COMPREHENSIVE ANALYSIS OF BPY2524M36 ABP DOCUMENTATION PACKAGE

INTRODUCTION

AUTHORSHIP

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PETITIONER

On july 2014, Mr Massimo Martelli (Milano, Italy), attorney who acts on behalf of Roman Kreuziger, asked Cristobal Belda Iniesta to draw up an expert opinion on the Biological Passport Documentation Package, identified with code BPY2524M36 that belongs to Mr. Kreuziger.

TOPICS COVERED IN THIS REPORT

The purpose of this expert report is to analyze the Biological Passport Documentation of Mr. Roman Kreuziger, from April 2008 to April 2013, based on current medical, biological, biochemical and mathematical knowledge.

This analysis will include a review of the expert reports submitted by the Cycling Anti- Doping Foundation (CADF) and a point-by-point analysis of every allegation and suggestion from CADF reviewers.

The structure of this manuscript attempts to provide readers, with different levels of biomedical education, a comprehensive understanding of Mr. Kreuziger Biological Passport and the biological evidences that will support our final conclusions. To achieve this aim, we have included different levels of scientific depth and terminologies covering different linguistic and educational varieties of experts that may have access to this report. It is clear, therefore, that it is not our intention to produce an inaccessible report to anyone beyond the field of Biomedicine and Molecular Biology. So we apologize if at any time descriptions or explanations of biological data are based on the fundamentals widely known by experts in Biomedicine. We intend to explain our findings in understandable language. In any case, the strength of the arguments is based on robust science and not on the complexity of language.

DOCUMENTS PROVIDED BY PETITIONER

- An expertise of Dr. G. D´Onoffrio, Dr. R. Parisotto and Dr. YO. Schumacher entitled "Athlete Biological Passport- Evaluation of the Athlete´s Arguments", identified with Conc.: Blood Profile BPY2524M36, dated on 12.05.2014 (English, 13 pages). This report is included as Appendix 1.
- An expertise of Dr. G. D´Onoffrio, Dr. R. Parisotto and Dr. YO. Schumacher entitled "Athlete Biological Passport- Evaluation of the Athlete´s Arguments", identified with Conc.: Evaluation of Blood Profile BPY2524M36, dated on 14.06.2012 (English, 3 pages). This report is included as Appendix 2.
- A Documentation Package including all analytical data and control quality of the Athlete Biological Passport ID BPY2524M36, dated on 23.05.2013 (English, 68 pages). Due to space limitations we have not included this document. It is available at request.
- An expertise of Dr. Locatelli (6 pages). This report is included as Appendix 3.
- An expertise by Dr. Douwe de Boer (19 pages). This report is included as Appendix 4.
- UCI Competition Schedule BPY2524M36, included as Appendix 5.

ACCOUNTS OF THE FACTS

The Union Cycliste Internationale (UCI) and the Cycling Anti-Doping Foundation (CADF) have included the Athlete Biological Passport as a tool for the identification, prevention and prosecution of illegal practices, doping in cycling. In June 2012, the CADF were informed that Mr Roman Kreuziger Biological Passport was identified with several abnormal features (Appendix 2). Following standard procedures, CADF informed the rider and offered him the opportunity to provide an explanation. Then a series of reports were submitted to CADF with the intention of explaining the "alterations" found in his Biological Passport (Appendix 3 and Appendix 4). However, CADF experts, in their report dated on may-2014, discarded Mr Kreuziger allegations and concluded that the only plausible explanation in light of the documentation available was the use of blood manipulating practices (Appendix 1). In July 2014, we reviewed all the documentation described above.

Recently, WADA launched the Athlete Biological Passport (ABP) (Sottas et al, 2011; Sottas et al, 2010; WADA, 2013). In brief, this is a longitudinal system that records hematological values of blood tests performed to athletes of several disciplines. This system is supported in the ADAMS (Anti-Doping Administration and Management System) platform. This platform provides a number of reports by means of a monitoring software called ABP. Specific reports are usually attached as documentation packages when an athlete is suspected of using prohibited techniques. From an operational point-of-view, the procedure is based on the extraction of blood samples of an individual and his/ her results are stored in a database (ADAMS platform). Then, the ABP software evaluates cumulative information of the athlete and, based on an adaptative, Bayesian like- modelling, computes an individual range of reference values for hemoglobin concentration, percentage of reticulocytes and other parameters. If the software detects alterations in the longitudinal sequence of the data, a suspicion is generated. This procedure is described in more detail in the next section. Anyway, many international and national associations comprising athletes from different fields have adopted this approach of persecution, prevention and identification of doping.

In the "Athlete Biological Passport - Operating Guidelines and Compilation of Required Elements" (v 4.0) (WADA, 2013), monitoring procedures and evaluation of anti-doping policies established by WADA are described (WADA, 2013). These procedures are based on creating an adaptive model from the initial hematological values of an athlete, which are entered into the ADAMS. Then, the ABP software, using these data, will predict expected range of future hematological values for this athlete, assuming a normal physiological condition and a few mathematical requirements. In addition, as more hematological data of an individual are introduced into the system, more reliable predictions the ABP software will make because new data will adapt prections to real values: an “adaptative model”.

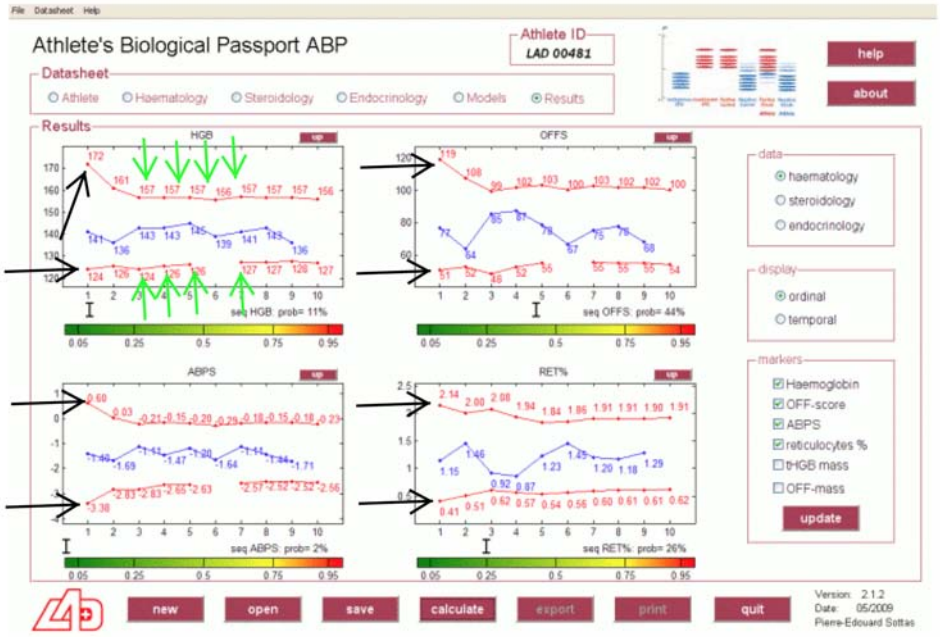

Figure 1. Screenshot from Laboratorie Suisse d´Analyse du Dopage website showing the longitudinal profile of hemoglobin, reticulocytes, OFF-score and ABPS for an athlete tested 9 times

(http://www.doping.chuv.ch/en/lad_home/lad-prestations-laboratoire/lad-prestations-laboratoire-passeport/lad-prestations-laboratoire-passeport-passeport-hemato.html)

The Athlete Biological Passport includes a few modules that explore different haematological, steroidal and endocrinological variables. Endocrinological module is currently in development and steroid module has begun to implement on January 1st, 2014. Figure 1 illustrates how the true values of the athlete (in blue) and margins (in red) are longitudinally plotted using the ABP software. In fact, expected margins are wider (black arrows) when a few samples are computed and get tighter (green arrows) as more samples are included. That is, information is enriched over time as the system learns athlete´s "limits of his/her biological behaviour" by monitoring hemoglobin concentration and many others hematological parameters.

Specifically, hematological module of ABP includes hemoglobin concentration, measured in g/L, reticulocyte percentage, calculated by dividing the absolute number of reticulocytes (measured in number per microliter) between the absolute number of red blood cells per microliter of blood and, finally, the OFF-hr score (Gore et al, 2003.)

The colour bar under every graph represents at which percentile the longitudinal sequence of hemoglobin, OFF-hr score or % of reticulocytes values from an individual athlete is in the probability distribution of sequences expected from a sample of controlled, clean athletes. Swiss Laboratory for Anti-Doping Analysis states, "a high percentile is suspicious of an abnormality and will deserve closer scrutiny". They consider that "a high value can be reached even in the conditions when no individual value has broken a single limit, typically

when the athlete is monitoring his blood profile via low doses of rEPO and/or IGF-1 and hemodilution" (www.doping.chuv.ch). For example, if we observe colour bars at figure 1, probabilities range from 11 to 44% based on the parameter we analyze. At figure 2, probabilities reach 99.99% and means that this athlete has a 99,99% of probability of harbouring an abnormal profile, when compared with a clean athlete population.

Once the data are included in the platform and computed, the ABP software provides a report (Figure 1) with a series of measurements obtained over time as well as a list of analytical determinations of the whole series of samples of the athlete. In cases of atypical variations, ie the values of the athlete represented in blue cross the margin values shown in red (see Figure 2), information obtained from the ABP software is refered to an expert.

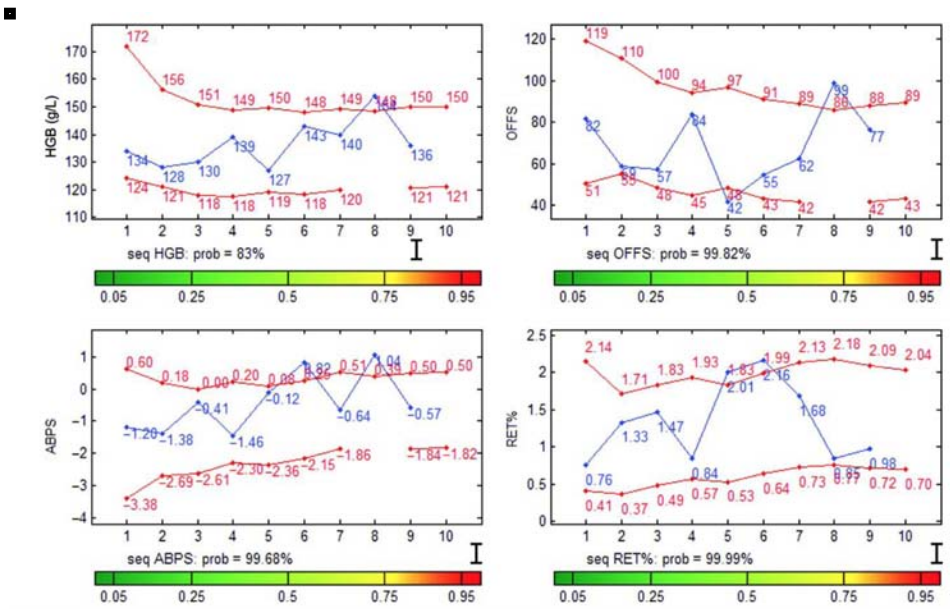

Figure 2. An example provided by LADS suggesting an abnormal ABP.

If the expert considers highly likely that the athlete has used illegal methods, the report is usually forwarded to two experts from a panel of experts. Finally if the group of three experts unanimously agree that the software ABP profile obtained and included in the reports, has a high probability of being linked to prohibited methods, the institution in charge for the anti-doping programme (CADF in this particular case) will be provided with a complete dossier (called ABP Documentation Package) which includes, besides the data processed by the software, the original analytical data. Upon reaching this point, the athlete is informed and receives the opportunity to explain the anomalies found in the data collected. Allegations provided by the athlete are, in turn, answered by the initial three experts. Then, they must reach a unanimous opinion on the possibility that the athlete has used illegal methods. Obviously, every single Federation follows specific procedures to handle with the ABP but the spirit described above is quite similar: ABP profile reviewed by three experts, information to the athlete who can provide additional explanations and a final, agreed by three experts, report.

In this particular case, we will refer only to the hematological module of Mr. Roman Kreuziger because it is the basis for CADF to consider Mr. Kreuziger “biological behaviour” as suspicious.

THE HEMATOLOGICAL MODULE OF MR. ROMAN KREUZIGER

The hematological module of Mr. Roman Kreuziger included 52 blood samples that met collection and analytical guidelines following WADA ABP guidelines (WADA 4.0, reference). Although there are additional data included at page 2 of the ABP documentation package, we agree with medical experts consulted by CADF to discuss about these 52 samples. In fact, ABP graphs do not include samples collected without observing WADA rules. We will discuss about valid samples. We will use numerical order of valid samples (represented by "Graph Number" at page 2 of ABP Documentation Package ID BPY2524M36).

First sample was collected in april 30th, 2008 and the last sample included was collected in april 9th, 2013.

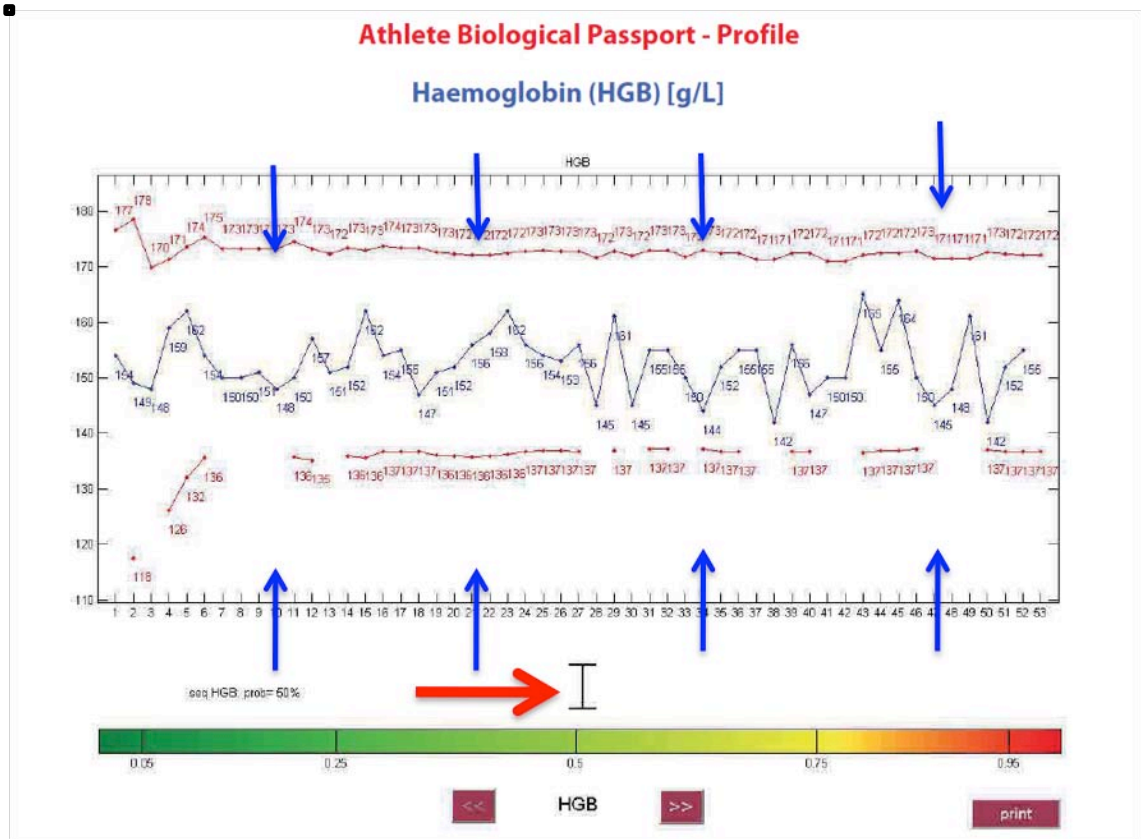

Figure 3. Hemoglobin concentration profile. ID BPY2524M36.

Hemoglobin concentration (Hb), plotted in figure 3, is allways within the red limits predicted by ABP software (blue arrows). Colour bars showed that Mr. Kreuziger hemoglobin concentration profile is within percentil 50 (red arrow) when compared with a clean athlete population that therorically matches his physical, training and demographic data.

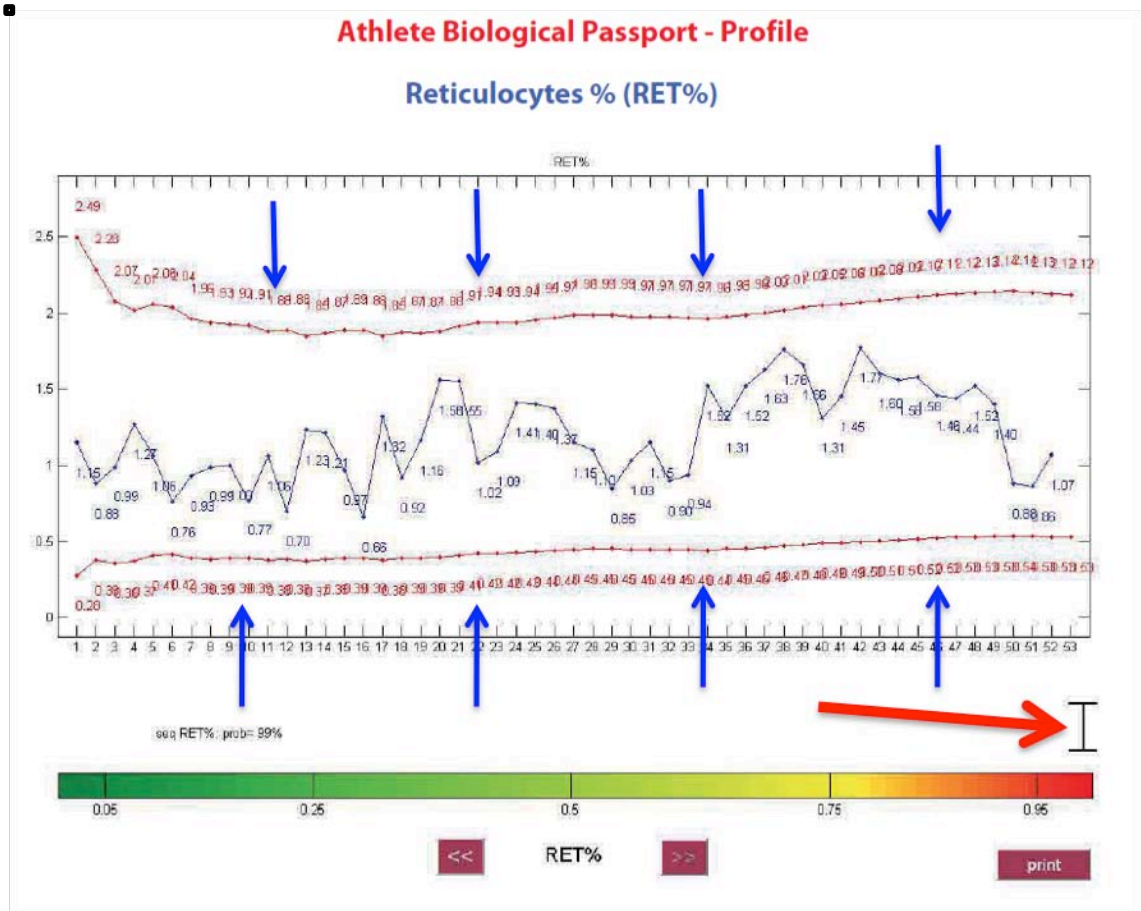

Figure 4. Percentage of reticulocytes profile. ID BPY2524M36.

