Photo: private

When the Rome-based, WADA-accredited anti-doping laboratory tested the race walker Erik Tysse (photo) for misuse of rEPO, good repeatability was definitely not achieved

Problems at a WADA-accredited anti-doping lab

Puzzling Discrepancy

Doping tests are very important to ensure fair competition but the test results should, of course, be correct and above doubt. Ironically, an accredited WADA laboratory seems to have major problems with the latter.

A noteworthy article by Garribba and co-authors, all at the World Anti-Doping Agency (WADA)-accredited laboratory in Rome, appeared in the June 2014 issue of *Bioanalysis* (6(12), 1605–15).

This article, entitled, *A modified procedure based on a vacuum-driven blotting system for the detection of erythropoietin and its analogs*, is not only noteworthy due to errors and an inaccurate presentation of results but also because the presentation is made by a professional and WADA-accredited analytical laboratory. It is disturbing that sloppiness and errors can dramatically influence the life and career of athletes, when tested for performance-enhancing drugs by this laboratory. We have earlier demonstrated (Nissen-Meyer *et al*., *Weak Evidence*. *Lab Times* 1/2013, pages 18–23) and will give further evidence here that these concerns must be addressed.

Detecting erythropoietin in urine

In their study, Garribba and co-authors examined the specificity and repeatability of WADA-approved methods – and slightly modified versions of these methods – for detecting recombinant erythropoietins (rEPOs) in urine. The amounts of rEPO in rEPO-spiked urine samples were measured and mean values, standard deviations (SD), and coefficient of variation (CV%) were reported. The authors concluded that the methods for detecting rEPOs

Information box: Same urine sample but different results

The adjacent Figure 1 shows three analyses for the presence of rEPOs in Erik Tysse's urine sample. The results are from the laboratory's documentation that was presented at the Court of Arbitration for Sports (see source below). The athlete's A-sample was tested three times using isoelectric focusing (IEF) and double-immuno-blotting.

We [the *Lab Times* authors] have cut out the athlete's lanes from the three IEF gels and placed the lanes side-by-side in order to more easily compare the results. The same urine sample was used in these three analyses, and the same concentrated urine sample was used in the first and second test (i.e. the samples applied to the gel were taken from the very same test tube).

Source: Laboratorio Antidoping–FMSI, Documentation Package Sample A code 3511158 (*www.eriktysse.com/Documents/The%20report%20for%20the%20A-sample.pdf*)

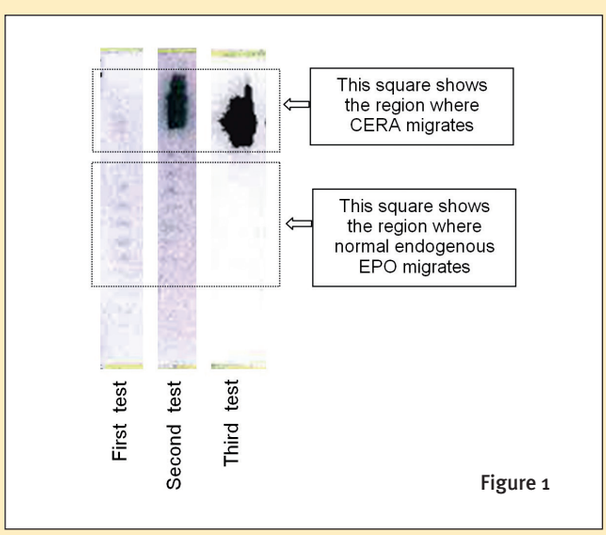


Figure 1

have good repeatability, specificity and accuracy and are consequently reliable.

Ironically, that conclusion is undermined by inconsistencies and mistakes in many of the numerical results presented to support the conclusion; there are, in fact, obvious and serious mistakes in five of the eight datasets shown for rEPO in the article's main table (their Table 1).

For instance, a mean value for the amount of rEPO is presented as 14,179,217 with an SD of 71,58,031 and a CV of 9%. Aside from the use of too many significant digits and the unconventional and confusing use of commas in the SD, at least one of these numbers must be wrong; 7.1 million is 50% of 14.1 million, not 9%. In this case the SD value is wrong.

An SD with exactly the same numerical value also appears in a completely different dataset in Table 2; the SD has most likely been "copied and pasted" from Table 2 to Table 1. The exact same mistake is encountered in the next dataset in Table 1, where the SD value (presented as 18,40,893) is wrong. This SD has also been erroneously copied and transferred from Table 2.

