WADA-accredited doping analyses cannot always be trusted

Weak Evidence

A Norwegian professional speed walker is convicted of doping. Nothing extraordinary there, one might think. However, the evidence presented by the World Anti-doping Agency (WADA) is fraught with errors and manipulations – especially regarding analyses of the athlete's urine using isoelectric focusing and SDS-PAGE.

Photo: wk

In June 2010, the World Anti-Doping Agency (WADA) laboratory in Rome reported the presence of CERA (Continuous Erythropoietin Receptor Activator) in the urine of the race walking athlete, Erik Tysse. The athlete denied any use of CERA and asked for his urine sample to be re-analysed at another WADA-accredited laboratory. The request was rejected. The athlete was charged and found guilty in The Tribunal of the Norwegian Confederation of Sports and later in The Court of Arbitration for Sports (CAS). He was deprived of the right to participate in competitions and organised training for two years.

Erik Tysse, doping offender or victim of a doping laboratory?

However, a number of scientists not affiliated with WADA later re-examined the data that formed the basis for the conviction and concluded that the results do not prove the presence of CERA in the athlete's urine. A declaration, stating this conclusion, was signed by more than 40 professors in the fields of analytical chemistry, biochemistry, molecular biology and physiology, including a Nobel Prize laureate.

In this article we present the relevant data and a discussion about what they show and which conclusions can be drawn.

Inconclusive results

WADA's purpose – to prevent the use of performance-enhancing drugs by athletes – is praiseworthy and has certainly reduced the incidence of drug abuse in sports. It is essential that WADA's tests for drug abuse are rigorous and completely trustworthy.

However, the situation presented below may signal that this is not always the case. The primary data presented by WADA are of poor quality and have been treated and interpreted in a deviant and superficial manner. If the procedures used in this case are common for WADA-accredited laboratories, athletes have good reasons to fear that they may be unjustifiably accused of and sentenced for drug abuse. We shall specify the reasons for our strong allegations below. But first a few words about erythropoietin (EPO) and CERA and the methods that WADA use for detecting these proteins.

EPO and its cousin CERA

EPO is a naturally produced protein hormone that stimulates the production of red blood cells, while CERA is a synthetic variant of EPO and is misused in sports as a performance-enhancing drug. EPO and CERA have the same amino acid sequence, but are modified differently. EPO is glycosylated whereas CERA is also pegylated, and these modifications generate isoforms of EPO and CERA that differ in charge (see Figure 1, page 19).

In the test that WADA has developed for detecting CERA, the proteins in urine or blood are concentrated by ultrafiltration and/or by the use of immunoaffinity columns and analysed by isoelectric focusing (IEF). CERA- and EPO-isoforms are visualised by a double-immunoblotting technique using a monoclonal antibody that recognizes the protein moiety of EPO and CERA (Lasne F *et al.* [2009], *Haematologica* 94: 888-890; Figure 1).

WADA's document sets the criteria

When evaluating whether or not the immunoblot images reveal drug abuse WADA's Technical Document TD2009EPO clearly sets the limits and criteria. The acceptance criteria include, *"Comparison to the reference samples shall allow assignment of corresponding migrating bands in the athlete's sample"* and that the presence of, *"spots, smears, areas of excessive background or absent signal in a lane that significantly interfere with the application of the identification criteria shall invalidate the lane"*.

Furthermore, in the lane for the athlete's sample, *"…there must be at least 4 consecutive bands corresponding with CERA reference substance"*.

We shall demonstrate below that WADA is not adhering to its own Technical Document.

Unambiguous detection of EPO-derived performance-enhancing drugs (such as CERA) is challenging due to the presence of various isoforms of endogenous EPO in blood and urine. Cleavage of almost any of the glycosidic bonds in the carbohydrate chains attached to the protein moiety of EPO will remove one or more negatively charged sialic acid units that decorate the ends of most of the carbohydrate chains. This will, in turn, cause EPO to appear in more basic regions of the IEF gel where EPO-derived performance-enhancing drugs also may appear.

