantification method of the optical density measurements.

It was then decided to make a graphic representation which can be easily understood, to our point of view, by the court in the arbitration hearings. The bands were numbered as shown on the Figure 4. The band 0 was defined by the less basic isoform of the standard EPO beta (dashed line). The bands closer to the anode were positive numbered, the basic ones being

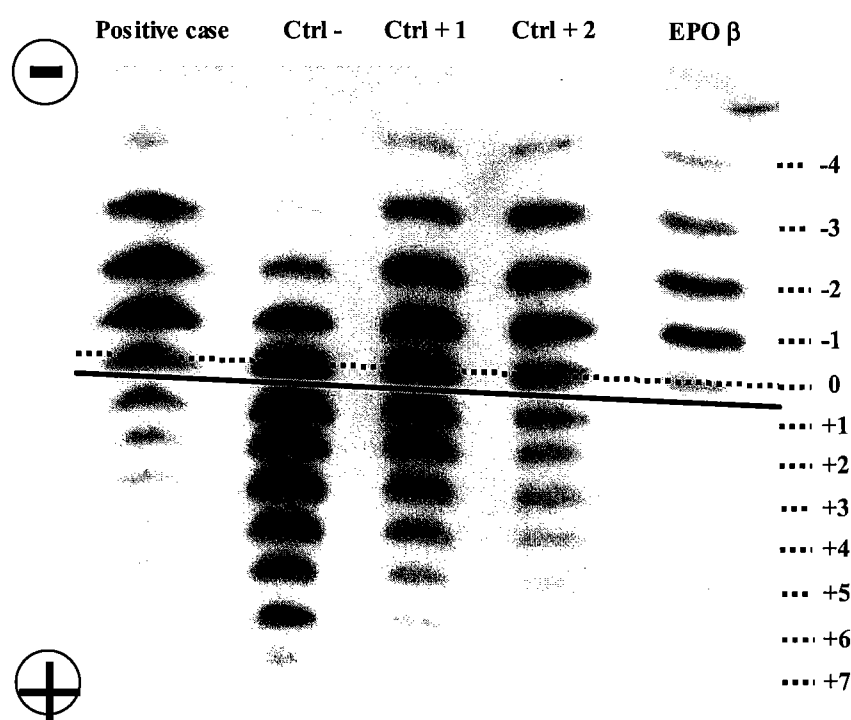


Figure 4: IEF gel with several controls and a positive case. The dashed line defines the band 0. The full line defines the separation between the basic and the acid field

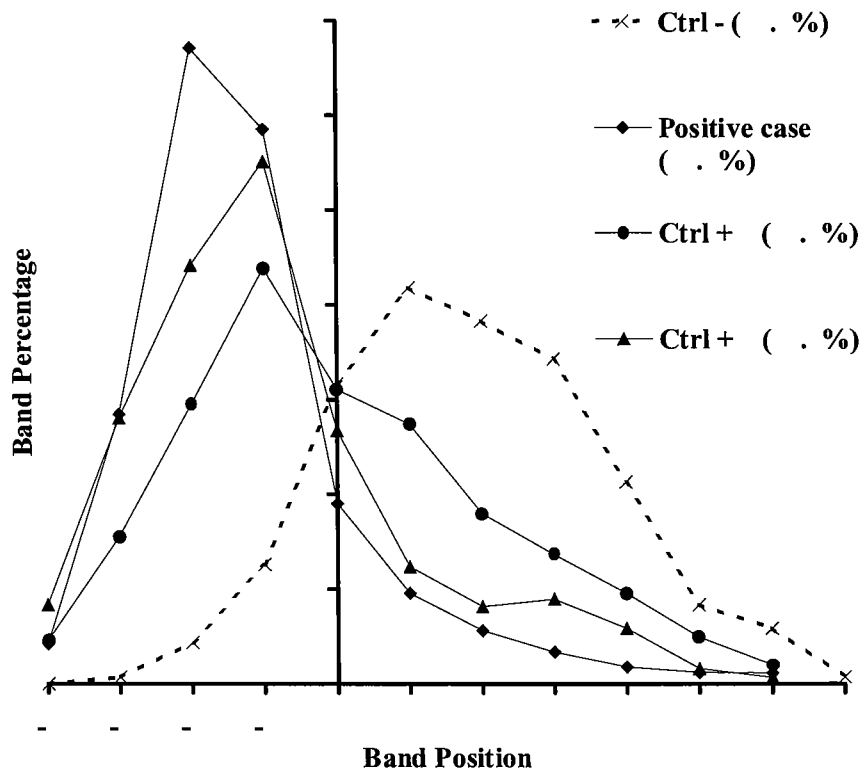


Figure 5: graphic representation of the gel from Fig.4. (%) with classical calculation.

identified with negative numbers. Each band corresponds to a certain percentage of the total chemi-luminescence signal. Each lane could be then represented by curves defined on a graph with the band number in the x-axis and the percentage of total signal in the y-axis. (see Figure 5).