For another dataset in Table 1, it is the mean value for the amount of rEPO (presented as 198,886,367 with an SD of 3,783,711) that is wrong; it is 10 times too large and should – judging from the electrophoresis data – be about 20 million.

Serious mistakes in five out of eight datasets…

One might think that this is not so serious. After one of us (JNM) made the editor aware of the mistakes, a correction appeared in May 2015 (issue 9) of *Bioanalysis* and corrections have now also been made in the electronic version of the relevant Garribba *et al.* article. It should be noted that there is no information in the new version that changes have been made; as the original version has been removed from the website, the numbers we refer to above can only be found in copies taken before the corrections were performed.

But why are we worried about these mistakes? Are we being too picky, considering that the mistakes and inconsistencies are so obvious that a reader can, at a glance, identify and correct them?

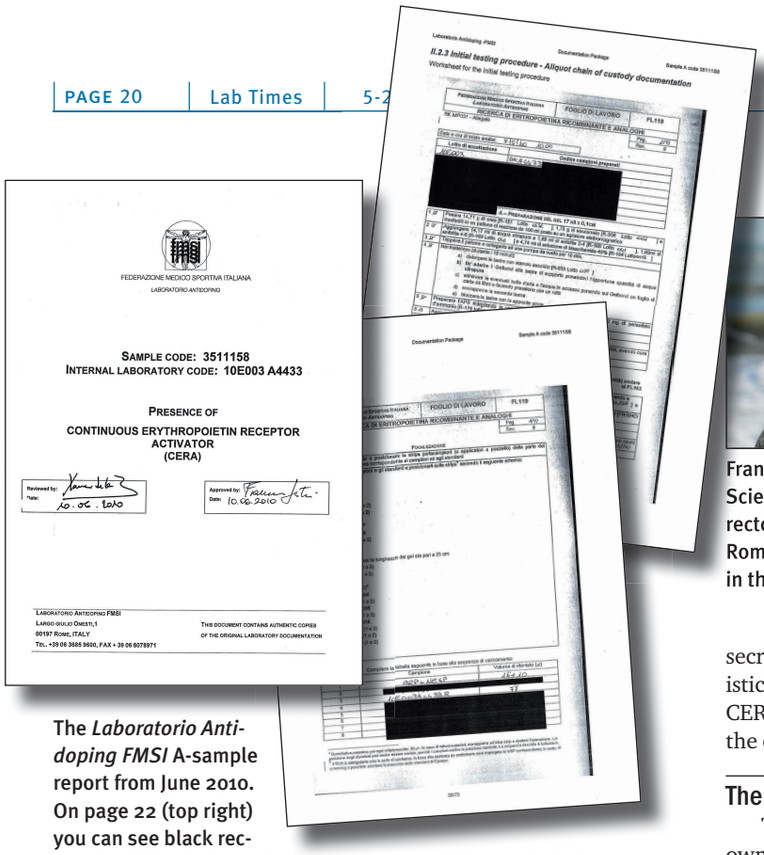
No. We rather find it is worrisome that these obvious inconsistencies and mistakes were not identified by any of the six authors, all working at a professional and WADA-accredited analytical laboratory that each year tests thousands of athletes for doping.

… not identified by any of the six authors

The good repeatability reported in the article was definitely not achieved when this Rome-based, WADA-accredited laboratory tested the race walker Erik Tysse for misuse of rEPO (see the adjacent information box and *Lab Times* 1/2013, pages 18-23, for details about the Tysse case).

This information box (see opposite page 18) shows the results of three repeated tests of Tysse's A-sample urine. Four of the six authors of the *Bioanalysis* article were involved in this test and they reported it as positive for CERA (a form of rEPO). The first test (first lane in Figure 1) is the one that most clearly reveals the athlete's normal endogenous EPO, whereas the bands in the CERA region are not clear (see *Lab Times* 1/2013, page 18ff, regarding interpretation of bands in the CERA region).