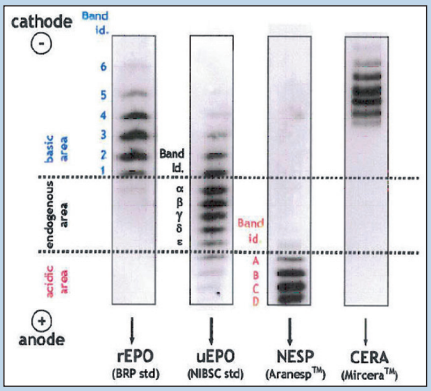
Disappearing bands

WADA's Technical Document specifies that in cases where the IEF results are ambiguous and additional scientific evidence is needed to arrive at a final conclusion, one may also analyse the sample by the use of SDS polyacrylamide gel electrophoresis (SDS-PAGE), combined with double-immunoblotting or equivalent methods, where protein separation is based upon a technique complementary to IEF.

In the first IEF analysis of the athlete's urine A-sample, the protein bands were weak and most of them corresponded to endogenous EPO, although some less intense bands could be discerned in the CERA region of the gel (Figure 2A, page 20).

Still unexplained, upon WADA's re-analysis of the same sample, is the fact that the intensity of the bands corresponding to endogenous EPO was reduced and there was a marked accumulation of staining in the CERA region of the gel (Figure 2B, page 20).

Fig. 1: Double-immunoblotting (standard pattern)



Double-immunoblot images obtained after isoelectric focusing (IEF), from the WADA Technical Document TD2009EPO. CERA and endogenous EPO as found naturally in urine (uEPO) together with two other commercial products of EPO (rEPO and NESP). Note that the EPO-isoforms are generally more anionic than the CERA isoforms.

In the third IEF analysis (the so-called confirmation test), the bands corresponding to endogenous EPO all but disappeared, while the staining in the CERA region remained (Figure 2C, page 20). It seems as if the endogenous EPO in the athlete's ▸▸

▸▸ A-sample became less acidic and more readily detectable by the immunoblotting procedure, all of which could be caused by cleavage in one or more of the carbohydrate chains attached to EPO's protein moiety.

In the appeal case in CAS it was suggested by WADA that this lack of reproducibility and the peculiar relocation of bands from the EPO to the CERA region of the gel might, *"…be a consequence of the use of applicator strips which occasionally leak and it cannot be excluded that lane 3* [should be lane 2, which is the athlete's sample lane] *was contaminated with retentates from the neighboring lane. […]*

In any case, due to quality issues, the data from the first screening was not accepted by the laboratory and the IEF analysis was repeated from the same retentate. Consequently, the data from this initial screening was nullified and the data from the second screening was considered as the valid data for assessment purposes" (quoted from *Expert Opinion* by Günter Gmeiner, Laboratory Director, WADA-accredited Doping Control Laboratory, Seibersdorf, Austria).

Poor quality using standard procedure

WADA thus simply ignores the troubling results obtained in the first IEF analysis, and justifies this by alluding to poor quality and the possibility that the athlete's sample may have been contaminated when analysed using WADA's standard test procedure. It is obvious that there are quality problems but it is not clear why the merging of lanes and the smudged CERA control lane, all on the right side of the gel (lanes indicated by short closed and long open arrows, Figure 2A, see above), justifies nullifying the results obtained in the athlete's sample lane on the left side of the gel (lane indicated by star in Figure 2A).

The latter lane seems to be of acceptable quality and clearly shows that all the strongest bands in the athlete's sample are due to endogenous EPO.

Contamination not excluded

Furthermore, by issuing this statement WADA acknowledges that contamination of urine might occur, which is worrisome in itself and questions the quality control of their analyses.

It should be noted that the level of endogenous EPO is significantly reduced and almost absent in the third IEF analysis, whereas it was clearly detected in the second IEF analysis (compare lanes indicated by a star in Figure 2B and C). The lack of distinct endogenous EPO bands combined with heavy staining in the CERA region in the third IEF analysis (lane indicated by star in Figure 2C) is noteworthy, because the pore size of the renal filtration system should result in EPO (apparent molecular weight of about 30,000) being excreted to urine much more efficiently than CERA (apparent molecular weight 60,000-100,000). To our knowledge, all published IEF-analyses of urine samples that reveal distinct and well-documented CERA bands also reveal clear and distinct endogenous EPO bands.