Percentage of reticulocyte (%Ret) concentration, showed in figure 4, is allways within the red limits predicted by ABP software (blue arrows). However, colour bars showed that Mr. Kreuziger %Ret profile is within percentil 99 (red arrow) when compared with a clean athlete population that therorically matches his physical, training and demographic data.

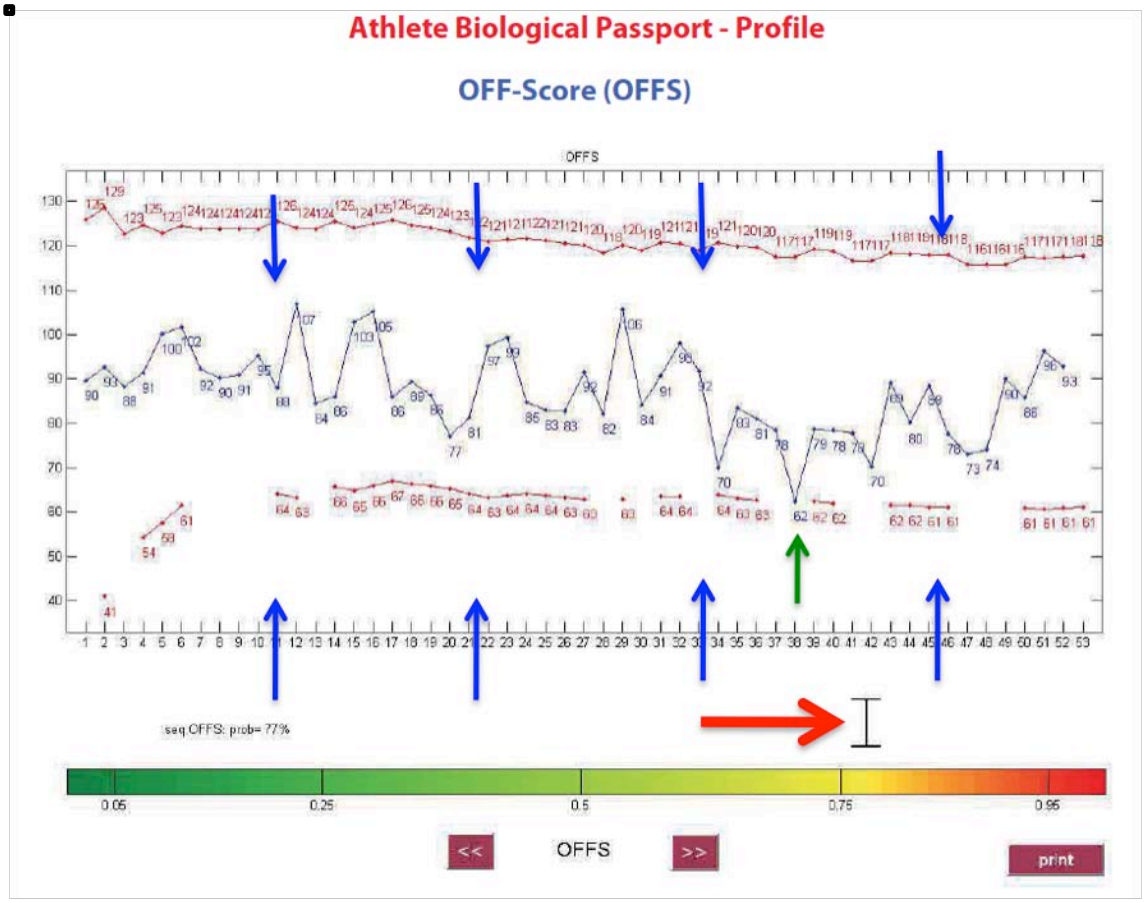

Figure 5. OFF- hr Score profile. ID BPY2524M36.

OFF-hr score profile, showed in figure 5, is mostly within the red limits predicted by ABP software (blue arrows) but there is a value of 62 points (sample 38, green arrow), overlapping the lower limit. Unfortunately, at that point, ABP software did not predict any lower limit. However, colour bars showed that Mr. Kreuziger OFF-hr Score profile is within percentil 77 (red arrow) when compared with a clean athlete population that therorically matches his physical, training and demographic data.

Regarding values included at page 2 of the ABP documentation package, medical experts consulted by CADF, suggest a few abnormalities listed below:

- An abnormal in-competition haemoglobin pattern in the 2012 Giro d´Italia.
- An abnormal Reticulocyte pattern from March 2011 onwards.

There are no differences between arguments and conclusions from CADF experts when we compare initial and final reports. However, we will analyze arguments and conclusions from both reports.

By one side, they observed an abnormal hemoglobin concentration pattern during the "Giro" 2012. This abnormal pattern is based on the asumption that exists a basal sample, namely sample 46, collected in May 3rd, 2012 and a final sample, namely 49, collected in may 24th, 2012. They stated that there is not a physiological pattern between sample 46 and sample 49 (see Box 1).

1- Giro d´Italia 2012

During the Giro 2012, the athlete displays the following blood values:

3/5/12	Hb 150g/l, Reticulocyte% 1.46
14/5/12	Hb 145g/l, Reticulocyte% 1.44
20/5/12	Hb 148g/l, Reticulocyte% 1.52
24/5/12	Hb 161g/l, Reticulocyte% 1.40

It is clearly visible that the Haemoglobin concentration in this athlete does not follow the physiological pattern expected after such arduous endurance exercise, namely a significant decrease in Haemoglobin

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concentration (1–7). In the present rider, the Haemoglobin concentration remains unchanged after 15 days of racing and is even higher than at the start on day 20.
The pattern with a higher Haemoglobin concentration at the end of the stage race than at the start in the Giro 2012 is in clear contrast to other stage races of the rider, where he showed a physiological decrease (see for example Tour de France or Vuelta 2010).

Box 1. Arguments regarding hemoglobin concentration included at the initial expert report from CADF medical experts (p1-2)

By other side, CADF experts considered that %Ret increases from March 2011 onwards. Indeed, they plotted %ret values per year showing an increase since 2010 (Box 2).

2- Abnormal Reticulocyte pattern from March 2011 onwards

The second notable observation made on review of the samples is related to Reticulocyte% levels. There appears to be a significant trend upwards in these values from March 2011 and then from April 2012 onwards, showing a time correspondence with important stage races. The average level from this point onwards is above 1.5% which is far beyond the athletes previous levels.

The gradual increase in Reticulocyte% for all samples is outlined in the figure below

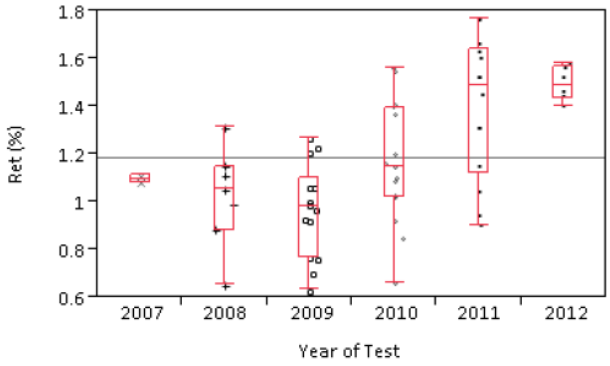

Figure 1: Distribution of Reticulocyte% in profile BPY2524M36 per year.
The red boxes represent the 25th and 75th percentile, the horizontal line represents the median, the whiskers illustrate the outliers)

Box 2. Arguments regarding %Ret profile from March 2011 onwards.

Finally, they concluded in their initial report (Box 3) and, after evaluating and arguing against expertises of Dr. Locatelli and Dr. de Boer, confirmed in their final report (Box 4) that it was highly likely that Mr. Kreuziger used a prohibited method or an illegal substance.

Summary

In our unanimous opinion, the likelihood of the described abnormalities being due to blood doping, such as the use of blood transfusions, is very high. In contrast, the likelihood of such deviations being caused by altitude exposure, a medical condition or any other cause is low and we therefore recommend requesting the rider's explanation for his blood values, specifically regarding the patterns observed during the Giro d´Italia 2012 (samples 56-59) and the abnormality of his Reticulocyte pattern from 2011 onwards.

We conclude that considering the available information contained within the passport at this stage and absent a satisfactory explanation from the athlete, it is highly likely that a prohibited substance or a prohibited method has been used and unlikely that the profile is the result of any other cause.

Box 3. Conclusions extracted from initial CADF expert report, dated on 14.6.2012

Conclusion

Based on the facts outlined above, we conclude that the justifications provided by the athlete in the expertise of Drs. De Boer and Locatelli do not explain the variations observed in his profile.

Based on the information in the Passport, it is therefore the unanimous opinion of the panel that it is highly likely that the Athlete used a prohibited substance and/or a prohibited method, likely involving blood transfusion and erythropoiesis stimulating agents, and that it is unlikely to find the passport abnormal assuming any other cause.

Box 4. Conclusions extracted from CADF final expert report, dated on 12.5.2014

Bases to discard, Dr. de Boer and Dr. Locatelli arguments, are highlighted in Box 5 and 6.

Summary Expertise Dr. de Boer

In his explanations, Dr. de Boer tried to explain the abnormalities highlighted in our previous reports by subclinical hypothyroidism and its treatment with increasing doses of L-thyroxin (50µg -75µg -100µg). Based on the available scientific literature, his arguments must be dismissed, as there is no evidence

that in non-anaemic, iron-replete subjects, the application of thyroid hormone would have any clinically relevant effect on the blood picture.
It is of note that the explanations of Dr de Boer do not provide any distinct clarification of the abnormal in-competition Haemoglobin pattern during the Giro d´Italia 2012, which is one of the key abnormalities of the profile.

Box 5. CADF expert arguments to discard Dr. de Boer explanations of ABP profile, dated on 12.5.2014.

Summary Expertise Dr. Locatelli

In his explanations, Dr. Locatelli tries to highlight analytical shortcomings in Reticulocyte analysis and normal physiological variation in Hb level during stage racing in line with the cited literature as the reasons to explain the two main abnormalities observed in the profile.
From our analysis, it becomes clear that the Ret analysis of the samples were correct and the regulations were misinterpreted by Dr Locatelli. Upon further scrutiny, the literature cited by Dr Locatelli to defend the abnormal Hb levels of the athlete during the 2012 Giro d´Italia in fact confirms the striking abnormality of the pattern observed towards the end of this race.
Therefore, both explanations can comfortably be dismissed, as they are not supported by the available scientific data.

Box 6. CADF expert arguments to discard Dr. Locatelli explanations of ABP profile, dated on 12.5.2014.

In brief, CADF experts discarded Mr Kreuziger allegations because they consider that hypothyrodism therapy does not modify any hematological blood value in absence of overt iron- deficiency and because the only explanation for the abnormal hemoglobin profile during the 2012 Giro are blood manipulation techniques. So, data that concur to suggest blood manipulation are:

1. Colour bars at %Ret graph, suggesting Mr. Kreuziger %Ret profile is at the 99% percentile of being different when compared with a sample of similar athletes (Figure 2).
2. OFF-hr score from sample 38 that overlaps a theoretical lower limit, although not plotted by the ABP software (Figure 3).
3. Abnormal pattern of hemoglobin concentration during Giro 2012. CADF medical experts consider that there is no scientific support to increase hemoglobin concentration under physiological conditions, at the end of a 3-weeks race when compared with basal values. They considered a basal value dated on May 3rd, 2012 and a final value on May 24th, 2012 (Box 1).
4. Increasing levels of %Ret since 2011 but also evidenced since 2010 (Box 2).
5. Hypothyroidism and its therapy do not modifiy any hematological blood parameter in non-anaemic patients.

To address all these issues, our report is arranged in two parts. Firstly, we will analyze hypothyroidism, differences between subclinical and overt hypothyroidism based on doses of sustitutive

therapy that Mr Kreuziger is receiving, and its relationship with hemathological and plasma volume values, mainly with reticulocytes in non-anaemic patients, under several conditions of exercise, temperature and, as well, time-course of thyroid activity requirements under these above mentioned conditions. Secondly, we will analyze scientific data regarding hemoglobin concentration and its variation within short period of time. Finally, our conclusions, although based on previous data, will discuss CADF arguments point-by-point.

HYPOTHYROIDISM AND MR. ROMAN KREUZIGER MEDICAL HISTORY.

Hypothyroidism is an endocrine disorder in which the thyroid gland does not make enough thyroid hormone. The thyroid gland is an important endocrine organ located at the front of the neck. This organ makes thyroid hormones that allow cells to modulate, to tunning their own metabolism. Most frequent cause of hypothyroidism is thyroiditis. Among several causes of thyroiditis, auto- immune thyroiditis is the most common etiology. Auto- immune thyroiditis is a disease in which the immune system attacks its own thyroid gland. Additional information for non- medical readers is published elsewhere (http://www.nlm.nih.gov/medlineplus/ency/article/000353.html).

There is a broad clinical spectrum of hypothyroidism although physicians frequently encounter patients with very mild thyroid dysfunction, combining increased levels of TSH and normal levels of FT4 and FT3. Such patients are often identified through routine screening. In fact, Mr Roman Kreuziger was diagnosed with subclinical hypothyroidism (TSH elevated and T4/T3 normal; green arrow in Figure 6) by chance in 2004 because his family has a variety of inherited hypothyroidism. The potential benefits and risks of therapy for subclinical hypothyroidism have been debated for decades. However, clinical guidelines published in New England Journal of Medicine in 2001 (the most relevant medical journal worldwide),

recommended sustitutive therapy for those patients with increased TSH levels and a positive test for antibodies against thyroperoxidase (Cooper, 2001). In this regard, Mr. Kreuziger showed a positive test against thyroperoxidase (TPO). Additionally, many authors recommend sustitutive therapy for those patients with high levels of physical activity. Finally, it is widely know that there is a increased risk of progression to overt hypothyroidism for patients who had been diagnosed with subclinical hypothyroidism and have elevated serum levels of antithyroid antibodies (Tunbridge et al 1977; Vanderpump et al 1995). It was stablished in 80s that sustitutive therapy with an initial dose of L-Thyroxine of 50 or 75 mcg per day is enough to normalize TSH serum level (Cooper et al 1984; Nystrom et al 1988).

In this regard, Mr. Kreuziger met all criteria to start a sustitutive therapy with low doses of L-Thyroxine. Therefore he started to take 50 ug of L-Thyroxine per day since 2004. However, in 2011, a blood test showed an increased TSH serum level in spite of sustitutive therapy. His endocrinologist increased L-Thyroxine dose to 75 mcg per day. That therapeutic intervention allowed TSH to normalize. Unfortunately, in 2012, another blood test evidenced a newly increased serum level of TSH. Similarly, a dose of 100 mcg of L- Thyroxine per day was initiated.

At that point, we would like to recall that sustitutive therapy for patients diagnosed with subclinical hypothyroidism is about 0.6- 0.7 mcg per kg of mass body per day (50 mcg per day assuming a man who weighs about 70 kg). In this regard, Mr. Kreuziger weigh ranged

67-70 kg during 2011-2012 and between 66 and 69 kg since 2013. That means that Mr Kreuziger needs doses ranging from 1.42 to 1.5 mcg per day per kg of body mass to keep his TSH serum levels within limits of normality. In fact, Mr Kreuizger needs the same replacement dose of L-Thyroxine as patients diagnosed with overt hypothyroidism after a complete resection of their thyroid gland. So, Mr Kreuziger is diagnosed with an overt hypothyroidism that has progressed from a subclinical hypothyroidism, previously diagnosed in 2004. There is no subclinical hypothyroidism treated with 100 mcg of L-Thyroxine.

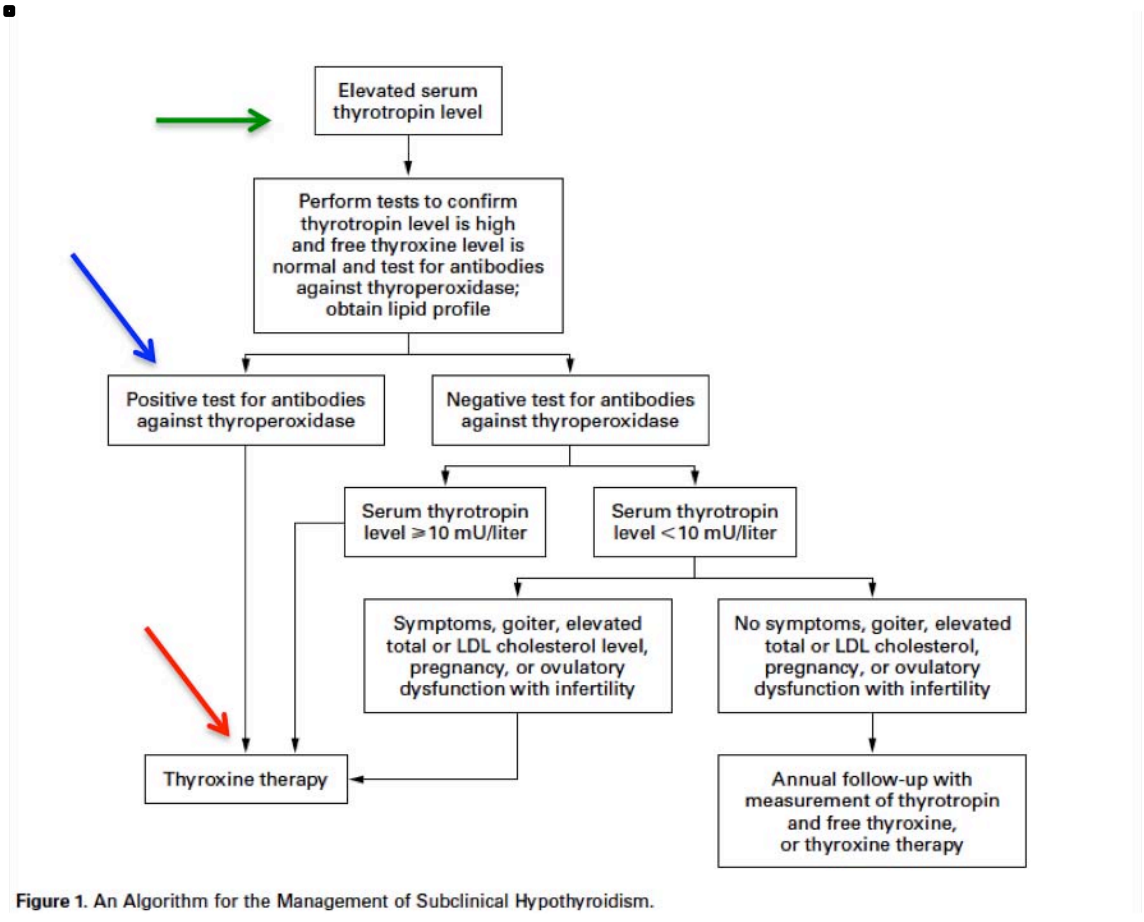

Figure 1. An Algorithm for the Management of Subclinical Hypothyroidism.