The second test (second lane in Figure 1) reveals a large and unexplainable amount of material in the CERA region. Remarkably, the samples used in the first and second tests were from the very same test tube and the experiments were car- ▶▶



FEDERAZIONE MEDICO SPORTIVA ITALIANA
LABORATORIO ANTIDOPING

SAMPLE CODE: 3511158
INTERNAL LABORATORY CODE: 10E003 A4433

PRESENCE OF
CONTINUOUS ERYTHROPOIETIN RECEPTOR ACTIVATOR (CERA)

Reviewed by: Date: 10.06.2010
Approved by: Date: 10.06.2010

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THIS DOCUMENT CONTAINS AUTHENTIC COPIES OF THE ORIGINAL LABORATORY DOCUMENTATION

II.2.3 Initial testing procedure - Aliquot chain of custody documentation
Worksheet for the initial testing procedure

FOGLIO DI LAVORO
RICERCA DI ERITROPOIETINA RICOMBINANTE E ANALOGHE

The *Laboratorio Antidoping FMSI* A-sample report from June 2010. On page 22 (top right) you can see black rectangles hiding information about sample numbers; similarly on page 38 (bottom right) for the retesting of samples. We [the authors] believe that the 'R' after the sample number for Tysse (A4433) on this page means 'retesting'. Why are the other sample numbers hidden? No need for secrecy if everything is OK because you need the code to deduce from whom the urine samples were obtained.

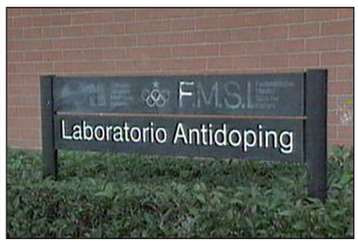

Photo: WAADS

Francesco Botrè was president of the World Association of Anti-Doping Scientists (WAADS) from 2006 to 2008 and is the acting Scientific Director of the WADA-accredited *Laboratorio Antidoping FMSI*, located in Rome, Italy. It is his laboratory that performed the analyses described in the article.

ried out in the same manner. The two results should, therefore, have been identical. In the third test of the same urine sample (third lane in Figure 1, page 18), hardly any of the athlete's normal endogenous EPO was detected but the relative amount in the CERA region had increased even further, resulting in an extraordinary 400-fold increase in the relative amount of CERA from the first to the third test. Considering how poorly CERA is secreted to urine, the results of the third test indicate an unrealistic amount of CERA in the urine; they imply a concentration of CERA in Tysse's blood that is almost 10,000 times greater than the concentration of his natural EPO.

The discrepancy is simply too large

The results shown in Figure 1 (page 18) do not fulfill WADA's own stability criteria, which say that the distributions of the most intense bands appearing in the initial testing procedure (i.e. the first and second test in Figure 1) and confirmation testing procedure (i.e. the third test in Figure 1) should be similar [WADA Technical Document – TD2009EPO]. It is indeed difficult to imagine that one and the same urine sample should give the results shown in Figure 1; the discrepancy between the tests is simply too large.

No one has been able to explain this discrepancy. At the Court of Arbitration for Sport (CAS), representatives from WADA-accredited laboratories stated that this lack of reproducibility might have been due to a contamination in the first test and that the discrepancy, regardless of cause, does not really matter:

"… it cannot be excluded that lane 3 [the athlete's sample lane] was contaminated with retentates from the neighbouring

Background: The doping cases against Erik Tysse and Alex Schwazer

Re-Analysis by Another Laboratory Rejected

The Norwegian athlete Erik Tysse participated in a race-walking competition in Sesto San Giovanni, Italy, in May 2010. Tysse came in second, after the Italian race-walker Alex Schwazer. Schwazer is well-known for winning the Olympic gold medal in race-walking in 2008 and for testing positive for rEPO before the Olympic Games in 2012. Subsequent investigations by Italian authorities indicate that he also used performance-enhancing agents before 2012 and that officials for athletic organisations might have been aware of this (see article in *The New York Times*: *http://mobile.nytimes.com/2013/06/19/sports/olympics/officials-accused-of-covering-up-italian-olympians-doping.html*).

Schwazer also used the Italian "doping-doctor" Michelle Ferrari as a "training-consultant" (as did cycling professional, Lance Armstrong) and, in fact, contacted Ferrari immediately after the competition in Sesto San Giovanni in 2010.

Urine samples from six athletes – among these were Tysse and Schwazer – who participated in the competition in Sesto San Giovanni were sent to WADA's laboratory in Rome. The laboratory reported the presence of CERA in Tysse's urine; he denied use of CERA and asked for his urine to be re-analysed at another WADA laboratory but the request was rejected.