Criteria not fulfilled

The IEF results presented by the Rome laboratory as evidence for misuse of CERA are overall of

Figure 2: Double-immunoblotting (obtained by WADA laboratory)

(A) Initial IEF test of the athlete's urine A-sample (the athlete's urine sample is divided into two vials, named the A- and B-sample); (B) repetition of the IEF test of the A-sample; (C) confirmation IEF test of the A-sample; (D) confirmation IEF test of the athlete's urine B-sample; and (E) repetition of the confirmation IEF test of the B-sample.

Black stars: the athlete's urine sample; long, white arrows: urinary EPO standard; short, white arrows: negative urine sample; long, black arrows: CERA standard in urine; short, black arrows: CERA standard.

(The figures are from the official reports from the WADA laboratory.)

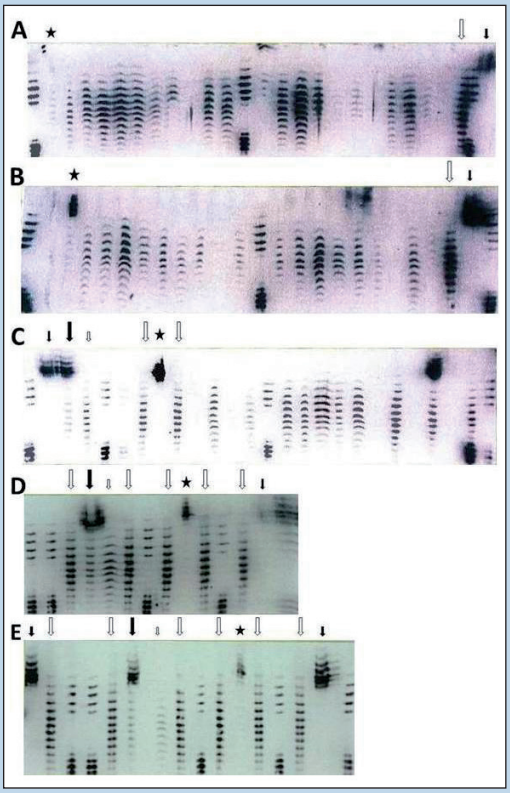


Figure 3: IEF results

A manipulated version of the IEF results presented to the CAS.
Lane 1: CERA standard in urine (long, black arrow, Figure 2C); Lane 2: the athlete's urine A-sample (star, Figure 2B); Lane 3: the athlete's urine A-sample (black star, Figure 2C); Lane 4: the athlete's urine A-sample (star, Figure 2E); Lane 5: the CERA standard in urine (long, black arrow, Figure 2E). To achieve the alignment of the athlete's bands with the CERA reference bands, the bands in the athlete's lanes were moved upwards and the scale stretched out (lane 3) or downwards and contracted (lane 4) relative to the CERA reference lanes present on the same gel.
(The figures are from* Expert Opinion *by Günter Gmeiner, Laboratory Director, WADA-accredited Doping Control Laboratory in Seibersdorf, Austria.)

very poor quality (Figure 2), nowhere near the quality required by WADA's Technical Document TD2009EPO (Figure 1, page 19) nor of a quality similar to the results presented by other laboratories (Dehnes Y. and Hemmersbach P. [2011], *Drug Test Anal* 3: 291-299).

It is indeed difficult to identify protein bands in the athlete's sample that exactly correspond with protein bands in the CERA reference.

Therefore, the acceptance and identification criteria in WADA's Technical Document are not fulfilled. When confronted with this problem in the CAS, WADA claimed of their own criterion that,

"…there must be at least 4 consecutive bands corresponding with CERA reference substance" (WADA's Technical Document TD2009EP) actually means that,

"… the 4 consecutive bands must be in the same general area as the CERA reference substance" (quoted from *Expert Opinion*).

This novel and *ad hoc* rule clearly reduces the reliability of the IEF test for detection of CERA. The term "the same general area" does in fact not appear anywhere in WADA's Technical Document TD2009EPO. The term is, of course, much too vague to be used as a specification in the Technical Document, as it does not give an accurate description as to how WADA-accredited laboratories should interpret their test results.

Does the only WADA-accredited laboratory for doping control analyses, the *Laboratorio Antidoping FMSI*, located in Rome, have serious quality problems?