Figure 6. From Cooper DS. NEJM 345 (4): 260- 265; 2001

General assessment of the provided Thyroid data

In the table below, the laboratory data of the thyroid hormone tests provided by the athlete is summarised:

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Date	TSH	FT3	FT4	Hb (g/dl)*	Laboratory
2.12.2010	**5.19**				A
16.06.2011	2.55	4.1	11.6		D
2.11.2011	4.29	4.9	17.3		C
2.12.2011	3.69				B
*19.01.2012	***5.78**			*15.2	A
7.03.2012	3.18	4.0	13.3		A
17.04.2012	2.81	3.8	13.0		A
11.06.2012	2.86	4.8	13.9		A

Table 1: Thyroid hormone levels reported by the athlete. Data outside the reported reference range indicated by the analyzing laboratories (A-D) are printed in bold. No comparison between laboratories was performed as the measurement units (not reported here) differ.

*In appendix 6C the TSH value is noted as 5.08 however in the actual laboratory report it is noted as 5.78, most likely a transcription error. * Hb levels only reported if analysed simultaneously by the same laboratory.
It is presumed that all FT4 levels have been re-calculated from the actual laboratory reports in which units are ng/dL however in Appendix 6C they would appear to be assigned pmol/L

It can be noted that the FT4 levels were always within the normal range of the reporting laboratory. Thus, the subclinical hypothyroidism of the athlete was probably of minor severity.

Box 7. From Final Report of CADF medical experts, dated on 12.5.2014

Comments from CADF Medical Experts regarding severity of Mr. Kreuizger thyroid disease are included in Box 7 and highlighted in yellow. In brief, they suggested that Mr Kreuziger subclinical hypothyroidism was of minor severity because of normal levels of free L-Thyroxine (FT4). In this regard, when serum levels of L-thyroxine (total or free: T4 or FT4) are evaluated, physicians allways keep in mind that a hypothyroid patient usually receives L-thyroxine as a sustitutive therapy. This means that patients take a drug exactly identical to the hormone produced naturally by the thyroid gland. As Mr. Kreuziger was taking L-Thyroxine since 2004, all L-thyroxine levels showed in Box 7 represent a pool of endogenous plus exogenous L-Thyroxine serum levels of Mr. Kreuziger. Really, they are indistinguishable. Finally, almost all patients receiving 100 mcg per

day of L-Thyroxine showing normal TSH levels, probably, do not show signs of any endogenous thyroid activity.

Taking that into account, the keys to interpret thyroid activity cannot rest on the levels of L-Thyroxine that Mr Kreuziger showed in his blood test. Furthermore, thyroid disease of Mr. Kreuziger should be analyzed in an evolutionary way because it is a disease that progresses over time: although Mr. Kreuziger was diagnosed with a subclinical hypothyroidism, this disease evolved to an overt hypothyroidism. A surrogate marker of this evolution is the L-thyroxine dose required to keep TSH levels of Mr Kreuziger within limits of normality. In this regard, 50 mcg of L-Thyroxine were enough since 2004 to 2010. Probably, his thyroid activity was within the concept of subclinical hypothyroidism at that time. However, his thyroid was running out when he needed two sucessive increases of L-Thyroxine dose (2010: 50 mcg; 2011: 75 mcg; 2012: 100 mcg). In fact, first change made in 2011 was not enough to control TSH levels in 2011 winter as showed in Box 7. As thyroid activity requirements in winter (or in a cold enviroment) are higher than in summer and decissions were made under TSH levels taken in winter, a question arises as to whether Mr Kreuziger was, and is, beeing overtreated. As TSH levels are very sensitive to the feed back loop orchestreated by T4 and T3 hormones, any L- thyroxine overdose would associate a TSH supression. At this point, any interested reader will arrive to the conclusion that TSH levels play a central role as a criterion of hypothroidism compensation. So, T4 blood levels by themselves do

not define severity of any hypothyroid patient when a patient is receiving T4 and there are additional criteria to modify sustitutive therapy, ie the quality of compensation of replacement therapy, described elsewhere (Cooper et al, 2001).

In summary, Mr Kreuziger is diagnosed with an overt hypothyroidism requiring a sustitutive dose of 100 mcg per day (1.5 mcg per kg of body mass per day). In 2004 he was diagnosed with a subclinical hypothyroidism that has progressed to an overt hypothyroidism, as predicted by familial history, age at diagnostic and antithyroid antibodies evidenced in his blood test.

HYPOTHYROIDISM AND EXERCISE.

Thyroid activity has a miriad of effects on several metabolic processes, in carbohydrate, fat and protein metabolism with the aim of optimizing the possibilities of energy supply and energy exploitation. Similarly, physical exercise is a complex stress situation, which requires numerous metabolic and hormonal adaptations, including thyroid activity. Physical exercise is known to affect thyroid hormones. This role is driven by the impact of these hormones on the degree of metabolic acceleration of body substrate oxidation and muscle function (McMurray et al 2000). Moreover, muscle conditioning influences thyroid metabolism. In fact, modulatory effects of exercise on thyroid hormone levels depend on individual

physiological requirements to react to a heterogeneous growing metabolic demand. In fact, thyroid hormones are involved in the regulation of VO2 and muscle function (Koistinen et al 1996). Molecular mechanisms by which this regulation takes place have been dissected in animal models. These hormones bind to response elements at regulatory areas of genes that codify for alpha actin, beta myosin heavy chain and sarcoplasmic reticulum ATPase CA21 (Edwards et al 1994; Hartong et al 1994; Muscat et al 1994). This causes a direct relationship between thyroid activity and shortening velocity of skeletal muscle fibers (Caiozzo et al 1993). Further information on the transcriptional effect that different thyroid hormones induce in muscle metabolism associated genes, is available elsewhere.

As we pointed out above, thyroid activity is associated with the regulation of VO2 kinetics, mainly at the slow component. Underlying mechanism is based on the recruitment of motor units made of low efficiency type IIb fibers once they have exceeded the lactate threshold (Coyle et al 1992). These data were replicated in well-trained cyclists showing that T3 serum levels were negatively correlated (Figure 7)with neuromuscular fatigue (Lucia et al 2001).

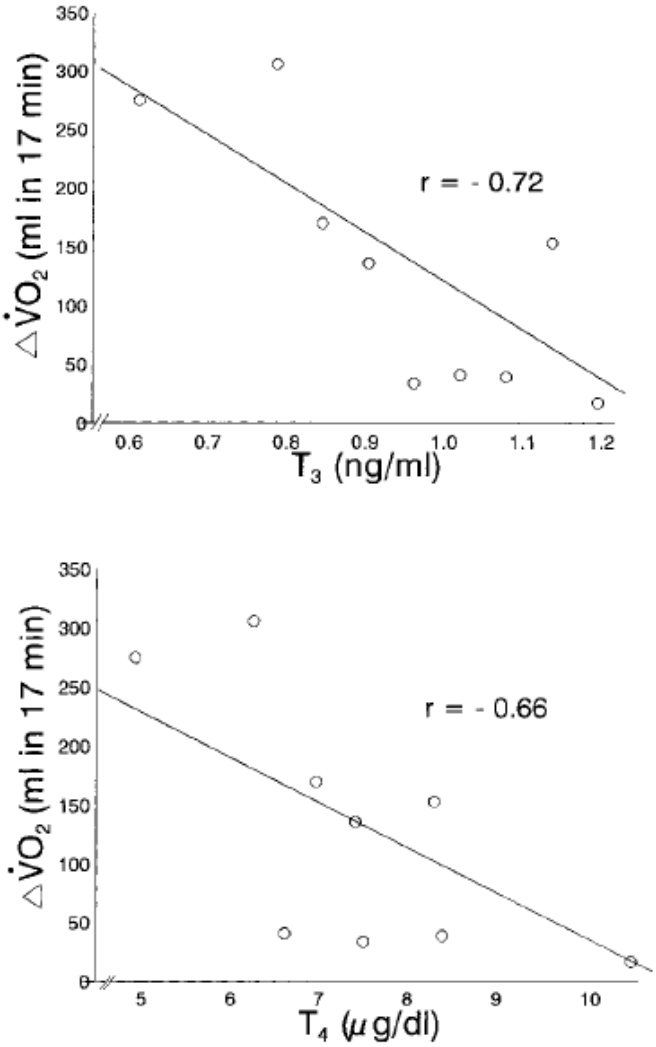


Fig. 1. Correlation ($p<0.05$) between $\Delta\dot{V}O_2$ and thyroid hormones.

Figure 7. From Lucia et al 2001.

Finally, it is known for years that thyroid hormones play biological roles on both the central and peripheral nervous system (Gambke et al 1983; Yuan et al 2005). In the peripheral nervous system, hormonal activity modulates motor neuron responses evidenced by electromyography as well (Khedr et al 2000).

In conclusion, scientific data published over 50 years, evidenced that thyroid activity is inversely associated with the onset of muscle fatigue by both direct mechanisms on muscle cells and neuro-humoral mechanisms exerted on motor neurons.

THYROID ACTIVITY VARIATION DURING EXERCISE

Studies in euthyroid individuals can give us valuable information on physiological requirements of thyroid hormones under different athletic disciplines and diseases (Rone et al 1992). For example, it has been proved that regular exercise positively affects the mechanisms of action associated with the physiologic deterioration and transition from subclinical thyroid disease (Hackney et al, 2012). In fact, many patients diagnosed with subclinical hypothyroidism are treated with mild exercise to delay progression to overt hypothyroidism. However, prolonged-intense endurance exercise (e.g., >25 km competitive runs) or low-moderate intensity activity for extended periods (e.g., days of military field operations) causes a transient non-pathological hypothyroidism (i.e., a deficiency of thyroid hormone) lasting for 24 hours up to 72 hours (Moore et al 2005). In fact, high-intensity interval exercise results in a transient hypothyroidal state during the recovery period evidenced by reduced levels of FT3 at 12 hours into recovery (red arrow at Figure 8) (Hackney et al. 2012).

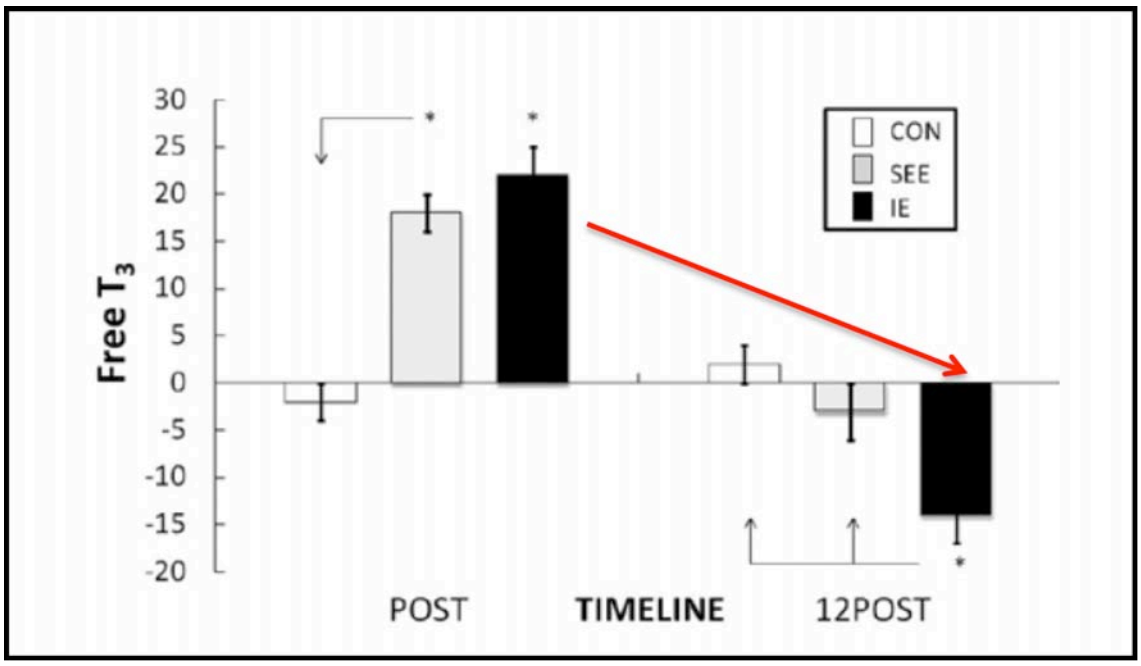


Figure 8. Free triiodothyronine (fT3) relative change (%) responses to high-intensity interval exercise (IE), steady-state endurance exercise (SEE) and a rest control session (CON) in highly trained males (n=15). Values are means ± SE. The * denotes significant ($p<0.05$) changes for respective session mean values from the mean value denoted by the arrow within a specific measurement time (POST or 12 POST). Hackney et al 2012; Hormones 11(1):54- 60).

Anyway, differences in the magnitude of responses between published studies are most likely due to the differences in the duration of the exercise conducted and the overall greater energy demand that increased T4 levels for at least 8 hours after exercise (Hackney 2011; Hackney et al 1994). Their results are better explained if we realize that increases in T4 concentrations were identified following aerobic-anaerobic exercise but T3 increased only after aerobic exercise. Additionaly, Refsum and Strømme showed in 1979 that TSH remained increased within the 4 days following extreme endurance exercise (70 km cross-country ski race). Caralis et al., in 1977, argued that during exercise, the rise in FT4 concentration becomes greater with increasing physical fitness. Further, Limanova et al. (Limanova 1983)

showed that thyroid hormones are also conditioned by adaptation phenomena and found greater pre-exercise levels of T3 and FT3 in athletes with respect to control subjects. These results are in general agreement with those described by Hesse et al in 1989 who reported higher T4 and TSH levels after exercise in faster endurance race runners than slower runners.

However, these modifications may vary according to the geographical conditions in which the exercise is performed. For example, if the exercise is performed in altitude, thyroid hormone requirements are different. Koistinen et al. observed that training at high altitude provoked a fall in total serum T3 while FT3 showed an increase. These authors found a fall in serum albumin parallel to the fall in T3 while T4 was altered to a lesser extent by the decrease in albumin (Koistinen et al 1996). In addition, if we combine altitude and cold weather, TSH, T3 and FT3 are described to increase (Hackney et al 1995). In this regard, variations of thyroid hormone profiles associated with tisular hypoxic insults are well documented for years (Humpeler et al 1980; Koistinen et al 1996; Sawhney et al 1991; Stock et al 1978).

Furthermore, physical exercise and prolonged, chronic exposure to extreme cold modulate subtle changes in thyroid hormones, which are also affected by season (Sawhney et al 1995). Additionaly, the thyroid axis of normal individuals can respond rapidly to acute cold exposure (O'Malley et al 1984). For example, acute exercise at different temperatures has been also associated with acute modifications of

plasma TSH and T4. A very interesting study, published in 1993 by Deligiannis et al, evaluated thyroid hormones on 15 elite male swimmers, immediately before and after 30 min swimming, at three water temperatures (namely 20, 26 and 32 °C). Their results showed an increase on T4 and TSH levels in lower temperatures and an opposite effect at 32 °C with no changes at 26°C. (Deligliannis et al 1993). That study evidenced the role of thyroid hormone in the acute physiological thermogenic response to cold.

THYROID ACTIVITY AND CYCLING

There are a few studies dealing with thyroid activity and cycling. Chicharo et al published one of the most important papers in 2001. They evaluated basal levels of thyroid hormones and TSH in 16 professional cyclists over a 3-week tour competition, the 1998 "Vuelta a España". They showed an increase in basal serum thyroid hormone levels (T4, FT4 and FT3) recorded at the end of an extreme endurance cycling competition of three weeks duration, with no change in TSH or T3 (see Figure 9). Intriguingly, a significant rise in serum concentrations were observed during the last week of competition, suggested to be related to the performance of the cyclist or may even be the result of accumulated physical stress. To minimize the effects of variation in plasma volume on the changes observed in thyroid hormone levels, hormone data were corrected using a method widely used to achieve this attempt (Dill et al 1974). In fact, previous

studies published controversial results due to lack of corrected volumes in individuals with dehydration from strenuous exercise (Terjung et al 1971).

Table 1. TSH and thyroid hormone levels (mean ± SD) during the 3-week competition

	t_0	t_1	t_2	t_3
TSH, $\mu IU \cdot ml^{-1}$	1.1 ± 0.6	1.5 ± 0.6	1.5 ± 0.7	1.4 ± 0.8
T_4, $\mu g \cdot dl^{-1}$	7.3 ± 1.1	6.5 ± 0.9	6.8 ± 1.5	8.3 ± 1.5[a, b]
FT_4, $pmol \cdot l^{-1}$	5.2 ± 1.5	15.3 ± 1.4	16.0 ± 1.6	17.5 ± 2.1[b]
T_3, $ng \cdot ml^{-1}$	1.3 ± 0.7	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.2
FT_3, $pmol \cdot l^{-1}$	5.2 ± 1.0	4.9 ± 0.6	5.0 ± 0.6	6.0 ± 0.9[b]

[a] $p < 0.05$, significant difference between values recorded at t_3 and t_0; [b] $p < 0.01$, significant difference between values recorded at t_3 and remaining times.

TSH = Thyrotropin; T_4 = thyroxine; FT_4 = free T_4; T_3 = 3,5,3′-triiodothyronine; FT_3 = free T_3; t_0 = pre-competition control; t_1 = end of first week; t_2 = end of second week; t_3 = end of third week.

Figure 9. From Chicharro et al 2001

Furthermore, a proper functioning of the hypothalamic-pituitary-thyroid axis, determines the magnitude of the slow component of VO2 kinetics in euthyroid cyclists (Lucia et al 2001). Similar results were published by O'Connell et al after 3.5 h of moderate exercise bicycle, suggesting an accute demand of thyroid activity (O'Connell et al 1979).

Finally, the effect of different intensity levels of acute aerobic exercise on any thyroid hormone concentration is evidenced at the anaerobic threshold (about 70% of maximum heart rate). At 90% of

maximum heart rate, ratio of T4 and FT4 continues to rise but T3 and FT3 begins to decrease (Ciloglu et al 2005). This means that in a non-linear exercise, thyroid response follows the specific metabolic requirement.

In summary, thyroid hormone profiles reported by many authors, for many years, dealing with different situations of altitude, hypoxia, exercise type, cold and combinations of all these factors, both acute and chronic, show that thyroid activity is tailored to muscle, non-linear requirements. In this sense, fine-tuning of thyroid activity is never linear and many authors have described acute modifications. Finally, in a 3- week tour competition, T4, FT4 and FT3 increased, mainly in the last week of competition suggesting that an adequate thyroid reserve is needed for long-term races.

PLASMA VOLUME AND HYPOTHYROIDISM

Early studies performed on hypothyroid rats evidenced an exaggerated natriuresis after saline infusion due to a decreased renal sodium reabsortive capacity, probably associated to a proximal and distal tubules impairment secondary to a disminished thyroid activity (Holmes et al 1970). Those data were confirmed and completed when Gillum et al evidenced that there was a modification in systemic and glomerular hemodynamics. It was remarkable that proximal tubular reabsorption fell maximally within 1 wk after thyroid impairment

(Gillum et al 1987). In addition, Parving et al published a seminal paper in 1979 dissecting pathophysiology of plasma volume disturbance before and after L-Thyroxine therapy (Parving et al 1979). Namely they find a low plasma volume (P less than 0.05), a reduced rate of albumin synthesis and catabolism (P less than 0.01), an increased transcapillary escape rate of albumin (P less than 0.01), a remarkable increase in the extravascular mass of albumin (1500 micronmol; P less than 0.01) and a longer mean transit time through the extravascular spaces, when those parameters were compared with other causes of edema. A few years before, Wheatley and Edwards showed an increassed capillary permeability to proteins, retention of salt and water that were reduced with thyroxine treatment (Wheatley et al 1983). Their data were confirmed by others (Villabona et al 1999).