Screening tests indicated that the urine of another athlete also contained rEPO, however, this was not reported by the laboratory as an adverse finding because the signal in the following confirmation test, which was run on the same gel as Tysse's confirmation test, was reported to be not good enough. Judging from the band-tailing and streaking that was obtained in this test, it appears that the sample was either destroyed after the initial screening test (which was positive) or that it was inadequately applied to the gel.

lane... In any case, due to quality issues, the data from the first screening [i.e. the first test shown in Figure 1] was not accepted by the laboratory and the IEF analysis was repeated from the same retentate. Consequently, the data from this initial screening [i.e. the first test shown in Figure 1] was nullified and the data from the second screening [i.e. the second test shown in Figure 1] was considered as the valid data for assessment purposes."

[a quote from Expert Opinion given by Günter Gmeiner, Laboratory Director, WADA-accredited Doping Control Laboratory, Seibersdorf, Austria].

Same urine, different results

It is not at all clear to us how contamination of the first test is compatible with the results shown in Figure 1. If contamination did in fact cause the discrepancy, it must instead have been the second and third tests that were contaminated.

Also, it is not clear to us why the first test should be "nullified". Of the three test results shown in Figure 1, it is the first lane in the figure that seems to be most reliable, since Tysse's natural EPO (which everyone produces and is thus present to a lesser or greater extent in one's blood and urine) is most clearly detected in this lane (Figure 1).

The above quote from Expert Opinion implies that the second test was initiated *after* the results of the first test were known. Francesco Botré (Director of the WADA-accredited laboratory in Rome and corresponding author of the article in *Bioanalysis*) also stated (both orally at CAS and in a written witness statement) that the second test was initiated because of the results they obtained in the first test. However, the laboratory's Documentation Package reveals that the second test was started *before* the results of the first test were known.

The laboratory was, thus, either sloppy when writing their laboratory report, or Botré's statement is incorrect and it needs to be clarified.

Only two credible explanations

In view of the apparent good repeatability that is reported in the *Bioanalysis* article, we see only two credible explanations as to the complete lack of reproducibility in the results obtained in the three tests shown in Figure 1.

Either…

1. the IEF analysis method and/or the work performed in the laboratory was so unreliable – possibly due to unspecific staining or degradation of proteins in the sample – that the entire test must be discarded; it is then likely that the staining seen in the CERA region is not due to CERA,

or…

2. someone in the laboratory has – on purpose or by accident – altered or manipulated some of the concentrated urine samples, in which case the laboratory in Rome may be accountable for serious misconduct. ▶▶

Photo: private

Erik Tysse is interviewed during a break at the Court of Arbitration for Sport (CAS) trial in Lausanne by the two main Norwegian TV channels. In this trial, representatives from WADA-accredited laboratories stated that the lack of reproducibility, regardless of cause, does not really matter. A declaration, signed by more than 40 scientists, offers the opposite opinion.

The documentation package that covers the Tysse case reveals several other irregularities that indicate bad laboratory procedures and may, in the worst case, suggest serious misconduct. Some of this has already been discussed in a cover article in *Lab Times* 1/2013, so in the following we will present just two additional examples.

Two additional examples of irregularity: the first...

The first example: In the laboratory's Documentation Package, one of the original gel images was inverted (i.e. presented upside-down and right-side to left). As a consequence, the order of the sample lanes in the original image is inverted relative to the lanes in the computer-processed version of the image. A more serious consequence of the inversion of the original image is that it made it more difficult to discover that the laboratory had, in the computer-processed version used for evaluating the results, suppressed the staining intensity of the negative control lanes and simultaneously increased the staining intensity of the lane that contained the athlete's sample (Source: Laboratorio Antidoping-FMSI, Documentation Package Sample B code 3511158; *www.eriktysse.com/Dockuments/The%20report%20for%20the%20B-sample.pdf*).

The Rome laboratory thereby camouflaged the fact that the negative control lanes contained the same bands as the athlete's lane (i.e. the negative control sample which did not contain CERA was virtually identical to the athlete's sample) and that this analysis did not present any evidence for the presence of CERA.

In a letter (dated 29th June 2011) to all participants at the CAS hearing, Werner Franke, a highly recognised biochemist from Heidelberg University, Germany, who played a major role in revealing the doping culture in the former GDR, characterised one such processed version that was presented at CAS as a, "falsification of a document used in a court case". Franke also wrote,

"In conclusion, there is no scientific evidence contained in these documents (i.e. the Documentation Package) which proves the presence of CERA in the athlete's (i.e. Erik Tysse's) urine." A declaration stating the same has been signed by more than 40 professors and scientists in chemistry, biochemistry and molecular biology, including one Nobel Prize winner.