To support their interpretation of the IEF results, WADA also presented to the CAS an altered version of these results. In this manipulated version (shown in Figure 3, page 20 below), the protein bands in the athlete's lanes, as originally shown in Figures 2B, 2C and 2E, were aligned with the CERA reference bands from Figure 2C and 2E. In order to achieve this alignment, the bands in the athlete's lane in the original gel (Figure 2C) were moved upwards and the scale expanded about 40% relative to the CERA reference lane (indicated by long black arrow) on the same gel.

Scale expanded about 40 percent

Moreover, the CERA reference lane (indicated by long black arrow) on the gel shown in Figure 2E was moved upwards and expanded about 20% relative to the athlete's lane (indicated by star) on the same gel. The athlete's lane in Figure 2B (indicated by star) was similarly adjusted so that the bands in the lane aligned with bands in the CERA reference lanes on the gels shown in Figure 2C and 2E.

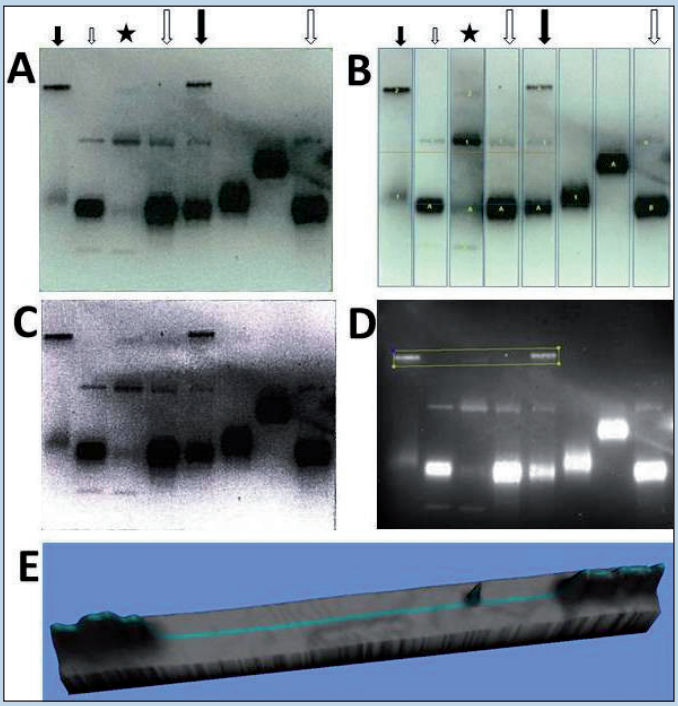
The use of such a cut-and-paste method is a deviant and unreliable way ▸▸

Figure 4: SDS-PAGE analysis

(A) The double-immunoblot image obtained after SDS-PAGE analysis of the athlete's urine B-sample;
(B) the same image as in A but after the laboratory in Rome had processed the data for subsequent quantification of the bands;
(C) the same image as in A, but after photo-shop intensification performed by us [the authors];
(D) the same image as in A (now white bands on black background);
(E) three-dimensional expanded image of the marked area in (D).

Black stars: the athlete's urine sample; **long, white arrows:** urinary EPO standard; **short, white arrows:** a negative urine sample with no EPO added; **long, black arrows:** CERA standard in urine; **short, black arrows:** CERA standard.

(Figures 4A and 4B are from the official reports from the doping laboratory. Figures 4D and 4E are taken from Expert Opinion *by Günter Gmeiner, Laboratory Director, WADA-accredited Doping Control Laboratory in Seibersdorf, Austria.)*



▸▸ of treating and presenting data. Only those not acquainted with the relevant techniques, such as the arbitrators in the CAS, are likely to be deceived by the presentation shown in Figure 3 (page 20 below).

The athlete's urine sample was also analysed by SDS-PAGE, since WADA's Technical Document allows the use of this or equivalent methods in cases where the results obtained by the IEF method are not conclusive.

SDS-PAGE: Unwanted bands ignored

The results obtained (Figure 4A, this page above) were indeed very different from what one would have expected if the intense staining obtained in the CERA region of the IEF gel was in fact due to CERA. None of the protein bands in the athlete's lane in the SDS-PAGE analysis (indicated by star in Figure 4A) corresponded to the CERA reference band (Figure 4A). On the contrary, the band pattern in the athlete's lane appeared to be nearly identical to the band pattern in the negative control lane (i.e. urinary EPO standard indicated by the long white arrows).

