Report on the Haematology Aspects of the Roman Kreuziger Case

By

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The case:

The CADF in two documents entitled Athlete Biological Passport Expert evaluation, evaluation of blood profile BPY2524M36 dated 14.06.2012 and in the Athlete Biological Passport Evaluation of the athlete's argument, dated 12.05.2014 have alleged that the biological passport shows a number of abnormalities that suggest the use of an illegal method. The rider has responded with reports of Dr Douwe de Boer dated 25 August 2013 and Dr Locatelli Massimo dated 1 August 2013. I have been asked by the rider to provide a further report.

The abnormalities:

There are two abnormalities highlighted in the two reports. The first are a consistently sustained even increase in haemoglobin level during competitive racing, especially during the Giro Italia 2012. This relates to samples taken before the Giro Italia 2012 on 5 May and after stage 9 on the 14 May stage 15 on 20 May and stage 18 on 24 May.

The second abnormality is the elevated reticulocyte values from March until August 2011 as well as from April 2012 until the end of the Giro Italia. This is because the reticulocyte series was abnormal but there was no suggestion that the haemoglobin or off scores were abnormal.

It is necessary for the CADF expert panel to propose a doping scenario for the observed abnormalities. In the case of the Giro Italia 2012 in their summary they propose that the likelihood described methods being due to blood doping such as the use of blood transfusion is very high. In the original document dated 14.06.2012 they make no proposal as to an illegal method used for raising a reticulocyte count, then in the document dated 12.05.2014 when discussing the expertise of Dr Locastelli Massimo they suggest that the raised reticulocyte values in 2011 and 2012 very likely represent the effect of an erythropoietin stimulation that has been introduced in 2011 and also applied during the competitive season of the following year 2012.

Giro 2012

The case against the athlete is that that his haemoglobin did not consistently fall throughout the race. The samples in question are as follows:

1. Date 03.05.12. Haemoglobin 15.0, haematocrit 45.1%, retics 1.46.
2. Date 14.05.12. Haemoglobin 14.5, white count 43.2, reticulocyte count 1.44%.
3. Date 20.05.12. Haemoglobin 14.8, haematocrit 44.8%, retics 1.52.
4. Date 25.05.12. Haemoglobin 16.1, haematocrit 48.1%, retics 1.44.

In support of their case they quote in the letter dated 14.06.2012 seven references. It is my contention that their hypothesis is not supported either specifically by the references they have quoted or by the available scientific literature. Reference 1 is in a journal I have not yet been able to access, there does not appear to be an electronic version of it and I am currently awaiting a paper copy or electronic copy from the British Library so cannot comment further, but will be able to do so in a short time.

Reference 2 is a paper by Schmitt et al, this looks at the haematocrit values in a number of settings. The numbers are small and not really applicable to the rider in this case. The first group is 8 subjects studied for 23 hours after a 1 hour bout of cycle exercise. The second group is 7 subjects subjected to 20 minutes of head down tilt. The third group is 4 subjects in a standing position for 60 minutes. The fourth group is 10 subjects doing a maximum exertion tests on a cycle ergometer on one occasion and the only group of relevance in this paper is 4 elite cyclists who participated in a 10 day competition covering 1,700 metres.

The third reference is a paper by Schumacher et al, called Haemoglobin mass cyclists during stage races. They measured haemoglobin mass by a CO-rebreathing method in 7 elite cyclists. Samples were taken pre race and at the end of the race only. They state plasma volume of blood volume tended to increase. Haematocrit dropped and haemoglobin concentration dropped. The starting haemoglobin in this group of only 7 riders was 15.8 +/- 0.9g/dL. At the beginning of the end 14.7 +/- 0.7 if subjects are normally distributed the mean of +/- 1 standard deviation will cover 66% of riders and the mean +/- 2 standard deviations will cover 99% of observations. The starting haemoglobin of 15.8 -1 standard deviation of 0.9 gives a figures of 14.9 which is comfortably lower than the final haemoglobin concentration of 14.7 +1 standard deviation of 0.7 = 15.4g/dL. Therefore, this small study has very limited power to prove the hypothesis held by the CADF.