Obviously, there are only a few studies exploring acute effects of T4 withdrawal because they are unfeasible in humans. So we need to review studies that recruit patients that had undergone thyroidectomy for thyroid cancer that accutely stopped T4 therapy before to an early follow-up iodine gammagraphy. A very illustrative paper was published by Brown et al. They recruited this subtype of patients (commonly attended in many cancer centers worldwide) that had stopped thyroid sustitutive therapy for 2-3 weeks (Brown et al 1989). They evidenced an acute, significant increase of noradrenaline levels and a decrease in blood pressure. These findings were quite different from those identified in patients diagnosed with chronic hypothyroidism. So, a

lesson that many learned when that study was published was that acute suppression of sustitutive therapy in a previously treated hypothyroid patient associates metabolic effects different to those evidenced in chronic, never-treated hypothyroid patients. Finally, there are many trials exploring acute effects of thyroid hormone supplementation reflecting a decrease of noradrenaline, N-terminal pro-B Type natriuretic peptide and aldosterone as well an increase of stroke volume and other cardiac outcome parameters (Pingitore et al 2008). However, T4 supplementation for 7 months in patients diagnosed with overt hypothyroidism was able to normalize basal vasopressin and atrial natriuretic peptide release and vasopressin suppression but not atrial natriuretic peptide by an acute water load. This finding reflected that free water clearance might be impaired in patients with an adequate T4 therapy (Ota et al 1994).

In summary, there are many plasma volume modifications described in hypothyroid patients and acute suppression of L-thyroxine therapy may induce additional disturbances that affect plasma volume management.

ERYTHROPOIESIS AND L-THYROXINE THERAPY

Regulation of erythropoiesis is not fully known, but there are robust data evidencing a strong dependence on oxygen demand and on the availability of substrates, namely erythropoietin and iron, along with a

range of nutrients such as vitamin B12 and folic acid. Besides them, there are many endocrine factors, including thyroid hormones, which play an important physiological role in erythropoiesis. Unfortunately these mechanisms are only partially known. They can be direct, by the direct action on beta 2-adrenergic receptor of erythroid progenitor cells of bone marrow; or indirect, by their effect on both the production of erythropoietin and/or iron metabolism (Sullivan et al 1992). The role of the hypothalamus-pituitary-thyroid axis in the regulation of erythropoiesis has been studied in humans and has been confirmed in different animal models. In animal models, initial studies were published by (Fisher & Crook, 1962) on hypophysectomized rats that were supplemented with TSH or T3 to investigate their effect on erythropoiesis. These authors showed that T3 was the most potent hormone to stimulate erythropoiesis althoug a significant effect was also evidenced with TSH alone. Those studies, published in 1961, were critical to identify additional sources of erythropoietic promotion beyond both the renal production of erythropoietin: hypoxic events were able to stimulate a number of hormones to be released, including thyroid hormones. All these actors, when coordinated, stimulate erythropoiesis (Fisher & Birdwell, 1961).

As expected, studies that block the activity of thyroid hormones show the opposite effect and decrease erythropoietic activity as demonstrated by studies in mice deficient for thyroid hormone receptor alpha isoform (TRa KO). As TR alpha isoform is expressed exclusively in the erythroid compartment of the bone marrow, their

study is essential to understand the impact that thyroid hormones have on erythropoiesis. Thus, TRα KO mice harboured altered erythrocyte maturation with reduced hematocrit and an altered stress erythropoiesis response to hemolytic anemia, confirming the above-mentioned direct mechanism of thyroid hormones on red progenitor cells (Kendrick et al 2008). Authors explored erythroid efficiency indices and erythropoiesis in the presence of phenylhydrazine-induced hemolytic anemia. That was one of the most interesting findings as they revealed a deficient response to stress erythropoiesis on TRa KO animals (see Figure 10: reduced BFU-Es formation –red arrow- whereas total CFU-Es were increased – blue arrow- suggesting an inefficient transit through differentiation).

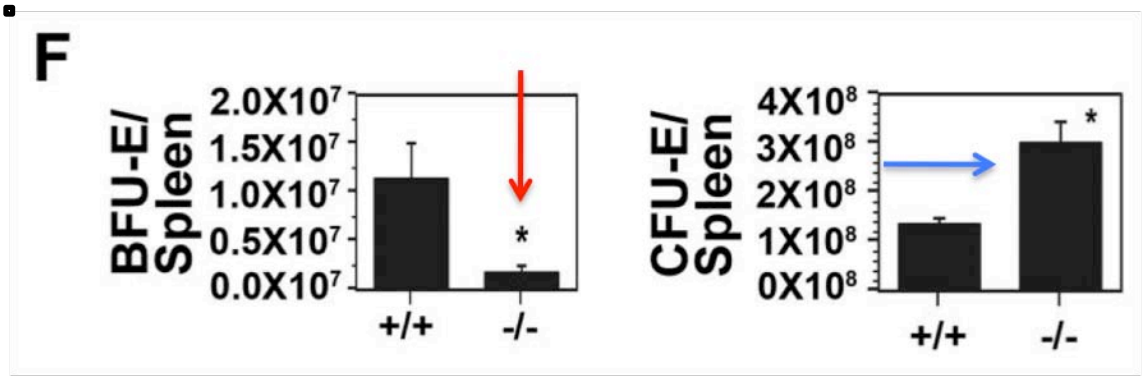


Figure 10. Figure 2F from Kendrick et al.

In addition, flow cytometric analyses, using a CD71 marker, revealed a significant accumulation of immature R1 compartment cells, combined with a marked reduction in more mature erythroblasts of the R3 compartment (Figure 11; red arrow). As described elsewhere, mature, CD71 positive erythroblasts are the final precursor of reticulocytes. So that, a marked reduction in R3 compartment will be

undoubtely associated with a marked reduction of circulating reticulocytes.

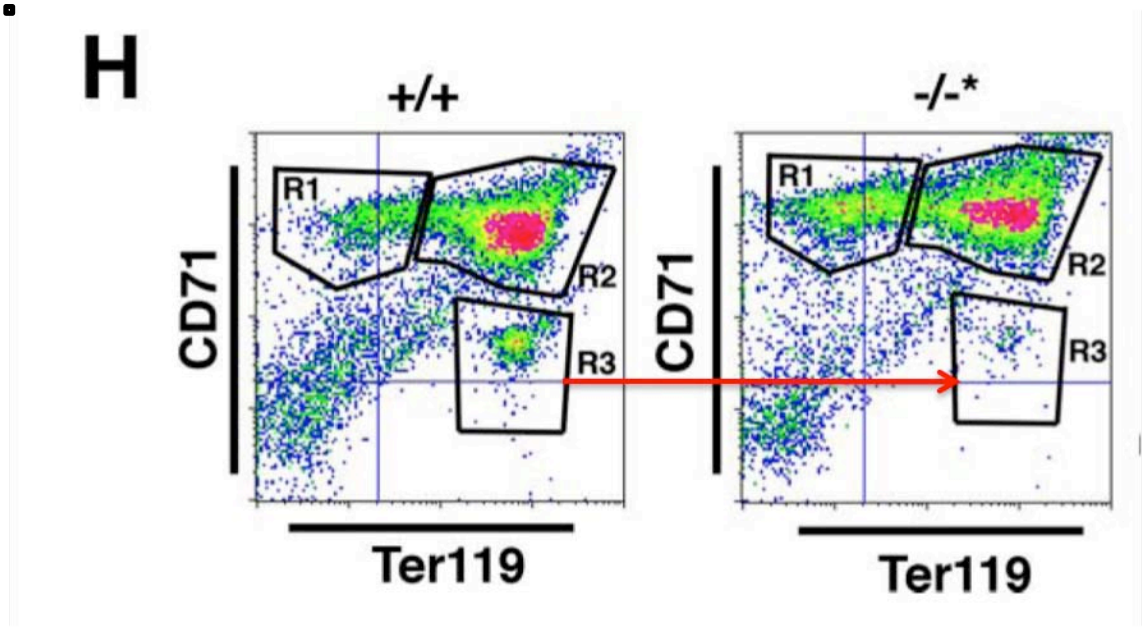


Figure 11. Figure 2H from Kendrick et al 2008.

As stated above, thyroid hormones stimulate erythropoiesis through indirect mechanisms. By one side, thyroid hormones can stimulate erythropoietin secretion both basal and in response to external stimuli. (Fein & Rivlin 1961; Christ-Crain et al 2003). By other side, thyroid hormones facilitate transport and utilization of iron (Fein & Rivlin 1961, Donati et al 1973. Cinemre et al 2009). Thus, relationship between thyroid hormones and iron is complex and two-way, so that thyroid hormones increase the erythrocyte absorption and incorporation of iron, while iron deficiency alters the secretion of thyroid hormones (Zimmermann & Kohrle, 2002). Moreover, when iron deficiency and hypothyroidism coexist, combined treatment with

thyroxine and oral iron is much more effective than oral iron alone to correct anemia (Cinemre et al 2009).

Additionally, effects on critical enzymes of reticulocytes have been proposed to depend on specific levels of testosterone or estrogen (Lawrence et al 1984). In fact, this stimulatory effect induced by intravenous infusions of T3 and T4 on bone marrow cellularity, was described in normal and nephrectomized rats as well as in groups of nephrectomized rats receiving rabbit antiserum against erythropoietin. So that, animal models evidenced in 1975 that thyroid hormones stimulate directly bone marrow erythropoiesis. This stimulation was noticeably apparent when high levels of free thyroid hormones were present in plasma (Malgor et al 1975). In this regard, we would like to highlight that no animal included in the control group of that study was anaemic. In fact, control rats showed an increase in total erythroid cells without significant differences using T4 or T3, probably due to sample size because a trend favouring T3 infussion was observed. Those results were concurrent with those published in 1967 exploring the role of thyrosine on the biosynthesis of hemoglobin in vitro (Krause et al 1967).

However, all these biological data do not deserve merit for anything if there were not a single paper exploring reticulocyte reponse after L-thyrosine therapy in non-anaemic, hypothyroid subjects. In this regard, Kim et al published in 2010 a very interesting paper devoted to explore levels of HbA1C before and after L- Thyroxine supplementation in non- diabetic patients that were also diagnosed

with overt hypothyroidism. They evidenced a significative increase of reticulocyte counts before and after L-Thyroxine therapy (see Figure 12). In addition, they showed a slight but significative decrease in the hemoglobin level after L-Thyroxine supplementation. None of those patients were anaemic (please, observe hemoglobin and ferritin levels included at table 1 of the referenced manuscript).

Table 1—*Laboratory characteristics of 30 nondiabetic patients with overt hypothyroidism before and after thyroid hormone replacement in study 2*

	Before thyroid hormone replacement	After thyroid hormone replacement	*P*
Age (years)	47.3 ± 12.3	—	
Sex (male/female)	7/23	—	
A1C (%)	5.57 ± 0.26	5.37 ± 0.32	<0.001
GA (%)	13.18 ± 1.35	12.52 ± 1.16	0.027
GA-to-A1C ratio	2.38 ± 0.29	2.35 ± 0.28	0.587
Glucose (mmol/l)	4.95 ± 0.41	5.24 ± 0.92	0.174
1,5-AG (μg/ml)	20.03 ± 6.92	21.42 ± 7.45	0.086
Hb (g/dl)	14.05 ± 1.35	13.64 ± 1.23	0.004
MCV (fL)	88.29 ± 4.73	90.78 ± 4.75	<0.001
MCH (pg)	29.48 ± 2.04	30.25 ± 1.95	<0.001
Reticulocyte (%)	0.64 ± 0.18	1.09 ± 0.34	<0.001
Ferritin (ng/ml)	74.34 ± 64.30	76.31 ± 57.58	0.689
EPO (mIU/ml)	12.93 ± 4.97	16.41 ± 6.44	0.022
Albumin (g/l)	43.5 ± 1.7	42.6 ± 1.6	0.009
Total cholesterol (mmol/l)	6.21 ± 1.23	4.37 ± 0.75	<0.001
Triglyceride (mmol/l)	1.57 ± 1.01	1.62 ± 1.15	0.802

Data are means ± SD. Hb, hemoglobin.

Figure 12. Table 1 from Kim et al 2010.

However, scientific evidence is also based on the reversibility of findings. This means that if a researcher showed a positive correlation in presence of a factor, in its absence, the resulting effect should be the opposite. So, if L-Thyroxine is able to stimulate erythropoiesis by a non- erythropoietin mediated mechanism, low levels of L-thyroxine must be associated with a lesser efficient

erythrocyte production. Indeed, the presence of anemia in many of hypothyroid patients is not evident from hemoglobin and hematocrit values due to concomitant reduction of plasma volume, reviewed above, and thus understimated. More relevant data come from erythrokinetics in hypothyroid patients that have provided evidence of significant decline of the erythropoietic activity of the bone marrow (Das et al 1975). Important, additional information on this topic emerges from the same papers included above. Nevertheless, there are many papers dealing with clinical management of patients unable to secrete renal erythropoietin, mostly diagnosed with both renal insuficiency and overt hypothyroidism. Conclusions from those manuscripts are quite similar: hypothyroidism therapy reduces requirement of EPO supplementation. So, erythropoietin and L-Thyroxine cooperates to modulate erythropoiesis and L-thyroxine effects on erythropoiesis are not related with any anaemic status because it is widely described a direct effect of this hormone on bone marrow precursors. In fact, it has been described and deposited in public transcriptomic databases that early erithroid progenitors, namely reticulocytes, express thyroid hormone receptors. Additionally, there are a few reports evidencing that thyroid hormones can modulate the synthesis of delta- globin in non- anaemic patients diagnosed with overt hypothyroidism as reported Kuhn et al in 1983.

So, with all the due respect, we must conclude that scientific evidence in vitro, in animal models and in human studies published in the last 40 years does support that L-thyroxine increases reticulocyte

levels in non- anaemic patients diagnosed with overt hypothyroidism. In fact, intimate, molecular mechanisms supporting biological plausibility of clinical, erythroid events associated with L-thyroxine effects on non- anaemic hypothyroid patients showed in clinical studies have been described as well (Zenke et al 1990).

SUMMARY

First, we have evidenced that hypothyroidism and its therapy modify hematological blood parameters in non- anaemic patients diagnosed with overt hypothyroidism. In fact, the biological plausibility for this effect is very sound as includes in vitro, animal models and human studies published and replicated since 1960 in very high- impact medical journals.

Second, we have shown that % Ret increases in patients diagnosed with overt hypothyroidism treated with L-Thyroxine.

Third, we have proved that Mr. Roman Kreuziger is diagnosed with an overt hypothyroidism that has progressed from a subclinical hypothyroidism diagnosed in 2004 in spite of increasing doses of L-Thyroxine. Now, he is taking 100 mcg of L- Thyroxine per day. This dose is the same he would receive if he had undergone a thyroid resection.

Fourth, we have demonstrated that temperature, aerobic and anaerobic exercise, hypoxia and many other factors influence on individual requirements of thyroid hormones. In fact, a 3-week race

results in an increased need of thyroid activity and it is widely described a transient hypothyroid status after extreme exercise in euthyroid individuals.

IN CONCLUSION

Reticulocyte data from Mr. Kreuziger are strongly influenced by hypothyroidism, developed over 10 years.

HEMOGLOBIN PATTERNS AND SEASONS

HEMOGLOBIN PATTERNS DURING 2012 SEASON.

CADF medical report suggests an abnormal hemoglobin concentration pattern during Giro 2012 (see table 1). That pattern started with a sample collected in May 3rd, 2012 (sample 46) and finished with a sample collect in May 24th, 2012 (sample 49). In this regard, CADF medical report considered a competition time between May 3rd and 24th 2012 even though Mr. Kreuziger took part in official competitions from April 23rd, 2012 to May 28th, 2012. Rest periods between the three competitions (Giro del Trentino, Tour de Romandie and Giro d´Italia) were always less than five days (please, see UCI Competition Schedule Appendix 5).

Sample (Graph number)	Date	Competition	Hb	%Ret	Comments
44	11-04-2012	No	15,5	1,56%	No previous competition
45	24-04-2012	Yes (Tour de Romandie)	16,4	1,58%	4 days after a 4-days race
46	3-05-2012	Pre (day -2) (Giro)	15	1,46%	4 days after a 5-days race (2

					consecutive races)
47	14-05-2012	Yes (Giro)	14,5	1,44%	Day +10
48	20-05-2012	Yes (Giro)	14,8	1,52%	Day +16
49	24-05-2012	Yes (Giro)	16,1	1,4%	Day +20

Table 1. Graph samples 44 to 49.

So, to accept May 3rd, 2012 sample as a baseline sample, we should assume two hypotheses as if they were true:

- Mr Kreuziger involvement in the 2012 Giro del Trentino and, after 3 days, in the 2012 Tour de Romandie (sample 45), does not have any impact on the sample collected 4 days later (sample 46).
- As sample collected in April 24th (sample 45) showed a higher level of hemoglobin than that obtained in May 3rd, 2012, we should consider that 15 g/dL was the basal hemoglobin for Mr Kreuziger.

However it is very difficult to support both hypotheses because many published data evidenced the opposite. For example, Schumacher et al, published the hematologic variables in 23 professional cyclists during a 5- day stage race. They showed a reduction in haemoglobin concentration after 5 days in the sample that was collected in the

morning and after 4 days if the sample was collected in the afternoon (Figure 13).

Table 1. Hematologic variables, erythropoietic serum markers (mean ± SD) and changes in vascular volumes (%) in 23 professional cyclists during a five-day stage race.

	Day 1	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Normal range*
Hemoglobin (g/dL)						
Morning	15.3±0.9	15.3±0.8	14.8±0.5	14.6±0.7	14.4±0.8*	*14-17.5*
Evening	14.8±0.7+	14.6±0.5	14.8±0.7+	14.1±0.8 *	14.6±0.8	

Figure 13. From Schumacher et al 2003.

Additionally, Voss et all, confirmed those results in 12 athletes (Voss et al 2014). However, Lombardi et al published a very relevant paper in 2013. They were able to recruit more than 200 cyclists that were involved in the 2010 and 2012 BioGiro (Lombardi et al 2013). Their sample size was the higher sample size ever published in a prospective study dealing with longitudinal values during a cyclist race. In this regard, we have to keep in mind that most papers published dealing with hematological variability in recent years had not recruited more than a few cyclists. Conclusions from that study evidenced a huge variability, higher than anticipated in small studies, but expected by many scientists out of the field of sport. In summary, they showed that 70% of participants showed a percentage variation through the race that exceeded the theoretical variability (Figure 14).

Table 2. Comparison between the theoretical biological variation and the intra-individual variations recorded in the study cohorts.

