A letter from the President of the World Association of Anti-Doping Scientists (WAADS)

"EPO Testing in Anti-doping Laboratories is No Joke"

Our last coverstory on, "another troubling doping case [that] is questioning WADA's credibility" and, "the credibility of the entire anti-doping system", provoked a response from Christiane Ayotte, President of the criticised testing labs organisation, WAADS. Here, we publish Ayotte's letter to *Lab Times*. And on pages 28-29, the *Lab Times* authors respond.

"This article [*Borderline Analysis, Lab Times* 5/2016] is the second one presenting the views of a group of scientists who are not only challenging the interpretation of an athlete's EPO test results, but discrediting 'WADA's credibility, again'.

While it may sound seemingly insignificant to refer to 'WADA's credibility', this one-side vitriolic opus is a charge against skilled, experienced scientists. The SAR-PAGE and IEF data presented are of excellent quality, the results clear and convincing. The methods, the interpretation of test results were published in the peer-reviewed scientific literature (more than 40 research articles from anti-doping scientists) and so were the criteria for issuing positive findings that are available on WADA's website (*www.wada-ama.org*) (1). It is worth noting that the four signatories never submitted any data in support to their position. After all, these techniques are not unique to EPO doping control tests and are common in many molecular biology laboratories. The antibodies and the EPO standards being accessible, nothing prevented the authors to demonstrate their point with simple experiments; they opted instead for voicing unchallenged theoretical objections, in a magazine.

The laboratory in Cologne tested Mr. Colvert's A- and B-samples five times with the two recognized and widely applied complementary techniques; each time the results were consistent with the presence of a recombinant EPO. The criteria for reporting an adverse analytical finding were objectively met and the conclusions reached by the laboratory were supported from the independent review made by the experts of a second laboratory located in Austria (2). The scientists from these two organizations have published on EPO testing, their expertise is recognised.

The tests, as applied for the past 16 years, target the known differences between human (endogenous) and recombinant EPO, the latter being the doping agent. The first method based on their different isoelectric profiles was published by F. Lasne in *Nature* (3), a prestigious scientific journal. Later, the discrimination based on their different apparent weight led to the development of the SDS-PAGE and finally SAR-PAGE approaches (4). Both laboratories involved in Mr. Colvert's case authored these publications.

The IEF test result

The initial test done on a batch of samples, including Mr. Colvert's, was with the SAR-PAGE: the laboratory determined that the profile of sample no. 7397 was suspicious and they decided to proceed with further confirmatory tests on other aliquots of the A-sample (N. B. the identity of the athlete is unknown to the laboratory).

The first confirmation data presented was from the IEF. In order to interpret the results, regions must first be delimited from the position of bands generated by reference standards analysed simultaneously: basic for recombinant, endogenous for human, acidic for NESP, as shown by the example provided in WADA Technical Document reproduced in Figure 1.

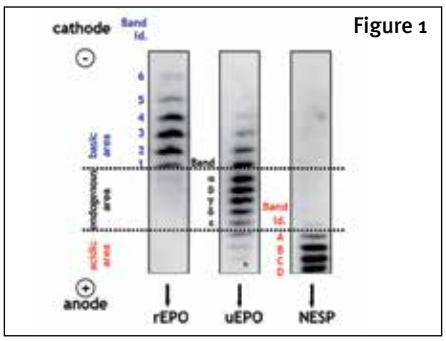

Figure 1

Image extracted from WADA Technical Document, showing the definition of basic, endogenous and acidic area from the analysis of standards (5).

Profiles composed uniquely of the recombinant EPO like in Figure 1 are not the norm. Doping regimes have evolved to "micro-dosing" and "biosimilar" recombinant EPOs have appeared on the market (1, 6). Their profiles of isoforms were shown to vary slightly from epoetin α and β. As a result, athletes' samples often show mixed profiles, as it is the case here e.g. a combination of endogenous and recombinant bands. The criteria for concluding to the presence of a recombinant EPO are currently as follows: i) the 2 most intense bands 'measured' by densitometry must be located in the basic area; ii) the second most intense band in the basic area has to be at least as intense as the most intense one located in the endogenous area 1.

These criteria were definitely met with Mr. Colvert's A-sample IEF test results, as shown by the images extracted from the documentation package (pp. 23 and 27), provided by the Cologne laboratory (Figure 2). The two, actually the three most intense bands (61.8 to 100 intensity) are located in the basic /recombinant area and are more intense than band α, the strong-

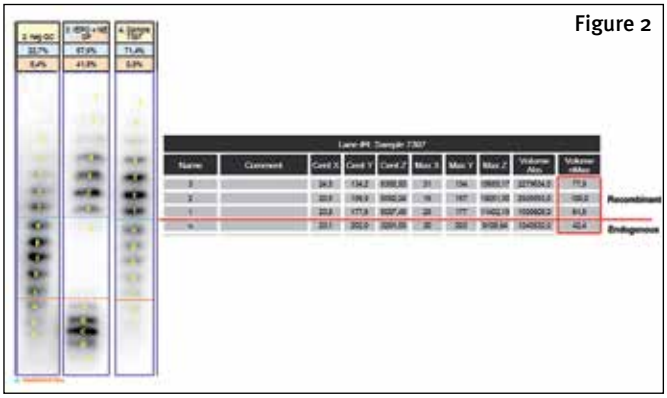

Figure 2

Image of Steven Colvert's A-sample – "no. 7397" IEF test result vs. negative and positive control samples (left) and relative abundances of bands as determined by the densitometric analysis (Gasepo software7) (right) as extracted from the documentation package pp. 23 and 27.

est one of the endogenous region (42.4 intensity).

