Review

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Bioanalysis

Analytical progresses of the World Anti-Doping Agency Olympic laboratories: a 2016 update from London to Rio

The 2016 Olympic and Paralympic Games, the biggest event in human sports, was held in Rio de Janeiro with more than 10,500 athletes from 206 countries over the world competing for the highest of sports honors, an Olympic medal. With the hope that the Olympic ideal accompanies all aspects of the XXXI Olympiad, WADA accredited antidoping laboratories use the spearhead of analytical technology as a powerful tool in the fight against doping. This review summarizes the main analytical developments applied in antidoping testing methodology combined with the main amendments on the WADA regulations regarding analytical testing starting from the 2012 London Olympics until the 2016 Olympic Games in Rio de Janeiro.

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The World Anti-Doping Agency (WADA) is an international independent agency, established in 1999, composed and funded equally by the Olympic movement and world governments. Its role is to promote, coordinate and monitor the fight against doping in sport through the implementation of the World Anti-Doping Code [1] that provides the antidoping policies, rules and regulations within all sports internationally.

The WADA updates at least yearly the Prohibited List [2], which specifies substances and methods forbidden in sports due to their potential performance enhancing, manipulating exercise and their potential toxicity effect. This list is the result of several expert group meetings with specialists from different fields and covers pharmaceutical, physiological and ethical aspects.

While emerging therapeutics represents one possibility of new substances with the potential to abuse, there are also substances illegally designed or methods developed specifically to increase performance. Often this kind of substances or methods represent a big health risk to athletes, as no clinical studies have been performed and the side effects are unknown.

Information on new substances/methods is gathered by collaborating with pharmaceutical companies, controlling body-building forums, receiving information from police or custom officers about confiscated material or getting hints by journalists or 'whistleblowers', as well as by analysis of confiscated materials carried out by the WADA-accredited laboratories (as for THG, desoxymethyltestosterone [madol] or TB-500 more recently) [3–5].

The biggest sports event in the world with the highest number of in-competition tests is the Summer Olympic Games. While there is often a delay between the appearance of a new doping substance on the market and a detection method officially introduced, the Olympic Games represent a good tool to measure analytical progresses in doping control. With every new edition of the quadren-

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nial competition, more substances have been added to the Prohibited List, more biological data of athletes have been collected for the passport approach, more international sports federations and national antidoping agencies follow the WADA and more sophisticated control methods have been developed.

Retrospective testing of samples as science advances is a powerful tool in the fight against doping. Following the code, it is possible to open a disciplinary proceeding within 10 years from the date an antidoping rule violation occurred. Recently, the International Olympic Committee announced the retesting of 1243 doping samples from both the Beijing Olympic Games in 2008 and the London Olympic Games in 2012. The selection of the reanalyzed samples is based on an intelligence-gathering program focused on athletes who could potentially compete at the Rio Olympic Games. As a result, up to 98 athletes from Beijing Olympic Games and London Olympic Games were banned from competing at the Rio Olympic Games [6,7].

Each Olympics had their antidoping milestones; be it the first hematological models or the erythropoietin (EPO) isoform test during Sydney Olympics 2000, the human growth hormone (hGH) and homologous blood transfusion test during the Athens Olympics 2004, the test for new pegylated EPO (Mircera) during Olympics in Beijing 2008 or the hGH biomarker test at the Games in London 2012.

One of the technologies used in antidoping, which witnessed, for example, a constant evolution over years, is MS where the detection levels of substances went from nanogram to picogram per ml of urine levels enabling a greater sensitivity and longer detection windows.

This review summarizes the analytical developments in antidoping testing methodology in conjunction with the major WADA regulations amendments for analytical procedures starting from the London Olympics 2012 until the 2016 Olympic Games.

Anabolic androgenic steroids

Anabolic androgenic steroids (AAS) are the most frequently misused substances in human sports representing about 60% of the adverse analytical findings worldwide [8]. AAS, synthetic analogs of testosterone (T), promote muscle growth but they can be used also for the treatment of steroid-hormone deficiency conditions (e.g., delayed puberty) and diseases resulting in loss of lean muscle mass (e.g., cancer) [9]. However, anabolic steroids are abused by athletes due to their potential performance enhancement action, that is, muscle growth, increase of strength, fast recovery, etc.

According to WADA Prohibited List [2], anabolic agents are classified into exogenous steroids, endogenous steroids when administered exogenously, other anabolic agents such as clenbuterol and selective androgen receptor modulators.

The administration of new designer and/or unapproved substances available on the black market, the extended use of endogenous steroids, the low detected concentrations of AAS's metabolites in samples are challenges that antidoping laboratories face when we refer to AAS detection [10,11].