Reference 4 is another paper by Professor Schumacher. In this case there are 23 subjects tested, pre and post are a 5 day race which is only a quarter the length of the tour of Italy. Again a detailed statistical analysis does little to support the case made by the CADF but I would note for later in the report that the reticulocyte count in these athletes was 1.6% on average.

Reference 5 is a paper by Gavican et al, Stability of haemoglobin mass during a 6 day UCI pro tour cycling race. Again there are only 6 professional cyclists in the active group. It is only a 6 day race and samples are only taken on stages 1, 3 and 6. Again this is not appropriate evidence to support the fact that the haemoglobin must fall during a 21 day stage race.

Reference 6 is a review by Fellman called a hormonal and plasma volume alterations following endurance exercise. As it is a review it brings in no new data. It addresses the combination of

exercise and heat accumulation and addresses a number of sports, in particular marathon running. Its relevance to professional cycling is not immediately obvious.

Reference 7 is another paper by Fellman et al, but this time an original article. It is called intracellular hydration induced by a 7 day endurance race. Again, there are only 9 athletes involved. Bloods were taken only at the beginning and at the end of the race and over 7 consecutive days the athletes performed 3 separate sports, namely running, cycling and cross country skiing. The distances covered were relatively short and the amount of time they exercised for was very variable. The results of this study, however, are quite interesting. In the figures in the article namely figures 1, 2 and 3, there is a relationship that shows changes in total body water, extra cellular water and intracellular water for each of the individual riders. In fact there are only 8 data points because one athlete's data was lost. What is clear is that even in these 8 athletes in whom you only have 2 data points, there is immense variation with some athletes showing very significant changes in body composition, in particular body water, whilst others show almost no change at all or even increases. Thus, the hypothesis that all athletes perform similarly which is implicit in the case made by the CADF, is disproved by this paper even though I do not believe it is strictly relevant to the case in question.

By far the most relevant paper is the paper by Roberto Coscetti et al, Haematological and iron metabolism parameters in professional cyclists during the Giro Italia 3 weeks stage race. In this study the doctors took samples from all 9 riders of the Liquigas-Cannondale team on 3 occasions only, namely at day -1 pre race, day 12 and day 22 during the race. The 2011 Giro Italia was 3 weeks long as usual and covered 3,524.5km. The 9 athletes finished 3rd, 57th, 60th 73rd, 82nd, 83rd, 104th, 139th, 142nd within the 159 cyclists, so a fair range of performances. In the summary, the authors say that the haemoglobin red cells and haematocrit decreased during the race was stabilisation in the second half but final values were lower than baseline. If, however, one looks at the figure 2, which shows for the 8 riders in whom data was available the comparison of the day -1 with day 12 and day 22 haemoglobins, it can be seen that between day -1 and day 12 all 8 riders had a small but significant fall in haemoglobin. Between day 12 and 22 there was on average still a fall in haemoglobin but looking at the specific points it can be seen that 4 of the riders' haemoglobin fell further. In 3 of the riders there was a small increase at day 22 back towards the starting levels but not attaining them, but in 1 rider the day 22 haemoglobin increased such that it was similar or even above the start in haemoglobin. Again this shows that even amongst a group of highly trained professional cyclists there is considerable variation in the physiological responses to a 3 week 21 stage tour event and that the use of group means rather than individual data points is mostly misleading.

What is missing amongst all this data is appropriate scientific evidence to support the hypothesis made by the expert group that anything other than a persistent fall in haemoglobin is abnormal.