		2010 (subjects exceding CV_{Bw})			2012 (subjects exceding CV_{Bw})		
	CV_{Bw} (%)	T2vsT1	T3vsT2	T3vsT1	T2vsT1	T3vsT2	T3vsT1
		(n = 144)	(n = 144)	(n = 144)	(n = 109)	(n = 109)	(n = 109)
WBC	11.2	61	95	92	55	58	71
RBC	2.1	141	126	112	104	83	92
Hb	3.4	136	118	81	95	56	77
Ht	2.4	134	137	87	106	81	81
MCV	1.3	60	44	104	28	16	36
MCH	1.6	21	40	38	26	19	18
MCHC	1.7	46	128	140	25	28	38
PLT	9.0	67	63	59	70	72	76
Ret%	13.0	93	106	92	75	80	67
Ret#	13.0	98	117	93	80	89	72

The table shows the theoretical intra-individual variability values (CV_{Bw}) and the number of subjects (out of 144 for the 2010 race and 109 for the 2012 race) for each GiroBio race event, whose percentage variation between time-points exceeded the theoretical CV_{Bw}.
doi:10.1371/journal.pone.0063092.t002

Figure 14. Lombardi et al 2013

In addition, they evidenced an striking hemoglobin pattern because hemoglobin was higher at the end of the race (Figure 15, red arrow) than at the halfway point (Figure 15, red circle). Their data about this issue were similar to those reported previously by Corsetti et al in 2012.

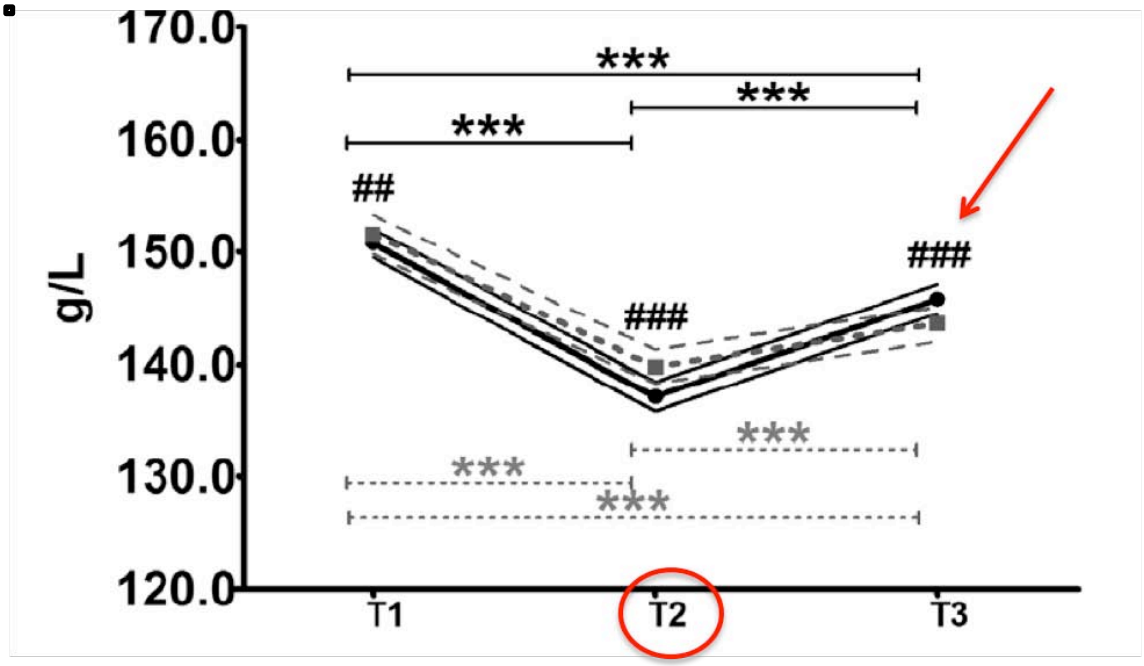


Figure 15. Lombardi et al 2013

They explored 9 professional cyclists during 2011 Giro d´Italia. They concluded that mean hemoglobin values decreased during the race with stabilization in the second half, although mean values were lower than baseline. However, when individual values are considered we can observe a different behaviour among individuals (red boxes and red circles in Figure 16). In fact, 4 individuals increased their hemoglobin when a comparation between day 22 and 12 is taken into account. It is critical to keep in mind that this study included hematological values during a 3- week race.

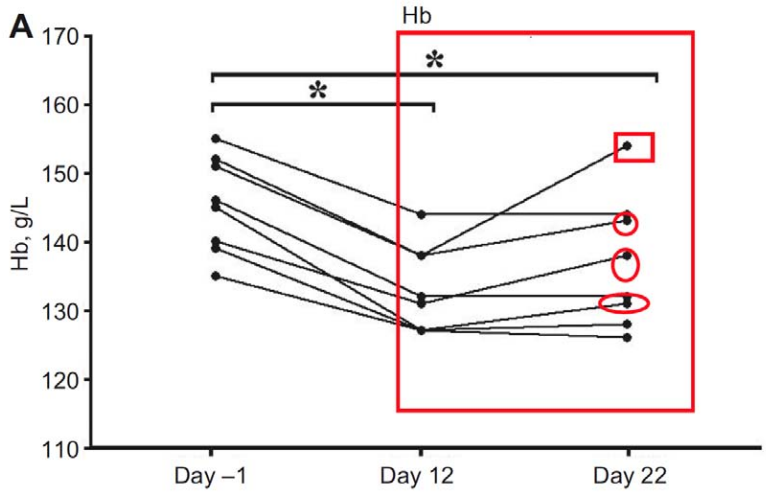


Figure 16. From Corsetti et al 2012

Another conclusion should be made from this study: day 22 hemoglobin level also depends on day -1 hemoglobin level. In this regard, Mr Kreuziger was involved in the Tour de Romandie from April 24th, to April 29th. A sample was collected on day 1 of the Tour de Romandie (sample 45, table 1; red arrow, Figure 17) and an additional sample was taken on May 3rd 2012 (sample 46, table 1). As

expected, hemoglobin levels were reduced after a 5- day stage competition (16.4 g/dL, red arrow to 15 g/dL, green arrow).

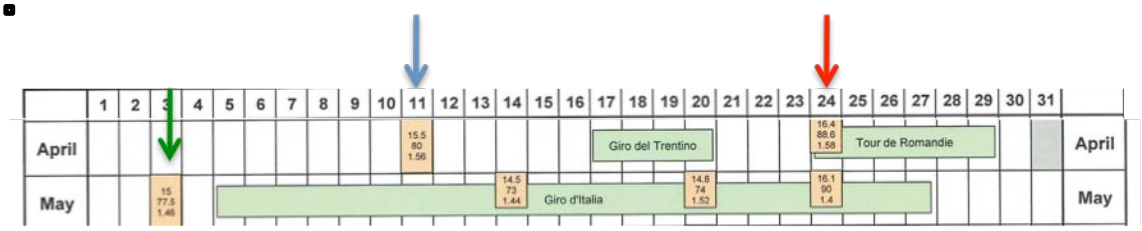


Figure 17. 2012 Season. April and March competition schedule. Blue arrow sample 44; red arrow sample 45; green arrow sample 46.

So, it is difficult to accept that sample collected on May 3rd 2012 could be a valid baseline sample for comparations with sample obtained during 2012 Giro. Probably sample collected on April 24th, 2012 could be more realistic but Mr. Kreuziger was also involved in the Giro del Trentino, a 4 days stage race, that was held between April 17th and 20th 2012, covering 506,6 kms. However, only a sample collected on April 11th 2012 is available. At that point, we can discuss about variation of hemoglobin levels from April 11th 2012 to April 24th 2012, keeping in mind that Mr Kreuziger was training in altitude for 14 days (between March 23rd to April 6th, 2012; Teide, Tenerife). It is out of the scope of this report to discuss about hemoglobin variation in altitude. However, it cannot be discarded that sample collected on April 11th 2012 was not affected by training schedules. Even if we assume that this sample (April 11th, 2012) could be the basal one, variations during Giro 2012 are similar to those reported by Corsetti et al and Lombardi et al without any kind of blood manipulation. What is indisputable is that May 3rd 2012 sample is not a baseline sample because it is affected by previous competitions. In fact, Mr Kreuziger

was involved in sequential competitions since April 17th 2012 to May 27th 2012, "resting" for a maximum of 5 consecutive days before Giro 2012 started. So, it is unnaceptable a baseline sample collected on May 3rd 2012.

Nevertheless, season 2012 means 8 resting days and 33 days in competition during 41 consecutive days and a combination of two consecutive 4-5 days races followed by a 3- week race. In this regard, there is no scientific evidence evaluating hematological changes after this schedule of competition. Thus, as CADF medical report suggests, we have to compare hematological profile of athlete during all seasons recorded at ADAMS to evaluate "normality" of Mr Kreuziger pattern through the years.

2008 SEASON.

There are only 3 samples that met WADA criteria to be included at ABP.

Sample (Graph number)	Date	Competition	Hb	%Ret	Comments
1	30/04/2008	Yes (Tour de Romandie)	15,4	1,15%	Day +2
2	03/07/2008	Pre (Tour de	14,9	0,88%	Day -2

		France)			
3	15/07/2008	Yes (Tour de France)	14,8	0,99%	Day +11

Table 2. 2008 Season. Graph samples 1 to 3.

It is impossible to put sample collected in April 30th 2008 (sample 1; table 2) into any context. It was taken during 2nd stage of the Tour de Romandie but there was no previous valid sample and the next official sample was collected in July 3rd 2008 (sample 2; table 2). This latter sample is interesting because it could be the baseline sample for an in-competition sample taken during 2008 Tour de France (Figure 18; red arrows). Both samples are virtually identical.

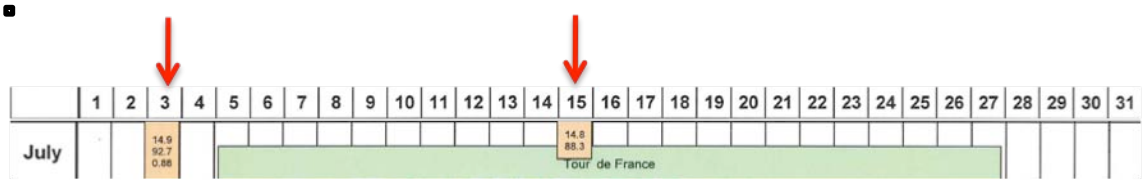


Figure 18. July 2008.

2009 SEASON.

During July 2009, Mr Kreuziger was involved in the 2009 Tour de France.

Sample (Graph	Date	Competition	Hb	%Ret	Comments

number)					
6	02/07/2009	Pre (Tour de France)	15,4	0,76%	Day -2
7	10/07/2009	Yes (Tour de France)	15	0,93%	Day +7
8	11/07/2009	Yes (Tour de France)	15	0,99%	Day +8
9	13/07/2009	Yes (Tour de France)	15,1	1%	Day +10
10	20/07/2009	Yes (Tour de France)	14,8	0,77%	Day +17

Table 3. July 2009. Graph Samples 6 to 10.

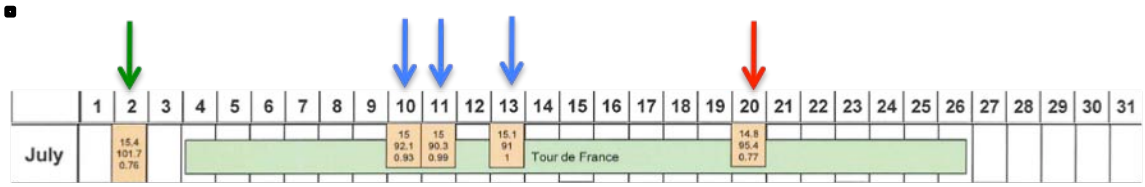


Figure 19. 2009 Tour de France Hb profile.

His samples evidenced a low decrease of hemoglobin levels until the end of the 2nd week of that race (Table 3; Figure 19). As there is no sample at the end of the race we are unable to evaluate the biological behaviour of his hemoglobin levels. However, if we consider first week, Mr kreuziger data concur with those published by Corsetti et al: a decrease in the first half (Figure 19, green arrows and blue arrows) and a stabilisation in the last phase (Figure 19, blue arrows and red

arrows). However, time-line of sampling is a little bit different from that designed by Corsetti et al. In addition, there is no sample at the end of the race and thus we are unable to analyze the entire pattern.

During last week of august and the first three weeks of September, Mr Kreuziger joined the 2009 "Vuelta a España" (table 4).

Sample (Graph number)	Date	Competition	Hb	%Ret	Comments
12	27/08/2009	Pre (Vuelta a España)	15,7	0,7%	Day -2
13	11/09/2009	Yes (Vuelta a España)	15,1	1,23%	Day +14
14	28/09/2009	No	15,2	1,21%	Collected one day after World Championships

Table 4. Graph samples 12 to 14

We can observe a similar pattern to that evidenced in the 2009 "Tour de France" (Figure 19). Once again, the absence of samples at the end of that particular race limits our conclusions. However, Mr Kreuziger was involved in the 2009 World Championships that began in Sept 23rd 2009, just 2 days after the end of the "Vuelta a España". Furthermore, a sample collected in Sep 28th (Figure 20, red arrow) evidenced a hemoglobin level identical to that collected after 2 weeks of competition in the "Vuelta a España" (Figure 20, blue arrow). In this regard, it has been described and reviewed in this manuscript

that competition reduces hemoglobin level (Voss et al 2014; Schumacher et al 2003). So, was the sample collected in Sep 28th (sample 14) affected by the 2009 World Championship? If we assume CADF medical report hypothesis as valid, this sample had to show a decreased value of hemoglobin when compared with a theorically value at the begining of the 2009 World Championship Competition.

Figure 20. 2009 Vuelta a España Hb profile.

If Mr Kreuziger hemoglobin values in the mid of the 2009 Vuelta a España (Figure 20, blue arrow) were 15.1 g/dL: What was the theoretical final value of Mr Kreuziger hemoglobin when finished 2009 "Vuelta a España" to explain that after 2009 World Championship, his hemoglobin value was 15.2? It is clear that the most probable explanation is that Mr Kreuziger hemoglobin value raised at the end of 2009 "Vuelta a España" close to Aug 27th values (Figure 20, green arrow). Corsetti et al have described this rise in hemoglobin levels in 4 out of 9 individuals in the last phase of a 3-week race (Figure 16). Unfortunately, the fact is that there is not a sample in the last days of both the 2009 Tour the France and Vuelta a España. So, we only can speculate with the value of a sample that was never collected. However, we can conclude that during the first two weeks of a 3-weeks race, Mr Kreuziger hemoglobin values decreased in 2009 Season. There is no data about his hemoglobin

levels in the last days of a 3-weeks race. So, anyone can conclude anything about his hemoglobin pattern in the last days of 3-weeks races based on data from 2009 Season.

2010 SEASON

Most samples collected during Jan to June 2010 were out of competition or around dates of 1-day competition. However, a few observations can be made.

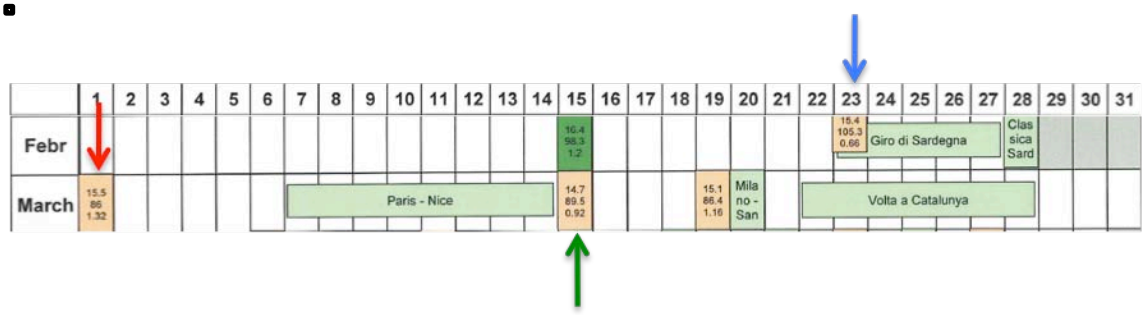


Figure 21. 2010 Feb plus March Hb profile.

If we compare hemoglobin level before (Figure 21, blue arrow) and after the Giro de Sardegna (Figure 21, red arrow), we can conclude that Mr Kreuziger hemoglobin level is identical (Table 5). However, after a consecutive, second 5- days race, hemoglobin decreased (Figure 21, green arrow; Table 5).

Sample (Graph	Date	Competition	Hb	%Ret	Comments

number)					
16	23/02/2010	Yes(Giro di Sardegna)	15,4	0,66%	Day +1
17	01/03/2010	No	15,5	1,32%	Collected one day after Giro di Sardegna and Clas Sardegna finished
18	15/03/2010	No	14,7	0,92%	Collected one day after Paris- Nice finished

Table 5. Samples 16 to 18.

Unfortunately, during 2010 season there is not a complete profile of hemoglobin level during a 3-weeks race.

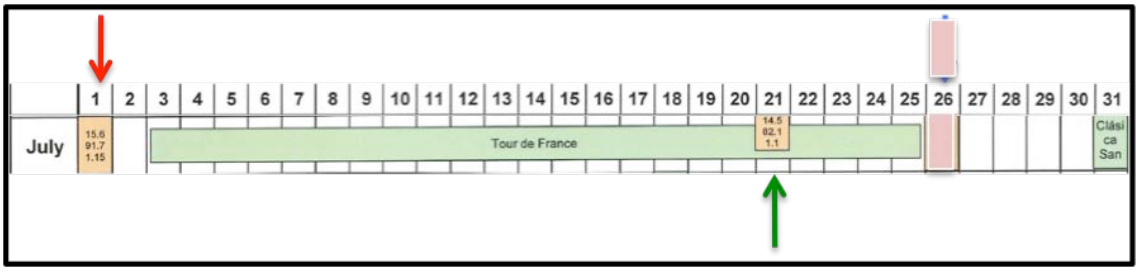


Figure 22. July 2010: Tour de France Hb profile. Soft red squares cover a mistake from the UCI competition schedule as sample 29 was collected in August 26th, 2010 but it is included in the schedule provided by UCI in July 26th.

Sample (Graph	Date	Competition	Hb	%Ret	Comments

number)					
27	01/07/2010	Pre (Tour de France)	15,6	1,15%	Day -2
28	21/07/2010	Yes (Tour de France)	14,5	1,10%	Day +19

Table 6. 2009 Tour de France. Samples 27 and 28.

When we compare data (table 6), it is obvious that Mr. Kreuziger hemoglobin levels decreased in the first 2 weeks (Figure 22, red arrow vs green arrow). Once again, there is no data at the end of the race. Unfortunately, during 2010 "Vuelta a España" there was a single sample that we are unable to decode within a rational context.

In conclusion, during 2010 we can observe a hemoglobin pattern after two consecutive 5-days races, similar to that observed in 2012 (Figure 17). As we have no samples collected at the end of any 3-weeks race, anyone can conclude anything regarding his hemoglobin pattern at the end of 3 weeks races using 2010 data. We have to keep in mind that there are no samples collected in the last days of 3-weeks races in 2008 and 2009.