The laboratory in Rome nevertheless concluded that the SDS-PAGE test revealed the presence of CERA in the athlete's urine sample by pointing to a very weak band that migrated faster than the CERA reference band. Not only did the laboratory disregard the difference in migration, they also seemed to ignore a similar weak band in the negative control (i.e. EPO in urine; Figure 4A).

Surprisingly, the Rome laboratory suppressed this band before further analysis by reducing the staining intensity of the negative control lanes (indicated by long and short white arrows, Figure 4B) and simultaneously increasing the staining intensity of the athlete's lane (indicated by a star,

Photo: Jan Petter Svendal/www.Nytt0s.com

The evidence presented by WADA accredited Rome laboratory was far from proving any guilt. Nevertheless, Erik Tysse was banned from competition for two years.

Figure 4B). For comparison, see the Photoshop intensification picture performed by us (Figure 4C).

Curious argumentation

When questioned about the unusual interpretation as well as the processing of the SDS-PAGE results, WADA representatives claimed that, *"Whilst the SDS-PAGE result could be clearer with respect to the presence of CERA in the sample, it does not in my opinion exclude the presence of CERA either"* (quoted from *Expert Opinion* by Günter Gmeiner). We believe that the analysis should, if possible, prove the presence of CERA. To exclude its presence is a daunting task and is not asked for.

Moreover, WADA also presented a three-dimensional and expanded image of the CERA region in the SDS-PAGE gel (Fig. 4E; expansion of the marked area in Fig. 4D). With reference to this image, in which the clarity of the relevant bands is – if anything – reduced, they claimed that there is no sign in the negative control lane of a weak band that corresponds to the alleged CERA band in the athlete's lane.

The evidence for whether or not the band is absent would clearly have been more convincing if WADA had presented the SDS-PAGE immunoblot image with an appropriate staining intensity for the negative control lane in Fig. 2B. It is not clear why this obvious option was not chosen.

The results obtained by SDS-PAGE analysis of the athlete's urine sample are clearly very different from what is expected if the intense staining obtained in the CERA region of the IEF gel was due to CERA. Therefore, the SDS-PAGE analysis presents no evidence for CERA in the athlete's urine and the athlete should therefore be considered innocent.

No evidence in the athlete's urine

Furthermore, WADA's interpretation of the IEF results is incorrect. The CAS nevertheless found the SDS-PAGE results irrelevant and relied entirely on WADA's opinion that, *"The SDS-PAGE analysis is not required to make a positive finding regarding the presence of CERA in a sample. The SDS-PAGE results are irrelevant and cannot contradict the clear evidence of an adverse analytical finding under the IEF method"* and, *"In any event, Prof. Botrè., Dr. Gmeiner, and Dr. Lasne* [Directors of the WADA-accredited laboratories in, respectively, Rome, Seibersdorf and Paris] *maintain that the SDS-PAGE results are positive for the finding of CERA"* (quoted from the Award delivered by the CAS).

It's remarkable that the CAS had complete faith in and relied entirely on WADA's interpretation of their own results, considering that WADA is directly implicated in the case and has a conflict of interest in its outcome. This decision clearly demonstrates that athletes accused of doping are not always guaranteed a rule of law.

A case such as this can never prove innocence, only guilt. Therefore, we do not know whether the athlete has taken any illegal drugs. But it is pretty clear that the evidence presented is far from proving any guilt. It is troubling that inconsistent data of poor quality followed by superficial and unscientific interpretations are considered as sufficient proof. The present case is an example of misuse of scientific methods and a lack of appropriate interpretations.

An example of misuse

WADA's behaviour in this case jeopardises their credibility. They must adhere to good scientific practice, as this is crucial for their efforts to prevent the misuse of performance-enhancing drugs and for gaining the respect and trust of athletes and the general public.

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▶ Lasne F., Martin L., Martin J.A., de Ceaurriz J. (2009), Detection of continuous erythropoietin receptor activator in blood and urine in anti-doping control. *Haematologica* 94: 888-890.