There is no study performed in professional cyclists over a 3 week stage race with sufficiently frequent sampling to show that their hypothesis is correct. It is in my opinion conjecture. I have reviewed the evidence that they have quoted and that together with the paper of Coscetti leads me to believe that there is considerable variation amongst individual athletes and that whilst in a population the overall means would be consistent with their hypothesis that plasma volume increases, this is not always so in individual riders. This is because statistics apply to populations not to individuals and the CADF argument is largely statistical in the absence of appropriate scientific evidence.

Finally, in the document of 12.05.14 no additional scientific evidence in support of their argument is introduced. They again quote two papers, namely the review by Fellman and the paper by Schmidt. They note the findings of the Corsetti paper as raised by Dr Locatelli Massimo but for some inexplicable reason dismissed this, although in my opinion it is the most appropriate comparator paper.

Using the formula for determining changes in plasma volume that is quoted in Mitton Rubini et al paper, reference 9, which is that changes in plasma volume can be calculated by

$$\frac{PV_2}{PV_1} = \frac{(1 - Hct_2)}{(1 - Hct_1)} \times \frac{Hb_1}{Hb_2} \times 100$$

It is possible to calculate the changes in plasma volume that would account for the different haemoglobins and haematocrits in the 4 samples in question in the 2012 Giro. Calling the sample on 3 May sample 1, 14 May sample 2, 20 May sample 3, and 24 May sample 4, then a comparison of sample 1 versus sample 2 shows that the plasma volume, assuming the haemoglobin mass stays constant, has increased by 7%. Sample 1 versus sample 3 it has increased by 2%. Sample 1 versus sample 4 shows a decrease of 12% and a comparison of sample 3 versus sample 4 shows a decrease of 14%. If one assumes that plasma volume in a 75kg man is 3 litres, then the rider weighed at the time of the race 63k giving him a plasma volume of 2.5 litres, a 12% change in plasma volume, that is the difference between samples 1 and 4 is a total difference in plasma volume of only 302ml.

I have eluded before to the evidence that there is variation between different individuals, even within the same cycling team or in the same endurance event in their physiological response to exercise. In a cycle team this is compounded further as many of the riders will have different roles and will be performing at maximal exertion at different times in the race, for instance sprint stages are more common in the first week when sprinters and the lead-out men will be exerting themselves maximally and will often perform slightly less intensively during individual time trials and mountain stages. In the second week riders who are neither sprinters nor GC contenders will either try and get in a breakaway or be charged with chasing down a breakaway and thus their period of maximal effort may well fall in

the second week while GC contenders and climbers will only be exerting themselves maximally during individual time trials and mountain stages and often will be protected by the team during other stages. Consequently, one might well expect the observed differences between different people within the same team depending on their specific role.

The rider in question had performed extremely well in the 2011 Giro. He had finished 6th overall, his best ever result in a grand tour. He had won the white jersey and he had finished only 11 minutes 28 seconds behind the overall winner. The 2012 Giro ran from 5th – 27th May.

Date			**Result**
5 May	Stage 1	Individual time trial	Finished 28th
6 May	Stage 2		Finished 42nd
7 May	Stage 3		Finished 33rd
8 May	Rest day		- -
9 May	Stage 4	Team trial	Team finished 3rd
10 May	Stage 5		Finished 47th
11 May	Stage 6		51st
12 May	Stage 7		18th
13 May	Stage 8		17th
14 May	Stage 9		60th
15 May	Stage 10		9th
16 May	Stage 11		56th
17 May	Stage 12		24th
18 May	Stage 13		36th
19 May	Stage 14		16th
20 May	Stage 15		8th
21 May	Rest day		- -
22 May	Stage 16		27th - 8 min 57 sec adrift
23 May	Stage 17		30th - 10 min 12 sec adrift
24 May	Stage 18		121st
25 May	Stage 19	Mountain stage	Won by rider
26 May	Stage 20		15th - 9 min 10 sec adrift
27 May	Stage 21	Individual time trial	83rd - 3 min 29 adrift