2011 SEASON

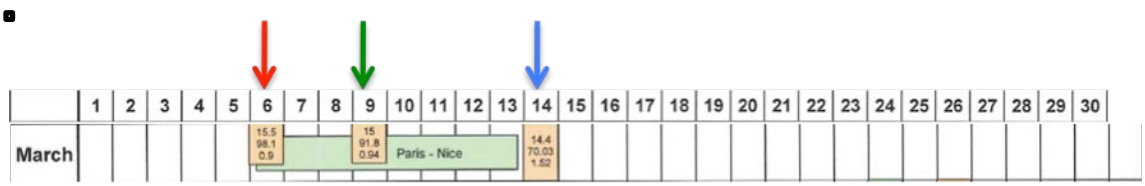


Figure 23. 2011 Paris- Nice Hb profile.

In this season, we can identify a few changes in Mr Kreuziger hemoglobin patterns. For example, during Paris- Nice race, we observe a decrease in his hemoglobin level (Figure 23, red arrow vs blue arrow) in absence of a previous 5-days race. However, that decrease in his hemoglobin level was of similar magnitud to that observed in 2010 (Figure 21).

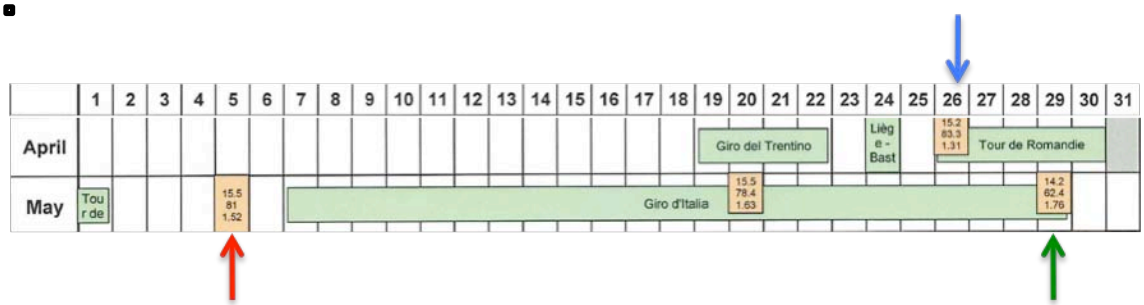


Figure 24. Giro 2011

In addition, before his first participation in the Giro d´Italia, Mr Kreuizger´s hemoglobin concentration increased after his involvement in the "Tour de Romandie" (Figure 24, blue arrow vs red arrow) but decreased at the end of the "2011 Giro de Italia" (Figure 24, red arrow vs green arrow).

This pattern is different to that observed in the "2011 Tour de France" when his hemoglobin level raised (Figure 25, red arrow vs blue arrow) and hold on (Figure 25, blue arrow vs green arrow) during the race.

Figure 25. Tour 2011

Unfortunately, we have no sample collected at the last days of the race. In conclusion, during 2011 Season there is a complete profile to analyze 2011 Giro but there is not a complete profile to analyze Tour de France. Regarding Giro we can conclude that Mr Kreuziger showed a decrease in his hemoglobin levels in 2011 at this race. No conclusions can be made regarding Tour de France as there is no sample at the end of the race.

2013 SEASON

There were 2 available samples that we cannot put in context.

PATTERNS OBSERVED

DESCRIPTION OF PATTERNS

Most of the samples collected into Mr Kreuziger ABP could be analyzed within the correct competition context. However, to provide a good analysis, it would have been very helpful that samples had been collected following a similar timeline through seasons. In fact, there

are only three 3-week races with a complete library of samples, namely 2011 (Figure 24) and 2012 Giro d´Italia (Figure 17). As stated by CADF medical experts those competitions showed a different hemoglobin pattern. Additionally, hemoglobin profile collected during 2011 Tour de France, showed an increased hemoglobin level when we compare control sample collected two days before Tour started and samples collected during competition. This pattern is the same that CADF Medical Report describes for 2012 Giro d´Italia. Regarding "Vuelta a España" profiles, we can only speculate with samples collected soon after the end of the race but affected by a 5-days competition. In addition, it is academically unacceptable to analyze sample collected in September 11th, 2009 without any reference to sample collected in September 28th, 2009.

Regarding 4-5 days race, patterns of hemoglobin are quite stable: hemoglobin remained unchanged or fell. We have identified a single exception: 2011 Tour de Romandie when a slight increase was observed.

In conclusion, we agree with CADF medical report suggesting that there is an abnormal pattern of hemoglobin evolution during Mr. Kreuziger ABP. However, the abnormal pattern was evidenced in 2011 Giro d´Italia and, possibly, during the 2011 Tour de Romandie. We all due respect, 2012 Giro pattern of hemoglobin is not abnormal when compared with Mr Kreuziger data.

ANALYSIS OF PATTERNS

We agree with CADF medical report that Mr Kreuziger´s hemoglobin displayed different patterns over the years 2008- 2012. Features evidencing different patterns are listed below:

- Hemoglobin concentration differences between the last in-competition sample and the baseline one, collected during 3-week races, followed opposite patterns. This is one of the most relevant finding of the ABP.
- Average hemoglobin increased from 2008 to 2010 and then it decreased.
- Hemoglobin concentration at baseline followed a trend during 2008-2012. Intriguingly, hemoglobin concentration at baseline was able to predict differences at the end of a 3-week race.

DIFFERENCES BETWEEN BASELINE AND LAST SAMPLE DURING A 3-WEEK RACE: HEMOGLOBIN RESPONSE TO EXTREME EXERCISE AND THYROID ACTIVITY.

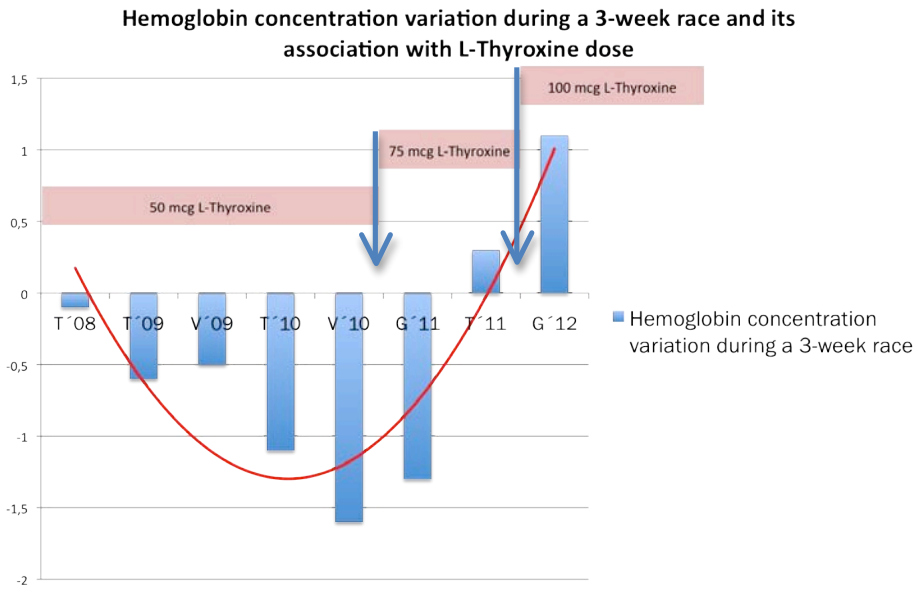


Figure 23. Hemoglobin concentration differences between baseline and last sample collected during eight sucessive 3-week races. T= Tour; V= Vuelta; G= Giro; two-digits represent year of competition.

We have plotted hemoglobin concentration differences between last and baseline samples collected during all 3-week races (figure 23), showing a 2nd order polynomial trendline. This concave-up shaped line evidenced an increased difference between hemoglobin concentration from 2008 to 2010, showing a turning point in 2011 Giro after maximal differences evidenced in 2010 Vuelta. If we incorporate data about thyroid insufficiency and time-to-effect of substitutive therapy into this figure (23), it is easy to observe that transitions between patterns are associated to L-thyroxine supplementation. In fact, the turning point started a few weeks after L-thyroxine substitutive therapy was increased. However, any modification in L-thyroxine supplementation needs a few months to be effective: full effect on hemoglobin response was evident in the 2011 Tour. Probably, L-thyroxine effect was amplified because of thyroid activity requirements in winter and spring are higher than in summer: the same dose could be inadequate in colder months but excellent in summer. Finally, maximal effect was observed when a new, increased dose of L-thyroxine was started in 2012. So, we can conclude that CADF medical report was right when considered that hemoglobin response was different between seasons. In fact we have evidenced a concave-up shaped trend line whose turning point was associated with L-thyroxine modification. However, to reach a robust conclusion we need a biological plausibility for this finding. Obviously, erythropoietic response under extreme conditions depends on many factors, including thyroid hormones. These proteins (both TSH and T3) have canonical, functional receptors on early eritroid progenitors and

mature red cells. Without adequate supplies of thyroid hormones, erythropoietic activity will be compensated by many factors. However, under extreme conditions (ie, last days within a 3-week professional cyclist race), all proteins involved in erythropoiesis have to be orchestrated and available, as evidenced in animal models. In humans, previous data published by Chicharro et al, evidenced that many riders become “transient hypothyroid patients” in the last days of a 3-week race, suggesting that thyroid reserve is depleted. As described above, it is easy to imagine what would happen with an hypothyroid individual depending on a static dose of exogenous L-thyroxine, without any ability to squeeze his/her thyroid gland under extreme efforts.

In this regard, negative differences started in 2009 and reached maximal values in 2010. Those differences anticipated an increased level of TSH and must be considered an unintended sign of future thyroid insufficiency.

Average hemoglobin increased from 2008 to 2010 and then it decreased.

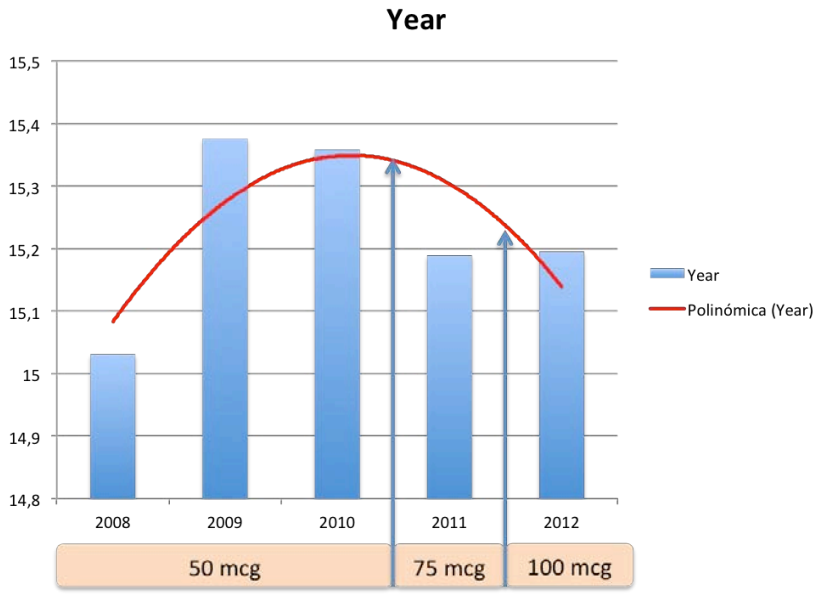


Figure 24. Average hemoglobin concentration between 2008 and 2012.

We have plotted average hemoglobin from 2008 to 2012 (Figure 24). A concave-down shaped trend line is observed, just opposite to that evidenced in previous figure. Again, turning point fits with L-thyroxine dose escalation. This event has been described, in non- anemic, non-iron deficient, hypothyroid patients when they are exposed to L-thyroxine therapy (figure 25: red squares).

Table 1—*Laboratory characteristics of 30 nondiabetic patients with overt hypothyroidism before and after thyroid hormone replacement in study 2*

	Before thyroid hormone replacement	After thyroid hormone replacement	*P*
Age (years)	47.3 ± 12.3	—	
Sex (male/female)	7/23	—	
A1C (%)	5.57 ± 0.26	5.37 ± 0.32	<0.001
GA (%)	13.18 ± 1.35	12.52 ± 1.16	0.027
GA-to-A1C ratio	2.38 ± 0.29	2.35 ± 0.28	0.587
Glucose (mmol/l)	4.95 ± 0.41	5.24 ± 0.92	0.174
1,5-AG (μg/ml)	20.03 ± 6.92	21.42 ± 7.45	0.086
Hb (g/dl)	14.05 ± 1.35	13.64 ± 1.23	0.004
MCV (fL)	88.29 ± 4.73	90.78 ± 4.75	<0.001
MCH (pg)	29.48 ± 2.04	30.25 ± 1.95	<0.001
Reticulocyte (%)	0.64 ± 0.18	1.09 ± 0.34	<0.001
Ferritin (ng/ml)	74.34 ± 64.30	76.31 ± 57.58	0.689
EPO (mIU/ml)	12.93 ± 4.97	16.41 ± 6.44	0.022
Albumin (g/l)	43.5 ± 1.7	42.6 ± 1.6	0.009
Total cholesterol (mmol/l)	6.21 ± 1.23	4.37 ± 0.75	<0.001
Triglyceride (mmol/l)	1.57 ± 1.01	1.62 ± 1.15	0.802

Data are means ± SD. Hb, hemoglobin.

Figure 25. Kim et al. Diabetes Care 2010.

So, data provided by average hemoglobin concentration during seasons and its variation after L-thyroxine therapy concur with that described by Kim et al.

Hemoglobin concentration at baseline followed a trend during 2008-2012.

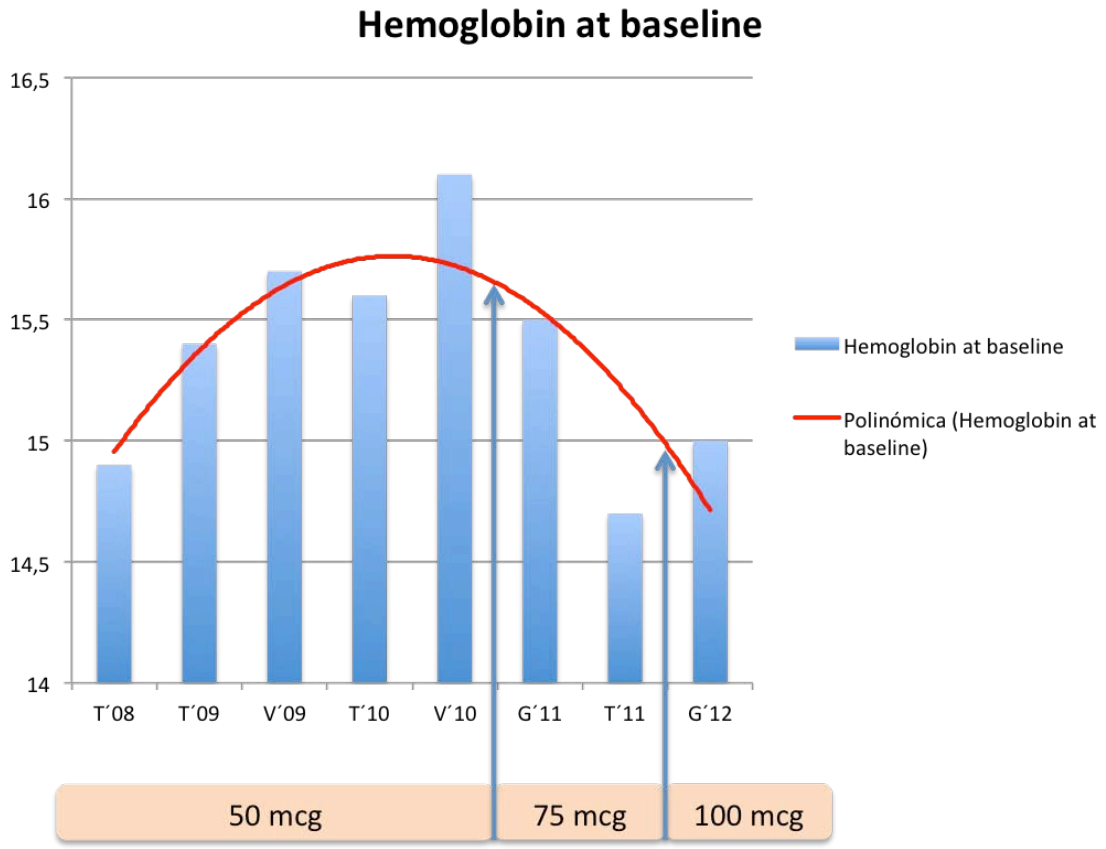


Figure 26. Hemoglobin concentration at baseline of 3-week races included in the ABP documentation package. T= Tour; V= Vuelta; G= Giro; two-digits represent year of competition.

A similar trend to that described for average hemoglobin during seasons is observed when hemoglobin at baseline is considered. Again, turning point is associated with L-thyroxine escalation. A decrease in hemoglobin concentration after L-thyroxine therapy is described by Kim et al.

SUMMARY

First, we have demonstrated that Mr Kreuziger hemoglobin values are influenced by L-thyroxine dose and its modifications over the time.

Second, we have demonstrated that previous researchers have evidenced that L-thyroxine impact on hemoglobin concentration in patients diagnosed with hypothyroidism in absence of anemia and any type of iron depletion.

Third, we have demonstrated that hemoglobin response during all consecutive 3-week races followed a concave-up shape trend line. The turning point appeared after L-thyroxine dose was increased.

Thus, there is both biological plausibility and clinical evidence that hemoglobin patterns of Mr Kreuziger are fully explained by hypothyroidism and its treatment.

SAMPLES 48 AND 49.

Samples 48 and 49 need an additional explanation because there was a 1,3 g/dL of hemoglobin difference between the two samples.

Date	Hb	% Ret
20-may	14,8	1,52
24-may	16,1	1,4

We also have to consider that % Ret values were almost identical. In this regard, a blood transfusion may well be the reason why this difference was evidenced. However, an increase of 1,3 g/dL within 4 days means that at least one unit of RBC was transfused in-competition. In this regard, we should observe a decrease in %Ret. As stated above, % Ret were identical (8% of absolute difference). However, many cheats use low- doses of EPO to counterbalance this very well known effect of blood transfusions. Nonetheless, Mr Kreuziger never had a positive urine EPO test during Giro 2012. Obviously, we could discuss about sophisticated transfusion protocols but the most likely explanation for these findings is that he was unable to recover his plasma volume properly after a strenuous exercise. In this regard, we have to remember that Mr Kreuziger is diagnosed with an overt hypothyroidism and free water clearance may be impaired in patients with an adequate T4 therapy (Ota et al 1994).

Additionally, a transient hypothyroidism occurs in euthyroid individuals during a 3- week race (Chicharro et al 2001). So that, Mr Kreuziger met all conditions to present a transient hypothyroidism that resulted in an impaired free water clearance when sample 49 was collected. He also pointed out "Disidratazione da attivita sportiva in data 23.05.12" in the Doping Control Form. Obviously, an euthyroid individual should be able to recover this situation without problems. However, Mr Kreuziger is diagnosed with an overt hypothyroidism and his ability to recover after extreme exercise relies on his pill of 100 mcg of L-thyroxine among others factors. We should keep in mind than euthyroid individuals suffer a transient hypothyroidism after extreme exercise (Chicharro et al 2001) in spite of a thyroid gland that can fully commit its duties during exercise. Mr Kreuziger depends on his dose of L-thyroxine. If his metabolism demands additional mcgs of L-Thyroxine, his thyroid gland will never supply any single molecule of L-Thyroxine. So, his ability to recover after extreme efforts is impaired and delayed.