Such a profile is not consistent with endogenous human urinary EPO and therefore, points to a recombinant EPO. The conclusion that was reached by the laboratory in Cologne was correct.

The SAR-PAGE test results

The second test for the A- and B-sample confirmations was the SAR-PAGE. Under these conditions, recombinant EPO show a 'characteristic band shape e.g. broad band' (1). As shown in WADA Technical Document, combined endogenous / recombinant profiles as Mr. Colvert's, result in a mixed band, 'consisting of endogenous EPO and rEPO' – 'a diffuse or faint area of the band above the corresponding endogenous band is also indicative for the presence of epoetin-α and-β'. As also stipulated in the Technical Document: 'The centroid or the boundaries of the width of the band can be used to ascertain that its position and shape differs from the position of endogenous EPO run in parallel'. The example provided in that regulatory document of a mixed endogenous/recombinant band is reproduced in Figure 3.

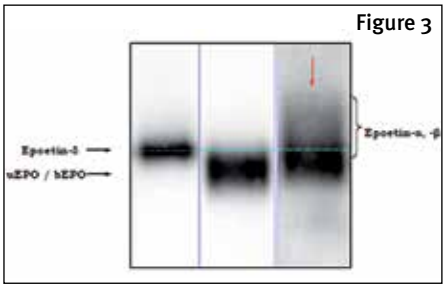

Figure 3

Excerpt of WADA Technical Document, representing the characteristically diffuse mixed endogenous /recombinant band (indicated by red arrow) (1).

Each time Mr. Colvert's samples were analysed, the mixed recombinant and endogenous populations were revealed by the diffuse and faint area above the corresponding signal of endogenous EPO, indicating the presence of recombinant EPO as shown in Figure 4 (sample no. 7397).

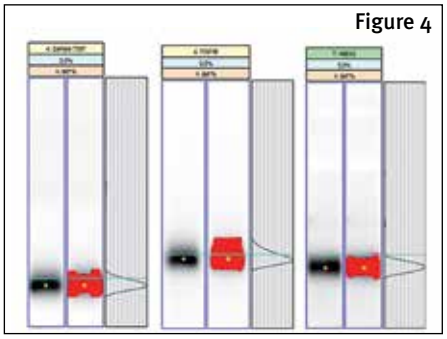

Figure 4

Gasepo densitometric analysis of A- (left) and B- (middle) samples SAR-PAGE confirmation tests (negative human EPO standard from the B-sample test is shown for comparison (right)).

Deducing the endogenous to recombinant composition of urinary EPO from the IEF and SAR-PAGE profiles of the Gasepo analyses is wrong, particularly for the latter. Both recombinant and endogenous bands are mixed, overlapped and not resolved (8). It is not possible to determine from such results the relative abundance of each specie. If the laboratory expert was correctly quoted, he made a mistake when he stated that the amount of recombinant was small when compared to the endogenous EPO.

With no hesitation, I support the conclusions of my colleagues from Cologne and Seibersdorf. These profiles depart significantly from human urinary endogenous EPO. Both the IEF and SAR-PAGE test results are evidence for the presence of a recombinant EPO in Mr. Colvert's A- and B-samples."

Christiane Ayotte
(President World Association of Anti-Doping Scientists & Director of the Laboratoire de contrôle du dopage INRS-Institut Armand-Frappier)

References and remarks

(1) TD2014EPO. 2014, *Harmonization of analysis and reporting of erythropoiesis stimulating agents (ESAs) by electrophoretic techniques.* Available at: *www.wada-ama.org/en/resources/science-medicine/td2014-epo.*

(2) In order to ensure that only positive findings are reported, a mandatory second opinion is sought from scientists listed in WADA Technical Document, following the review of the analytical data.

(3) Lasne F. & J. Ceaurriz, *Recombinant erythropoietin in urine. Nature*. 2000, 405, 635; Lasne F *et al.*, *Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered hormones. Anal Bioch*. 2002, 311:119;

(4) M. Kohler *et al.*, *Discrimination of recombinant and endogenous urinary erythropoietin by calculating relative mobility values from SDS gels. Int. J. Sports Med*. 2007, 29, 1; C. Reichel *et al.*, *SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood. Drug Test. Anal.* 2009, 1, 494.; Reichel C., *Recent developments in doping testing for erythropoietin. Anal Bioanal Chem*. 2011, 401:463.

(5) The borders of the basic and acid areas are defined as shown with standards, endogenous is therefore in between.

(6) Y. Dehnes *et al., Detection of recombinant EPO in blood and urine samples with EPO WGA MAIIA, IEF and SAR-PAGE after microdose injections. Drug Test. Anal*. 2013, 5, 861; Martin L. *et al., New insights for identification of doping with recombinant human erythropoietin microdoses after high hydration, Drug Testing and Analysis*, 2016, DOI 10.1002/ dta.2004.

(7) Bajla I *et al., Quantitative analysis of images in erythropoietin doping control. Med Biol Eng Comput*. 2005 May;43(3):403-9.

(8) The overlap of epoetin α/β and human urinary EPO isoforms is known: for example, Ph. Desharnais *et al., Desialylation improves the detection of recombinant erythropoietins in urine samples analyzed by SDS-PAGE, Drug Testing and Analysis*. 2013. DOI 10.1002/dta.1494.