...and the second example

Urine samples should, according to WADA's regulations, be handled in such a way that the likelihood for contamination and degradation is minimised. It is thus remarkable and bad laboratory practice that the seal on Tysse's urine sample, which the laboratory received Sunday, 2nd May 2010, was broken and aliquoted for EPO-testing on Monday, 3rd May 2010, four days before the test actually started (on Friday, 7th May 2010).

Furthermore, the test was started on a Friday, thus resulting in the concentrated urine sample being left over the weekend – two extra days – before the test was completed on the following Tuesday (the first lane in Figure 1, page 18, is from this test).

Moreover, after having obtained the results from the second screening test (the second lane in Figure 1 is from this test), the laboratory waited for one week before the confirmation test was started (the third lane in Figure 1 is from this test). And again, Tysse's urine was aliquoted for the EPO-test (on Monday, 17th May 2010) three days before the test started (on Thursday, 20th May 2010).

Just one month earlier (in April 2010), when reporting that the Rome laboratory found the B-sample from the Italian cyclocross rider Vania Rossi negative for CERA, despite a positive A-sample, Botré excused their negative B-test by saying that CERA is unstable and degraded in urine, more so than in blood. The delayed testing after seal breakage and sample aliquoting of Tysse's urine sample is therefore puzzling.

Puzzling delays

Because of these delays, more than three weeks elapsed from the time the laboratory received Tysse's urine sample (Sunday, 2nd May 2010) until their report was mailed (Wednesday, 26th May 2010) to the International Association of Athletics Federations (IAAF).

In contrast, when Alex Schwazer was tested positive for rEPO in 2012 (see the "background box" on page 20 for details about the Schwazer case) it took only seven days from the time his urine sample was collected and analysed in the WADA-laboratory in Cologne to reports of the positive test appearing in the press. WADA's regulations state that there should not be more than ten working days from when the sample is received to a positive finding being reported, such that a blood sample can be taken in order to confirm the results obtained with urine. Blood is, especially when testing for CERA, much more sensitive and reliable. The long delay meant that the following test of Tysse's blood, which was negative for CERA, could not be used as reliable evidence to prove that he was innocent. This seriously reduced Tysse's chances of clearing himself of the doping charges.

No will to deliver relevant information

We have contacted the laboratory in Rome as well as WADA, IAAF, the Norwegian Athletics Association and Anti-Doping Norway and its board of directors, in order to get access to some spe-

cific information that we think might clarify whether or not Erik Tysse used CERA and reveal the reasons for the inconsistent laboratory results. None of the above-mentioned organisations have, however, been willing to reveal any further relevant information. Thus, we have not received information about the amount of CERA standard protein applied to the gels, which would help to estimate the amount of CERA that, according to WADA, is detected on the gels.

Moreover, we have not been allowed to see information that is hidden by black rectangles at several places in the report for Tysse's A-sample (see images on page 20); we do not see any reason to hide this information if the analytical work has been performed as it should have been. We also wanted to know why 20 ml of Tysse's A-sample urine is missing. We can only find three possible explanations:

- the laboratory is sloppy when writing their reports,
- they have spilled some of the athlete's urine (exactly what is required for one analysis), or
- they have carried out an additional and unreported EPO test – the delayed testing mentioned above might indicate this.

It is unacceptable that the information we are seeking is not freely available, since it is required to establish clearly how the analyses were performed and should therefore be part of the laboratory report.

A ban on information, but why?

Finally, the recent investigations by Italian authorities that officials of athletic organisations were aware of Alex Schwazer's doping (see background information box on page 20), combined with the earlier report by Alessandro Donati describing involvement of officials for sport organisations and the previous anti-doping laboratory in Rome to hiding or assisting doping, makes it even more important that all information regarding the analysis of Tysse's sample is made available (Donati, A., Anti-doping; the fraud behind the stage. *www.playthegame.org/news/news-articles/2000/anti-doping-the-fraud-behind-the-stage*).

If not, one might suspect that the irregularities we have described in this case are the result of a cover-up action, taken to protect Schwazer, who delivered a urine sample for doping analysis in the same race as Tysse.

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Erik Boye (Oslo University Hospital & Univ. of Oslo),
Bjarne Østerud (University of Tromsø),
Tore Skotland (Oslo University Hospital & Univ. of Oslo)

Lab Times *readers who have information to contribute, questions or any other comment to make on this article should contact the corresponding author, Tore Skotland:* tore.skotland@rr-research.no