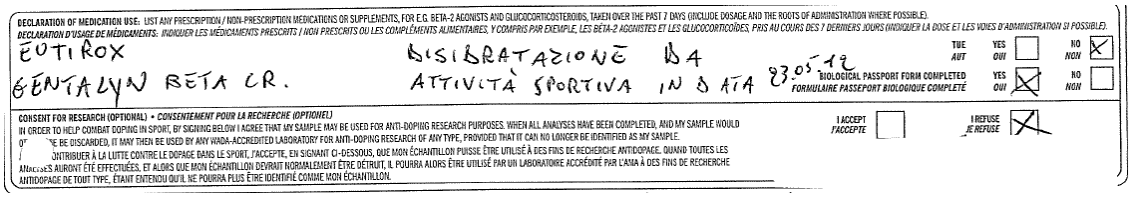

DECLARATION OF MEDICATION USE: LIST ANY PRESCRIPTION / NON-PRESCRIPTION MEDICATIONS OR SUPPLEMENTS, FOR E.G. BETA-2 AGONISTS AND GLUCOCORTICOSTEROIDS, TAKEN OVER THE PAST 7 DAYS (INCLUDE DOSAGE AND THE ROOTS OF ADMINISTRATION WHERE POSSIBLE).
DÉCLARATION D'USAGE DE MÉDICAMENTS: INDIQUER LES MÉDICAMENTS PRESCRITS / NON PRESCRITS OU LES COMPLÉMENTS ALIMENTAIRES, Y COMPRIS PAR EXEMPLE, LES BÊTA-2 AGONISTES ET LES GLUCOCORTICOÏDES, PRIS AU COURS DES 7 DERNIERS JOURS (INDIQUER LA DOSE ET LES VOIES D'ADMINISTRATION SI POSSIBLE).
EUTIROX
6 ENTALYN BETA CR.
DISIDRATAZIONE DA ATTIVITÀ SPORTIVA IN DATA 23.05.12
TUE / AUT YES / OUI NO / NON
BIOLOGICAL PASSPORT FORM COMPLETED / FORMULAIRE PASSEPORT BIOLOGIQUE COMPLÉTÉ YES / OUI NO / NON
CONSENT FOR RESEARCH (OPTIONAL) • CONSENTEMENT POUR LA RECHERCHE (OPTIONNEL)
IN ORDER TO HELP COMBAT DOPING IN SPORT, BY SIGNING BELOW I AGREE THAT MY SAMPLE MAY BE USED FOR ANTI-DOPING RESEARCH PURPOSES. WHEN ALL ANALYSES HAVE BEEN COMPLETED, AND MY SAMPLE WOULD
OTHERWISE BE DISCARDED, IT MAY THEN BE USED BY ANY WADA-ACCREDITED LABORATORY FOR ANTI-DOPING RESEARCH OF ANY TYPE, PROVIDED THAT IT CAN NO LONGER BE IDENTIFIED AS MY SAMPLE.
/ CONTRIBUER À LA LUTTE CONTRE LE DOPAGE DANS LE SPORT, J'ACCEPTE, EN SIGNANT CI-DESSOUS, QUE MON ÉCHANTILLON PUISSE ÊTRE UTILISÉ À DES FINS DE RECHERCHE ANTIDOPAGE. QUAND TOUTES LES ANALYSES AURONT ÉTÉ EFFECTUÉES, ET ALORS QUE MON ÉCHANTILLON DEVRAIT NORMALEMENT ÊTRE DÉTRUIT, IL POURRA ALORS ÊTRE UTILISÉ PAR UN LABORATOIRE ACCRÉDITÉ PAR L'AMA À DES FINS DE RECHERCHE ANTIDOPAGE DE TOUT TYPE, ÉTANT ENTENDU QU'IL NE POURRA PLUS ÊTRE IDENTIFIÉ COMME MON ÉCHANTILLON.
I ACCEPT / J'ACCEPTE
I REFUSE / JE REFUSE

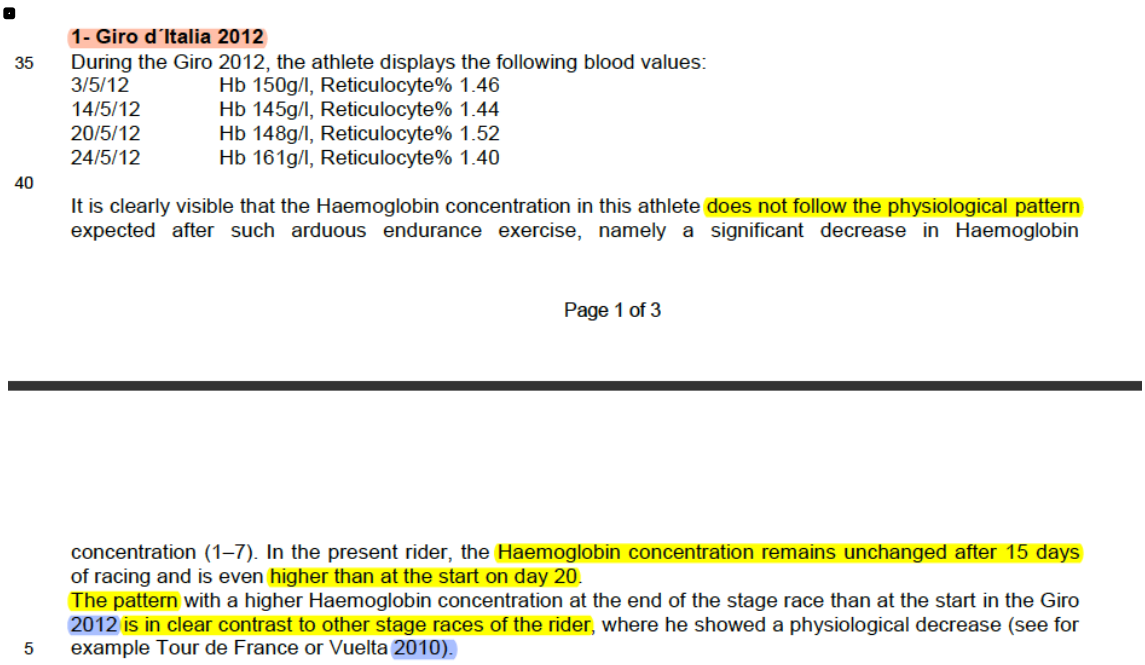
Figure 27. Extracted from Doping Control Form dated May-24th- 2012.

In conclusion, with all due deference to CADF Medical Reports, there are no supporting data to conclude any blood manipulations on 2012 Giro d´Italia.

POINT-BY-POINT ANALYSIS OF CADF REPORTS.

Initial report dated on May 24th, 2012

CADF Medical Report stated that:



1- Giro d´Italia 2012

During the Giro 2012, the athlete displays the following blood values:

3/5/12 Hb 150g/l, Reticulocyte% 1.46
14/5/12 Hb 145g/l, Reticulocyte% 1.44
20/5/12 Hb 148g/l, Reticulocyte% 1.52
24/5/12 Hb 161g/l, Reticulocyte% 1.40

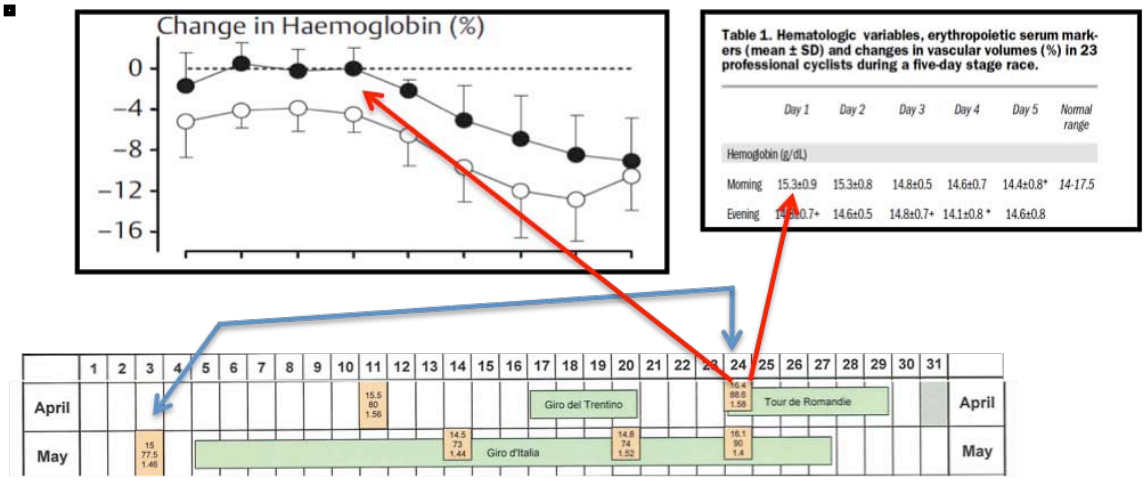
It is clearly visible that the Haemoglobin concentration in this athlete does not follow the physiological pattern expected after such arduous endurance exercise, namely a significant decrease in Haemoglobin

Page 1 of 3

concentration (1–7). In the present rider, the Haemoglobin concentration remains unchanged after 15 days of racing and is even higher than at the start on day 20.
The pattern with a higher Haemoglobin concentration at the end of the stage race than at the start in the Giro 2012 is in clear contrast to other stage races of the rider, where he showed a physiological decrease (see for example Tour de France or Vuelta 2010).

We have demonstrated that it is unacceptable that the sample collected on day May 3rd, 2012 was a baseline sample to make comparisons about hemoglobin patterns during 2012 Giro. Briefly, Mr. Kreuziger took part in official competitions from April 17, 2012 to May 28, 2012. Rest periods between the three competitions (Giro del Trentino, Tour de Romandie and Giro d´Italia) were always less than five days (make table). So, accepting May 3rd, 2012 sample as a baseline sample, forces us to assume two hypothesis as if they were true:

- Mr Kreuziger involvement in the 2012 Giro del Trentino and, after 3 days, in the 2012 Tour de Romandie, does not have any impact on the sample collected in May 3rd 2012. That is 4 days after the 2012 Tour de Romandie finished.
- Although sample collected in April 24th, 2012 showed a higher level of hemoglobin than that obtained in May 3rd 2012, we should consider that 15 g/dL was the basal hemoglobin for Mr



Kreuziger. That is the hemoglobin level in May 3rd 2012.

As we demonstrated above, it is impossible to support these hypotheses. Schumacher et al (2003), Voss et all (2014), Lombardi et al (2013) among others, have evidenced that during a 5-day stage race, hemoglobin levels of cyclist decreased.

In addition, Corsetti et al (2012), and Lombardi et al (2013) have evidenced an striking hemoglobin pattern because hemoglobin was higher at the end of the race than at the halfway point.

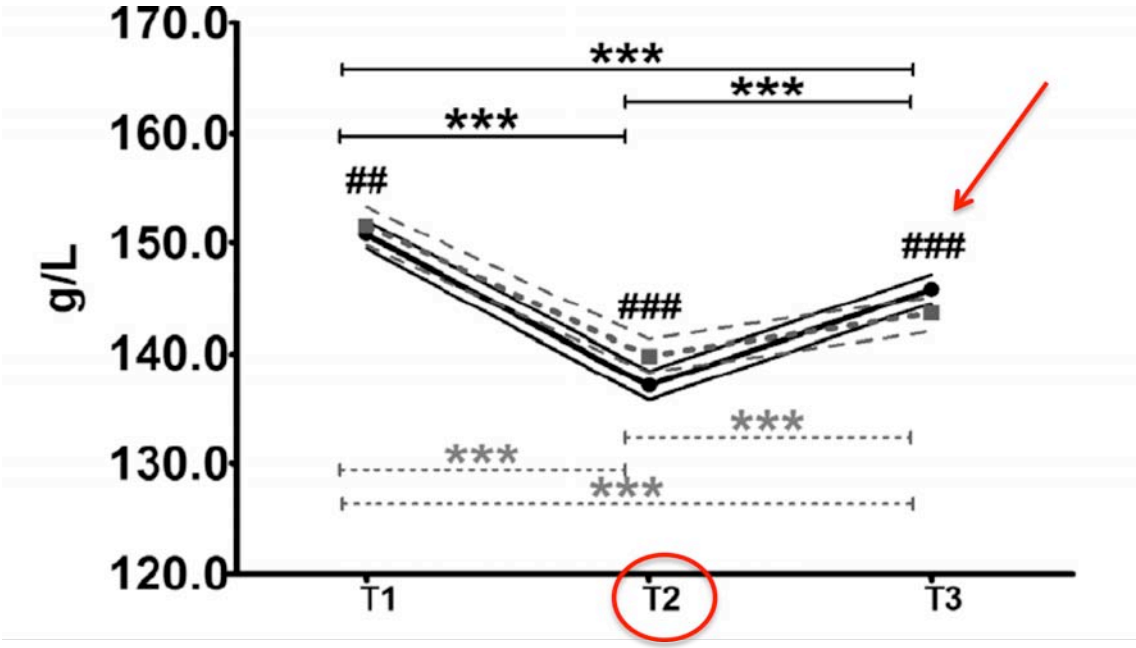


Figure 29. Lombardi et al. PLOSOne2013

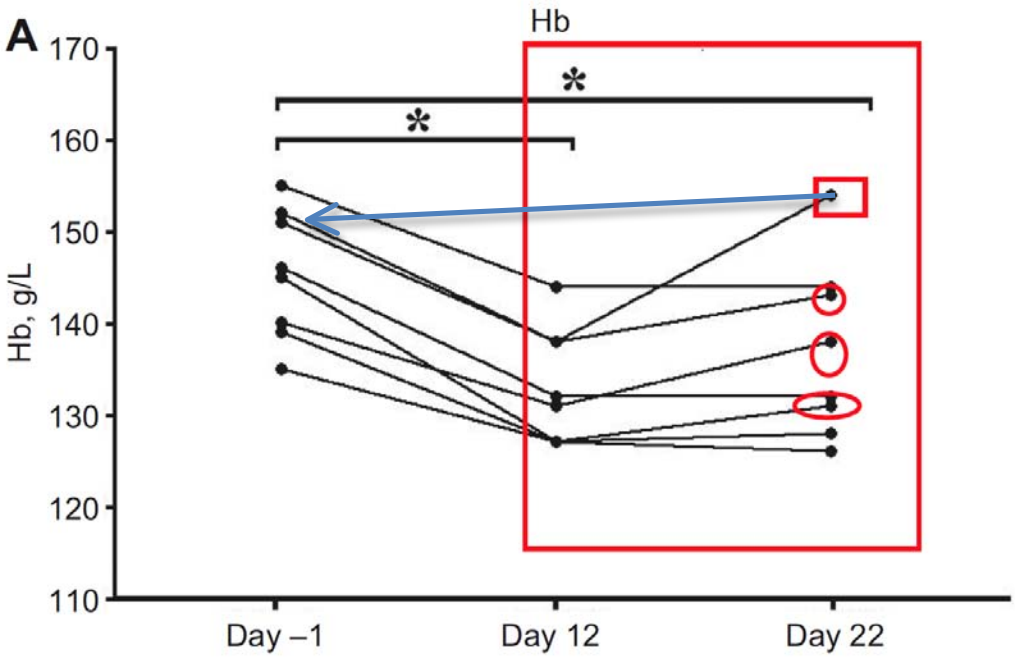


Figure 30. From Corsetti et al. Clin Chem Lab Med 2012;50(5):949–956

CADF medical report also stated as uncommon that hemoglobin levels were unchanged during 15 days of racing and even higher than at the

start. In this regard, Mr Kreuziger was involved in the Tour de Romandie from April 24th, to April 29th. A sample was collected on day 1 of the Tour de Romandie and an additional sample was taken on May 3rd 2012. As expected, hemoglobin levels were reduced after a 5-day stage competition. So, it is difficult to accept that sample collected on May 3rd 2012 could be a valid baseline sample for comparations with sample obtained during 2012 Giro because it was affected by previous competition (that finished 4 days later). Probably sample collected on April 24th, 2012 could be realistic but Mr. Kreuziger was also involved in the Giro del Trentino, a 4 days stage race, that was held between April 17th and 20th 2012, covering 506,6 kms. However, only a sample collected on April 11th 2012 is available. Even if we assume that this sample (April 11th, 2012) could be the basal one, variations during Giro 2012 are similar to those described in references included by CADF experts without any kind of blood manipulation. What is indisputable is that May 3rd 2012 sample is not a baseline sample because it is affected by previous competitions.

In addition, Samples 48 and 49 need an additional explanation because there was a 1,3 g/dL of hemoglobin difference between the two samples.

Date	Hb	% Ret
20-may	14,8	1,52
24-may	16,1	1,4

We also have to consider that % Ret values were almost identical. In this regard, a blood transfusion may well be the reason why this difference was evidenced. However, an increase of 1,3 g/dL within 4 days means that at least one unit of RBC was transfused in-competition. In this regard, we should observe a decrease in %Ret. As stated above, % Ret were identical (8% of absolute difference). However, many cheats use low- doses of EPO to counterbalance this very well- known effect of blood transfusions. Nonetheless, Mr Kreuziger never had a positive urine EPO test during Giro 2012. Obviously, we could discuss about sophisticated transfusion protocols but the most likely explanation for these findings is that he was unable to recover his plasma volume properly after a strenuous exercise. In this regard, we have to remember that Mr Kreuziger is diagnosed with an overt hypothyroidism and free water clearance may be impaired in patients with an adequate T4 therapy (Ota et al 1994). Additionally, a transient hypothyroidism occur in euthyroid individuals during a 3- week race (Chicharro et al 2001). So that, Mr Kreuziger met all conditions to present a transient hypothyroidism that resulted in an impaired free water clearance when sample 49 was collected. He also pointed out "Disidratazione da attivita sportiva in data 23.05.12" in the Doping Control Form.