The rider overall finished 15th in 2012 and did not ride the Tour de France or Spain that year. He was not in the top 10 and was at the end 19 min and 58 seconds adrift of the winner. If we specifically consider the period from the 21st, 22nd, 23rd, 24th May, that is the 4 days prior to the rider winning stage 19, a climb, and the day after 4th sample on 24 May was taken, we can see that on day 21 there was no strenuous exercise as it was a rest day. On stage 16 the rider was nearly 9 minutes adrift, the next day 10 minutes adrift of the winner. The next day 121st in a sprint finish. At this time the rider was 20th overall and 12 minutes and 53 seconds adrift of the leader. It was clear at this stage that 1) he could not win overall and 2) had not been performing at maximal intensity in the 4 days leading up to the mountain stage 19 on the 25th. Thus, this would further support the reason why his plasma volume was returning to normal as his exercise had not been as strenuous as since he could no longer win the tour he had decided on a strategy of a single maximal effort to win a single stage which he successfully did on stage 19 on 25 May, although he only won this by 3 seconds. Following this, again although there were only 2 stages left it can be seen that his effort was again not maximal, losing 9 minutes and 10 seconds on stage 20 and 3 minutes 29 seconds on stage 21. Consequently, I would argue that as the change in plasma volume is related to exercise intensity and is reversible, then the rider was not at maximal effort in the 4 days leading up to his win in stage 19 and therefore there would have been a lessening of the expansion of his plasma volume. As we have seen in the papers quoted above, haemoglobins can increase as the expansion in plasma volume decreases in the second half of the tour and it is my opinion that the increase in his haemoglobin on 24 May is due to a reduction in plasma volume rather than an increase in red cell mass due to an illegal method such as transfusion.

If the rider had been transfused prior to sample 4 on 24 May then according to the letter of Damsgaard et al reference 10, which investigates the effects of blood withdrawal and blood reinfusion on haemoglobin and reticulocyte count amongst other variables, then reinfusion should be associated with not only a significant increase in haemoglobin and haematocrit but a significant fall in reticulocyte count. In the experts' opinion, they suggest that the reticulocyte count has not fallen significantly because there has not been time but in this letter following reinfusion on day 0 the reticulocyte count is significantly different at $P<0.05$ on days 1, 3, 7 and 14. Consequently the suppression in reticulocytes is immediate if there is a sudden increase in red cell mass, although we have limited data in this case I see no evidence to support a marked fall in the reticulocyte count between samples 1, 2, 3 and 4 and this be further evidenced against the rise in haemoglobin being due to an increased red cell mass secondary to transfusion rather than due to changes in plasma volume. This letter to the editor also shows the effect of blood withdrawal where withdrawal is associated with a sudden fall in haemoglobin and a marked increase in reticulocyte count from levels of 1.1% up to 3% at day 7. I would note that although in the second part of this argument I will have to address the raised

reticulocyte counts at no time is a reticulocyte count of that level seen in this rider, but I accept that out of season blood withdrawal would be difficult to identify given the limited frequency of biological passport tests. However, out of season blood withdrawal would then necessitate approximately monthly reinfusion and withdrawal of blood until it was used by being reinfused in May. If there is no fall in reticulocyte count another explanation could be that there was not only transfusion but concomitant use of erythropoietin to prevent the suppression of the fall in reticulocytes but the athlete has had a large number of urine tests performed between 2010 and 2014, all of which are negative.

The raised reticulocyte count:

It is the hypothesis of the CADF that the reticulocyte count is raised from March 2011 due to the use of an erythrocyte stimulating agent, namely erythropoietin. There are in the documents boxed block plots showing a rise in reticulocyte count and an explanation for this has been given by Dr Douwe de Boer previously. I would like to make reference at this stage to a number of observations. Firstly, the rider's urine tests were performed in 2010 on 3 occasions, in 2011 on 4 occasions, in 2012 on 7 occasions, in 2013 on 7 occasions and in 2014 on 4 occasions – all of these are negative. Specifically in the Giro 2012 he had samples performed on stages 13, 19 and 21 which were negative.