The maximum peak of a positive urine is clearly in the basic side (negative bands) of the gel, whereas the maximum peak for a negative urine is in the acid field. This graphic representation was drawn in order to give arguments for convincing the judges of the qualitative aspects of the gel picture. This can certainly be completed by the actual new criteria proposed by the inter-laboratory protocol. On the Figures 4 and 5, it can be said that bands -1 and -2 should be present in the lane of the sample and that they should be more intense than the bands 1 or 2 by a factor to be determined. In that case, the band 0 is in fact ignored, because its status, to our point of view, is not very clear.

In Figure 5, the negative control shows clearly a maximum peak in the acidic portion of the gel (maximum at band 1). The positive case is the image of an athlete's urine which was

declared positive. The maximum peak is clearly in the basic field (band -2) and the percentage of basic bands (with the classical calculation) is 88.6%.

The two positive controls are obtained from a volunteer who received 5000 IU of EPO beta.

The positive control No 1 is .an urine sample collected 3 hours after the injection. The relative abundance of the bands are just in favor of the basic ones with a maximum peak on the band - 1. But the signals of the band 1 and 2 are not suppressed. This is also shown by the calculation of the percentage of basic bands (62.2%). This situation (at the beginning of treatment) is not met very often; whereas the same figure occurs at the end of the treatment when the “endogenous” bands reappear after being suppressed by a longer term treatment.

The positive control No 2 is an urine sample collected about 24 hours after the s.c. injection. The co-localization of the more intense bands with those from the EPO beta and the partial suppression of signal from bands 1 and 2 are there quite obvious.

In the Figure 6, the IEF curves A, B1 & B2 are A and B samples from an athlete’s doping control. The A curve was obtained from the gel of the A-sample analysis, the B1 and B2 curves being two duplicates (two lanes) from the same retentate of the B-sample , prepared one month later (the B sample being kept frozen in between).

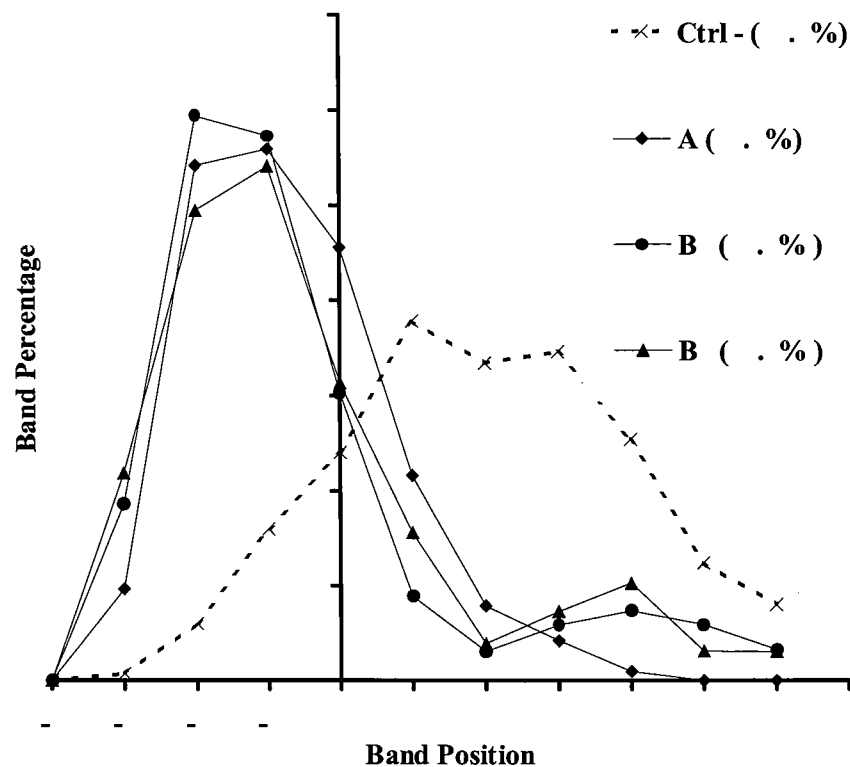


Figure 6: graphic representation of lanes coming from the same athlete. A and B samples were analyzed with one month interval . (%) with classical calculation.

The variability between the 3 lanes is acceptable. The B2 lane has been calculated at only 78.4 % of basic bands even if from the qualitative point of view, it looks not significantly different from the others (A & B1).

Reading these results with other criteria, the positivity appears clearly. The co-localization of the most intense bands (bands -1 & -2) and their relative intensity comparatively with bands in the acid field (band 1 or 2) are in all cases over 200 %.

Results over the 2001 season.

In cycling, we have declared 7 samples positive for rhEPO after urine analysis. The table 2 shows the relation between the hematological values and the urines tested positive. At the end of the season, it appeared that the situation changed and that the hematology was not so helpful in detecting the potential suspicious cases.

Cases	Hct (%)	Hb (g/dl)	Retic. (%)
1	45.6	15.7	1.9
2		No blood	
3		No blood	
4	42.6	13.8	3.6
5	44.6	14.9	3.3
6	45.0	14.7	3.5
7	45.7	15.0	2.4

Table 2. Hematological values (if any) of the positive cases for rhEPO found in our laboratory in cycling for the 2001 season. On 156 EPO urinary analyses, 7 were finally declared positive.

The need for definitive common criteria on the definition of positive results by IEF became more and more obvious. This should certainly lead to the adoption of the urine analysis alone as a burden of proof in case of EPO doping.

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