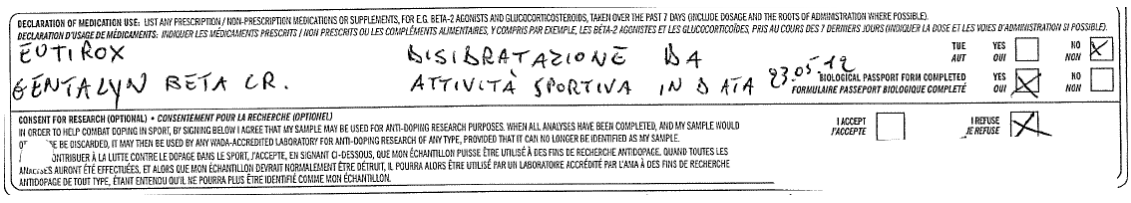

DECLARATION OF MEDICATION USE: LIST ANY PRESCRIPTION / NON-PRESCRIPTION MEDICATIONS OR SUPPLEMENTS, FOR E.G. BETA-2 AGONISTS AND GLUCOCORTICOSTEROIDS, TAKEN OVER THE PAST 7 DAYS (INCLUDE DOSAGE AND THE ROOTS OF ADMINISTRATION WHERE POSSIBLE).
DÉCLARATION D'USAGE DE MÉDICAMENTS: INDIQUER LES MÉDICAMENTS PRESCRITS / NON PRESCRITS OU LES COMPLÉMENTS ALIMENTAIRES, Y COMPRIS PAR EXEMPLE, LES BÊTA-2 AGONISTES ET LES GLUCOCORTICOÏDES, PRIS AU COURS DES 7 DERNIERS JOURS (INDIQUER LA DOSE ET LES VOIES D'ADMINISTRATION SI POSSIBLE).
EUTIROX
GENTALYN BETA CR.
DISIDRATAZIONE DA ATTIVITÀ SPORTIVA IN DATA 23.05.12
TUE / AUT YES / OUI NO / NON
BIOLOGICAL PASSPORT FORM COMPLETED / FORMULAIRE PASSEPORT BIOLOGIQUE COMPLÉTÉ YES / OUI NO / NON
CONSENT FOR RESEARCH (OPTIONAL) • CONSENTEMENT POUR LA RECHERCHE (OPTIONNEL)
IN ORDER TO HELP COMBAT DOPING IN SPORT, BY SIGNING BELOW I AGREE THAT MY SAMPLE MAY BE USED FOR ANTI-DOPING RESEARCH PURPOSES. WHEN ALL ANALYSES HAVE BEEN COMPLETED, AND MY SAMPLE WOULD OTHERWISE BE DISCARDED, IT MAY THEN BE USED BY ANY WADA-ACCREDITED LABORATORY FOR ANTI-DOPING RESEARCH OF ANY TYPE, PROVIDED THAT IT CAN NO LONGER BE IDENTIFIED AS MY SAMPLE.
JE CONSENS À CONTRIBUER À LA LUTTE CONTRE LE DOPAGE DANS LE SPORT, J'ACCEPTE, EN SIGNANT CI-DESSOUS, QUE MON ÉCHANTILLON PUISSE ÊTRE UTILISÉ À DES FINS DE RECHERCHE ANTIDOPAGE. QUAND TOUTES LES ANALYSES AURONT ÉTÉ EFFECTUÉES, ET ALORS QUE MON ÉCHANTILLON DEVRAIT NORMALEMENT ÊTRE DÉTRUIT, IL POURRA ALORS ÊTRE UTILISÉ PAR UN LABORATOIRE ACCRÉDITÉ PAR L'AMA À DES FINS DE RECHERCHE ANTIDOPAGE DE TOUT TYPE, ÉTANT ENTENDU QU'IL NE POURRA PLUS ÊTRE IDENTIFIÉ COMME MON ÉCHANTILLON.
I ACCEPT / J'ACCEPTE
I REFUSE / JE REFUSE

Figure 31. Extracted from Doping Control Form dated May-24th- 2012.

2- Abnormal Reticulocyte pattern from March 2011 onwards

The second notable observation made on review of the samples is related to Reticulocyte% levels. There appears to be a significant trend upwards in these values from March 2011 and then from April 2012 onwards, showing a time correspondence with important stage races. The average level from this point onwards is above 1.5% which is far beyond the athletes previous levels.

The gradual increase in Reticulocyte% for all samples is outlined in the figure below

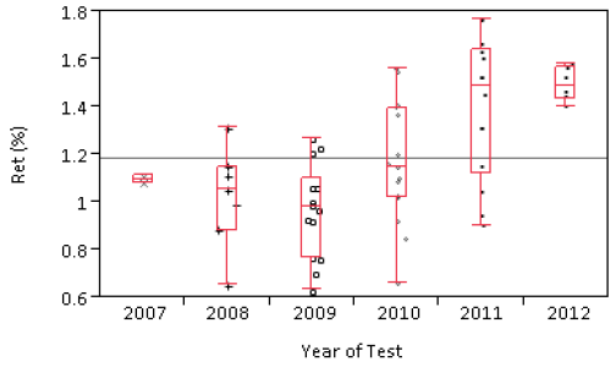


Figure 1: Distribution of Reticulocyte% in profile BPY2524M36 per year.
The red boxes represent the 25th and 75th percentile, the horizontal line represents the median, the whiskers illustrate the outliers)

CADF medical experts clustered % Ret year by year. However, if we cluster % reticulocytes month by month (Figure 33) we will observe a seasonal variation of % Ret. Higher levels of % Ret were evidenced in May.

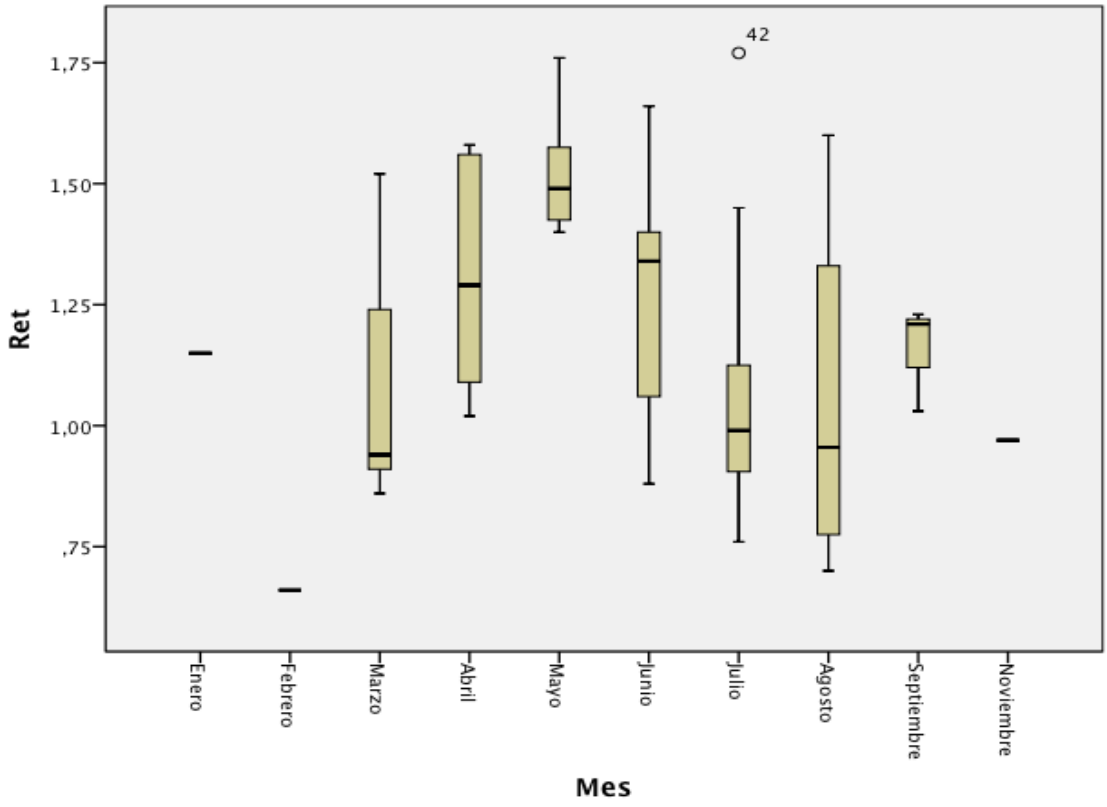


Figure 32. % Ret plotted month-by-month

Furthermore, if we plot samples per month and year (Figure 34), we will observe that samples obtained in 2012 were enriched in "May-samples" whereas samples collected in 2009 and 2010 were enriched in "July and April- Samples" respectively. Curiously, samples collected in 2011 were homogeneously distributed from March to July.

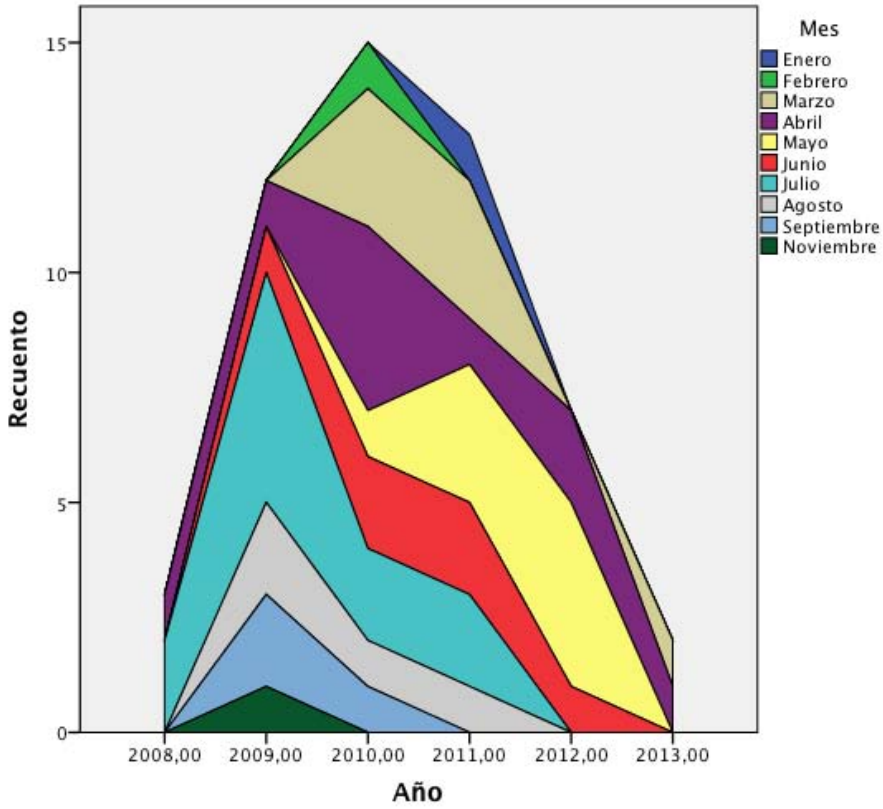


Figure 33. Samples per month plotted by year

In spite of these "statistical" considerations, % Ret is rising since 2011. In 2011, Mr Kreuziger endocrinologist advised his patient to increase L-Thyroxine dose from 50 mcg to 75 mcg because of an increased TSH level. One year later, L-Thyroxine dose was also augmented to 100 mcg because of another elevated TSH level. We have demonstrated above that L-Thyroxine supplementation in non-anemic, iron non- deficient hypothyroid patients increases % Ret values.

Table 1—*Laboratory characteristics of 30 nondiabetic patients with overt hypothyroidism before and after thyroid hormone replacement in study 2*

	Before thyroid hormone replacement	After thyroid hormone replacement	*P*
Age (years)	47.3 ± 12.3	—	
Sex (male/female)	7/23	—	
A1C (%)	5.57 ± 0.26	5.37 ± 0.32	<0.001
GA (%)	13.18 ± 1.35	12.52 ± 1.16	0.027
GA-to-A1C ratio	2.38 ± 0.29	2.35 ± 0.28	0.587
Glucose (mmol/l)	4.95 ± 0.41	5.24 ± 0.92	0.174
1,5-AG (μg/ml)	20.03 ± 6.92	21.42 ± 7.45	0.086
Hb (g/dl)	14.05 ± 1.35	13.64 ± 1.23	0.004
MCV (fL)	88.29 ± 4.73	90.78 ± 4.75	<0.001
MCH (pg)	29.48 ± 2.04	30.25 ± 1.95	<0.001
Reticulocyte (%)	0.64 ± 0.18	1.09 ± 0.34	<0.001
Ferritin (ng/ml)	74.34 ± 64.30	76.31 ± 57.58	0.689
EPO (mIU/ml)	12.93 ± 4.97	16.41 ± 6.44	0.022
Albumin (g/l)	43.5 ± 1.7	42.6 ± 1.6	0.009
Total cholesterol (mmol/l)	6.21 ± 1.23	4.37 ± 0.75	<0.001
Triglyceride (mmol/l)	1.57 ± 1.01	1.62 ± 1.15	0.802

Data are means ± SD. Hb, hemoglobin.

Figure 34. Reticulocyte counts before and after L-Thyroxine therapy. Kim et al 2010.

So, we can conclude without any doubt that %Ret behavior was secondary to L-Thyroxine dose.

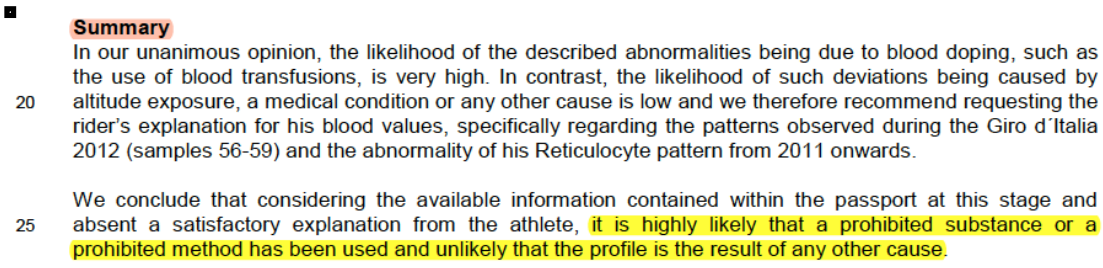
REFERENCES INCLUDED AT THE INITIAL REPORT

References included in that Initial Report deserve additional considerations because they hardly support CADF Medical Report conclusions. For example, Reference 1 is a letter-to-the-editor and thus invalid to support anything. In fact, a letter-to-the-editor is an academic commentary about a manuscript published by other. Reference 2, published by Schmidt et al in 2000, deals with hemoglobin levels during a 10- day stage cycling competition. Unfortunately, only 4 individuals were recruited. These very small sample sizes are too common in many studies in this field of Medicine. In this case, Schmidt et al use an ANOVA test of repeated measures to compare longitudinal values. We recommend authors to explore statistical requirements that a sample should met before use

this test. Please, read Keselman & Keselman (1988), Maxwell & Arvey (1982) or Girden (1992). Making a long history short, a very small sample size (and we can conclude without any doubt that 4 is a very small sample size) increases type I error, so thresholds for statistical significance should be modified. That means that a "p value" less than 0,05 guarantees nothing regarding type I error. Reference 3 is a study published by Schumacher et al, dealing with a sample size of 7 individuals. In fact, only two samples were taken throughout the competition. This study has no statistical power to support nothing out of that group of 7 individuals. It is out of the scope of this manuscript discussing about sample size, statistical power and scientific evidenced, but everyone is aware about strong limitations that emerge from very small studies. Reference 4 is another study from Dr. Schumacher Group, published as a letter-to-the-editor in Haematologica in 2003. They recruited 23 individuals during a 5-day stage race. Their conclusions support our conclusions regarding samples collected in April 24th 2012 and in May 3rd 2012. However, no conclusion should be made for a 3-week days race. They studied a 5-day stage race. Similarly, reference 5 describes a study during a 6-days race and has nothing to do with a 3-week days race but support our conclusions about samples collected in April 24th 2012 and in May 3rd 2012. Reference 6 is a review of findings published by others and has nothing to do with this topic. Finally, reference 7 is a paper by Fellman et al. They analyzed blood data from 8 individuals during a 7-day competition that involved running, cycling and a variety of sky.

They evidenced a wide range of findings that impaired any conclusion, probably due to sample size limitations.

So, with all due respect, we are unable to understand the role of references included in the Initial CADF Medical Report. In spite of letter-to-the-editor and reviews, original research studies (references 2, 3, 5 and 7) explored 5 to 7- day races with reduced sample size (ranging between 4 and 8 individuals). We have to keep in mind that we are discussing about hemoglobin variation before and after a 3-week race. In addition, Mr Kreuziger was also involved in previous races that finished 5 days before the 2012 Giro began. So, these references could be applied to 5- 7 day races.



Summary

In our unanimous opinion, the likelihood of the described abnormalities being due to blood doping, such as the use of blood transfusions, is very high. In contrast, the likelihood of such deviations being caused by altitude exposure, a medical condition or any other cause is low and we therefore recommend requesting the rider's explanation for his blood values, specifically regarding the patterns observed during the Giro d´Italia 2012 (samples 56-59) and the abnormality of his Reticulocyte pattern from 2011 onwards.

We conclude that considering the available information contained within the passport at this stage and absent a satisfactory explanation from the athlete, it is highly likely that a prohibited substance or a prohibited method has been used and unlikely that the profile is the result of any other cause.

CADF Medical Report stated that:

50 ***Summary Expertise Dr. de Boer***

In his explanations, Dr. de Boer tried to explain the abnormalities highlighted in our previous reports by subclinical hypothyroidism and its treatment with increasing doses of L-thyroxin (50µg -75µg -100µg). Based on the available scientific literature, his arguments must be dismissed, as there is no evidence

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that in non-anaemic, iron-replete subjects, the application of thyroid hormone would have any clinically relevant effect on the blood picture.
It is of note that the explanations of Dr de Boer do not provide any distinct clarification of the abnormal in-competition Haemoglobin pattern during the Giro d'Italia 2012, which is one of the key abnormalities of the profile.

We have demonstrated the application of thyroid hormone in non-anemic, iron replete hypothyroid patients is able to rise % Reticulocytes. Please, remember that a patient that needs 100 mcg of L-Thyroxine to keep his TSH level within limits of normality is not a subclinical hypothyroid patient: it represents an overt hypothyroidism. Finally, FT4 and T4 levels do not reflect grade of severity in a patient with thyroid insufficiency that receives exogenous T4. Grade of severity is determined by TSH levels and many physical and phsycological symptoms and thus, endocrinologists and oncologists modify L-Thyroxine supplementation accordingly.

Summary Expertise Dr. Locatelli

In his explanations, Dr. Locatelli tries to highlight analytical shortcomings in Reticulocyte analysis and normal physiological variation in Hb level during stage racing in line with the cited literature as the reasons to explain the two main abnormalities observed in the profile.
From our analysis, it becomes clear that the Ret analysis of the samples were correct and the regulations were misinterpreted by Dr Locatelli. Upon further scrutiny, the literature cited by Dr Locatelli to defend the abnormal Hb levels of the athlete during the 2012 Giro d´Italia in fact confirms the striking abnormality of the pattern observed towards the end of this race.
Therefore, both explanations can comfortably be dismissed, as they are not supported by the available scientific data.

CADF Medical Experts consider that the effect described by Corsetti et al, and Lombardi et al regarding a tendency of hemoglobin to increase at the end of races, is not observed by ample literature on the topic. However, it is difficult to accept that studies recruiting 4 to 8 individuals should be considered as "ample literature". In addition, references 11 and 14 are letters-to-the-editor that does not provide any experimental data but personal opinions about research studies published by others. By far, reference 13 is the most robust one and recruit 23 individuals. Obviously, a single group (as referred by CADF Medical Experts) has evidenced an "opposite effect" when recruit hundreds of individuals. This "sample-size" effect is widely described in current Science.

Additionally, they consider that it is highly unlikely that an athlete supported by a professional cycling team presents dehydrated in the morning after an extreme effort. We are completely agreed with CADF Medical Experts and this is true if we were discussing about an euthryoid individual. However, Mr Kreuziger is diagnosed with an overt hypothyroidism and his ability to recover after extreme exercise relies on his pill of 100 mcg of L-thyroxine among other factors. We should keep in mind than euthyroid individuals suffer a transient

hypothyroidism after extreme exercise (Chicharro et al 2001) in spite of a thyroid gland that can fully commit its duties during exercise. Mr Kreuziger depends on his dose of L-thyroxine. If his metabolism demands additional mcgs of L-Thyroxine, his thyroid gland will never supply any single molecule of L-Thyroxine. So, his ability to recover after extreme efforts is impaired and delayed until an additional dose is taking.

FINAL CONCLUSIONS

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