Next, I have analysed the data, not on reticulocyte count, but on haemoglobin levels in the UCI samples given in appendix 1 on page 10 and 11 of the 13 page report dated 12.05.2014. In 2008 there were 4 samples, mean haemoglobin 15.2. In 2009 there were 9 samples, mean haemoglobin 15.38. In 2010 there were 17 samples, mean haemoglobin 15.3 and at the beginning of 2011 there were 3 samples, mean haemoglobin 15.33. Considering these 34 samples together the mean haemoglobin is 15.39. From sample 43 dated 14.3.2011 the reticulocyte count increases, for the rest of 2011 there were a total of 10 samples with a mean haemoglobin of 15.16 and in 2012 up to sample 62 including samples 56, 57, 58 and 59 taken at the time of the Giro 2012 there are 17 samples with a mean haemoglobin of 15.04. Overall, in the 17 samples in this time period the mean haemoglobin is 15.11. Consequently, it can be very easily seen that the mean haemoglobin before the increase in reticulocyte counts was persistently higher than the mean haemoglobin afterwards. Since the purpose of giving an illegal method such as using recombinant erythropoietin is not to increase a reticulocyte count but to increase the red cell mass, which is reflected by an increase in haemoglobin as well as an increase in reticulocyte count, in order to increase the oxygen carrying capacity of the blood, if the rider was using recombinant erythropoietin throughout this period he was clearly unsuccessful as although his reticulocyte count was increased his haemoglobin was not, and hence there would be no increase in performance due to increased haemoglobin and hence increased oxygen carrying capacity. Consequently, alternative explanations for his increase reticulocyte counts should be sought and I refer to the report by Dr Douwe de Boer.

In the paper European Journal of Applied Physiology, M Ashenden et al, current markers of the athlete blood passport do not flag microdose epo doping, reference 11, there is a study that shows that small doses of erythropoietin given frequently can be given without triggering abnormalities of the athlete's biological passport. Ten subjects were given twice weekly intravenous injections of erythropoietin for up to 12 weeks in 5 treatment groups, the results of which are shown in table 1. It can be seen that there are significant increases in haemoglobin mass, there is a 10% increase in haemoglobin from 15.53 at baseline to 16.43 g/dL at the end, whereas the reticulocyte count starts at 0.63 and finishes at 0.66 in these 9 athletes across the whole period; thus, in this study the use of microdosing erythropoietin increased haemoglobin and red cell mass and hence oxygen carrying capacity without increasing reticulocyte count. The CADF expert panel, however, were suggesting in this athlete that erythropoietin has increased reticulocyte count without increase in haemoglobin. Certainly, my clinical expertise in the therapeutic use of erythropoietin to treat patients with anaemias, is that therapeutic doses of erythropoietin of either human recombinant or pegylated human recombinant erythropoietin is associated with an increase in the reticulocyte count, accompanied by significant increases in the haemoglobin. Indeed, if the erythropoietin only increased the reticulocyte count and did not increase the haemoglobin, it would have found no clinical use whatsoever. Similar points are made in the article in Blood in 2011 called Blood doping and its detection by Jackleman and Lungbe which is reference 12. Again this suggests that the use of erythropoietin either in full dose or smaller so called microdosing, is used to increase the haemoglobin not the reticulocyte count.

Summary:

I have reviewed the athlete's biological passport, the scientific evidence presented by the expert panel and the additional scientific evidence from the Corsetti paper which is perhaps more relevant to a 3-week stage race and conclude that it is not possible to be comfortably satisfied that the athlete's increase in haemoglobin in the sample on 24 May 2012 was due to the use of an illegal method as there is considerable variation between athletes which could account for this and also changes in plasma volume are not consistent between athletes or across all the stages of the race, and in part are dependent on race strategy and periods of exceptional exertion and periods of suboptimal exertion.