Antidoping community has also the mission to protect athletes from the so called 'doping traps'. Inadvertent doping can result from nutritional supplements adulterated with AAS, natural products containing endogenous steroids, cases of clenbuterol findings resulting of contaminated meat products [12,13].

Current strategies for the detection of AAS include the expansion of the detection window of exogenous AAS by identifying long-term metabolites and the athlete's biological passport (ABP) approach for the detection of misuse of endogenous steroids together with the implementation of isotope ratio MS (IRMS).

Expanding the window detection of exogenous AAS

During the London Olympic Games of 2012, >5000 samples were analyzed using a fast, sensitive and comprehensive method based on LC–high-resolution MS (HRMS) [14]. Full scan data in both positive and negative polarity were collected combined with positive fragmentation of a number of analytes for sensitivity improvement with a mass accuracy of 5 ppm. The high throughput of the method combined with a fast sample preparation treatment (direct enzymatic hydrolysis of glucuronide conjugates, SPE) allowed the detection of almost 200 prohibited substances. London Olympics were the first where LC–HRMS analysis was implemented. The GC–MS analysis (where the majority of anabolic steroids are screened) was performed with GC triple quadrupole (QQQ) MS instruments, also for first time in summer Olympic Games.

In 2013, WADA published the new version for the technical document (TD) Minimum Required Performance Limits (MRPL) of the laboratories, the TD2013MRPL [15]. By this TD, the MRPL for exogenous steroids decreased from 10 to 5 ng/ml, while the LOD was set to 2.5 ng/ml. This change forced the laboratories to use the state-of-the-art techniques (GCQQQ, LCQQQ or LCHRMS) and revalidate their methods [14,16].

The identification of new long-term metabolites of prohibited substances in combination with the progress in analytical instrumentation and sensitivity represents a powerful tool in sports drug testing. The prolongation of the detection window of exogenous

AAS is of utmost importance also in the context of retrospectivity of urine analysis.

Several methods have been developed for the expanded detection of exogenous AAS through the implementation of new long-term metabolites in screening routine analysis combined with the use of sensitive analytical technologies (LC–MS/MS, GC–MS/MS, etc.) [17,18].

The biotransformations of dehydrochloromethyltestosterone (oral turinabol), madol and oxymetholone have been studied by Sobolevsky *et al.* [19,20] in order to discover novel long-term metabolites valuable for antidoping analysis. Urine samples obtained from human administration studies were fractioned using HPLC and subjected to GC–MS and GC–MS/MS analysis. The novel metabolites of oxymetholone and madol can be detected for at least 14 days after administration, comparing to 5–7 days' detection of the previously reported metabolites [19].

The mass spectrometric characterization of oral turinabol has led to the detection of about 50 metabolites with some of them allowing a detection window for up to 40–50 days [20]. The implementation of highly sensitive detection methods with the introduction of novel long-term metabolites in the screening procedures resulted to an enormous increase of adverse analytical findings (AAF). The retrospective analysis of 133 routine doping control samples by Moscow laboratory including sports with a high risk of AAS abuse (weightlifting, athletics and powerlifting) for testing the significance of the new metabolites resulted in ten more positive samples. The introduction of three of oral turinabol long-term metabolites in the screening analysis of the Cologne laboratory [21] had added 82 more positive cases for the year 2013.

A longer detection window up to 18 days was achieved by the new oxandrolone metabolites synthesized and characterized by Guddat *et al.* [22]. Post-administration urine samples were analyzed using GC–MS/MS for the parent drug, the two common metabolites (epioxandrolone and 18-nor-oxandrolone) and the novel two metabolites. Urine samples were collected up to 18 days after administration. The two long-term metabolites were detectable for up to 15 and 18 days. To understand the significance of the prolongation of the detection window for oxandrolone misuse, the parent drug was detectable for up to 3 days while the metabolites, epioxandrolone and 18-nor-oxandrolone, for up to 3 and 6 days, respectively. Analysis of approximately 10,000 doping control samples was performed to examine the presence of the two new metabolites of oxandrolone using LC–MS/MS. In 2012, two positive findings were reported by Cologne laboratory for the new oxandrolone long-term metabolites while epioxandrolone and 18-nor-oxandrolone were not detected.

Belgium laboratory [23] developed a sensitive method for the detection of stanozolol glucuronides with 3'-OH-stanozolol glucuronide to be the most important metabolite excreted in urine. A fast and easy sample preparation procedure was followed based on a single SPE avoiding enzymatic hydrolysis or derivatization and directly detected by LC–MS/MS. The developed method allowed a detection window for up to 10 days after oral administration of 2 mg of stanozolol.

Schänzer *et al.* [24] reported also an LC high-resolution/high accuracy mass spectrometric method for the direct analysis of stanozolol glucuronides. The method allowed the detection of 3'-OH-stanozolol glucuronide including two new metabolites, stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide with a detection window for up to 28 days after oral administration of 5 mg of stanozolol. The introduction of these metabolites in the screening procedure of routine samples led to an increase of stanozolol AAF from 23 to 182 after the analysis of 659 samples from Cologne laboratory.

As shown at Figure 1, the introduction of new long-term metabolites of exogenous AAS into the routine screening and confirmation analysis expands the detection window of their misuse, which is of utmost importance in the context of retrospectivity of urine analysis.

As it was shown with the case of stanozolol, the direct detection of the intact conjugated steroid metabolites can lead to increased sensitivity and hence enhanced retrospectivity. A number of laboratories have focused their research to the sulfate-conjugated steroid metabolites. A sulfate-conjugated metabolite of methyltestosterone is detected 21 days after the administration, improving the retrospectivity by to two- to three-times compared with the previously reported metabolites of methyltestosterone [25]. Similar studies have been performed for methenolone [26], mesterelone [27], oxandrolone and danazol [28], clostebol [29,30] and other steroids [31]. Similar studies have been performed for the direct detection of intact glucuronide-conjugated steroid metabolites [32,33].

Endogenous androgenic anabolic steroids & the steroidal module of the ABP

One of the most difficult tasks for the antidoping laboratories is the identification and subsequent confirmation of the presence of synthetic endogenous androgenic anabolic steroids (EAAS) in athletes' urine. While for the exogenous AAS, only a qualitative analysis is enough to prove the presence of the target steroids or their metabolites; for the synthetic forms of endogenous steroids, both a GC–MS quantitative deter-