I have also reviewed the data from the athlete's biological passport on his reticulocyte count and related this to his haemoglobin. Although his reticulocyte count is increased the haemoglobin actually falls during this period which is not consistent with the effects of erythropoietin even at therapeutic or microdosing and therefore not consistent with an increase in performance by use of an illegal method. Dr Douwe de Boer has provided an alternative explanation that temporally fits with the changes observed.

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He graduated MB, ChB (with honours) from the university of Leeds and at the same institution was a Welcome Research Training Fellow, whilst obtaining his MD in the Hormonal control of Haemostasis and Fibrinolysis.

He is a Fellow of the Royal College of Physicians and the college of Pathologists, and has a diploma in Medical Education from the University of Sheffield.

He has published in excess of 50 papers in peer reviewed journals, as well as invited editorials, subject reviews and book chapters.

He was awarded Young Investigator Prizes by both the BSHT and ISTH

He has worked in an expert capacity for UK Athletics and UK Anti-doping in the past on the use of illegal methods and biological passports.

References:

1. Gore CJ, Morkeberg J, Schmidt W, Garvican LA, FellmanN. Plasma volume shifts during multiday racing. Clini. Chem. Lab. Med. Cclm Fescc. 2012 Dec 6;1-3.
2. Schmidt W, Biermann B, Winchenbach P, Lison S, Boning D. How valid is the determination of haematocrit values to detect blood manipulations? Int. J. Sports Med. 2000;21(2):133-8.
3. Schumacher Y, Pottgiesser T, Ahlgrim C, ruthardt S, Dickhuth H-H, Roecker K. haemoglobin Mass in Cyclists during Stage Racing. Int. J. Sports Med. 2008 Apr;29(5):372-8.
4. Schumacher YO, Temme J, Bueltermann D, Schmid A, Berg A. The influence of exercise on serum markers of altered erythropoiesis and the indirect detection models of recombinant human erythropoietin abuse in athletes. Haematological. 2003 Jun;88(6):712-4.
5. Garvican LA, Eastwood A, Martin DT, Ross MLR, Gripper A, Gore CJ. Stability of haemoglobin mass during a 6 day UCI ProTour cycling race. Clin. J. Med. Off. J. Can. Acad. Spor Med. 2010 May; 20(3):200-4.
6. Fellmann N. Hormonal and plasma volume alternations following endurance exercise. A brief review. Sports med. Auckl. Nz. 1992 Jan;13(1):37-49.
7. Fellmann N, Ritz P, Ribeyre J, Beaufrére B, Delaître M, Coudert J. Intracellular hyperhydration induced by a 7-day endurance race. Eur. J. Appl. Physiol. 1999 Sep;80(4):353-9.
8. Roberto Corsetti, Glovanni Lombardi, Patrizia Lanteri, Alessandra Columbini, Rosella Graziani and Gluseppe Banfi. Haematological and iron metabolism parameters in professional cyclists during the Giro d'Italia 3-weeks stage race. Liquigas Cannondale Medical Board, Sesto al Reghena, Italy.
9. Milton E Rubini, Charles R Kleeman, Ezra Lamdin. Studies on alcohol diuresis. 1. The effect of ethyl alcohol ingestion on water, electrolyte and acid-base metabolism. The Journal of Clinical Investigation.
10. R. Damsgaard, T Munch, J Morkeberg, S P Mortenson and J Gonzalez-Alonso. Effects of blood withdrawal and reinfusion on biomarkers of erythropoiesis in humans: implications for anti-doping strategies. 1006, Haematologica, the Haematology Journal, 2005; 91(7).
11. Michael Ashenden, Clare E Gough, Andrew Garnham, Christopher J Gore, Ken Sharpe. Current markers of the Athlete Blood Passport do not flag microdose EPO doping. Euro J Appl Physiol. DOI 10.1007/s00421-011-1867-6.
12. Wolfgang Jelkmann and Carsten Lundby. Blood doping and its detection. Blood, 1 September 2011, volume 118, number 9.