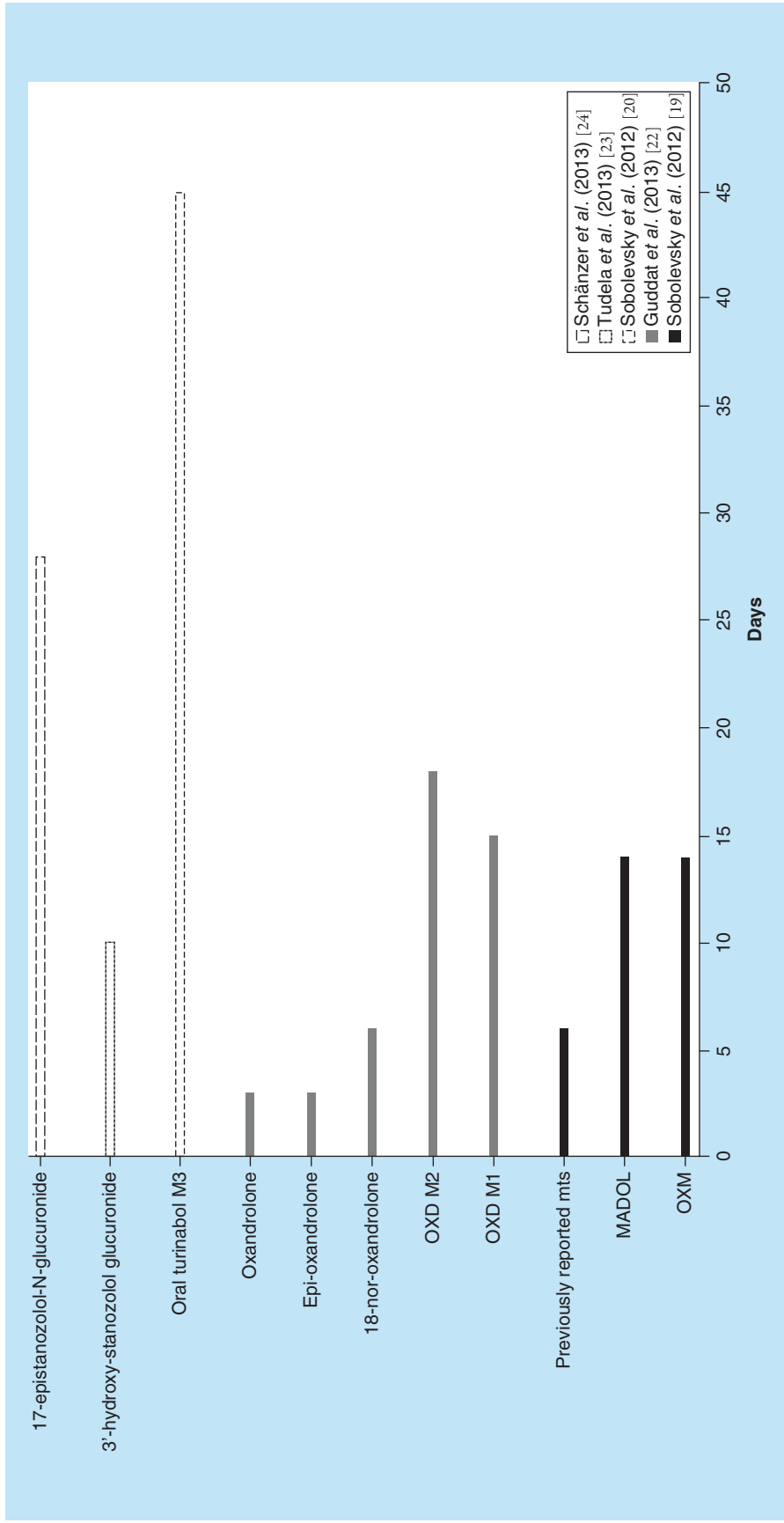

Figure 1. Detection window data for oxymetholone, desoxymethyltestosterone, oral turinabol, oxandrolone and stanozolol glucuronide long-term metabolites.

mination and a laborious gas chromatography-carbon-isotope ratio mass spectrometry (GC-C-IRMS) analysis are required to prove the 'abnormal' concentration and the exogenous origin of them [34]. The IRMS technique is able to differentiate between natural and synthetic endogenous steroids by measuring the stable carbon isotope ratios ^{13}C–^{12}C [35].

The great difference in the detection of EAAS between London and Rio comes from the fact that in 2012 the antidoping laboratories still followed the TD2004EEAS [36], a document published just before the Athens 2004 Olympic Games in order to be implemented in those Games, while in 2016 the laboratories are following the completely revised TD2016EEAS [34].

The misuse of endogenous steroids is traditionally monitored by measuring the steroid profile (SP) in athletes' urine [34]. The SP consists of the metabolites of T, its precursors, as well as epitestosterone (E). For a number of years, the main marker of testosterone misuse was the famous T/E ratio. Based on population data, initially every sample, which had T/E ratio above six was considered suspicious [37], while later this ratio was decreased to four. This marker has been historically proven a robust tool in the arsenal of the antidoping laboratories to differentiate between normal and suspicious SPs. Nevertheless, the main drawback of this marker was that athletes with physically elevated T/E ratios were targeted, while furthermore, athletes who, due to a deletion polymorphism in the gene coding UGT2B17, showed decreased T/E ratio even after the administration of synthetic forms of endogenous AAS, were escaping detection [38]. Based on the above remarks and gaining knowledge from endocrinological studies, the application of individual reference values combined with population-based reference limits was considered as a more effective tool [39,40].

The approach of monitoring individual athletes' markers for doping control was at first implemented by International Cycling Union in 2008 for the values of hematological parameters obtained in whole blood [41]. The hematological module of the ABP was initiated based on longitudinal evaluation of hematological profiles of individual athletes, based on an adaptive model developed by Sottas *et al.* [42]. The steroidal module of the ABP stems from the hematological module as far as the bayesian model is concerned, but, actually, it is basically a more standardized evolution of the urinary steroid profile first developed by Donike and coworkers in the 80s [37].

The implementation of the 'TD2014EAAS' [43] by the antidoping laboratories, enabled the harmonization of the analysis among the accredited laboratories worldwide, and hence the application of the steroidal module of the ABP. The steroidal profile parameters that are currently monitored by the steroidal module of ABP are: T, E, 5α and 5β androstane-3α, 17β-diols, (5αdiol, 5βdiol), androsterone (A) and etiocholanolone, as well as the ratios T/E, A/T, A/etiocholanolone, 5αdiol/5βdiol and 5αdiol/E. The longitudinal SPs of each athlete are collected, monitored, combined and assessed through the WADA's database called 'Anti-Doping Administration & Management System' (Figure 2). For the evaluation of the SP, many confounding factors are considered, such as aging, gender, genetics, drugs and medication, ethanol intake and bacterial contamination [44]. The laboratories along with the SP of every sample have to report also any signs of microbial contamination, the concentration of the conjugated metabolite of ethanol (ethanol glucuronide) if above 5 μg/ml, the presence of 5α-reductace inhibitors and ketoconazole. Especially for the quantification of ethylglucuronide the laboratories had to either add a 'dilute and shoot' fraction to their existing LC–MS screening procedure, or to modify the GC–MS method used for the detection of polar compounds like hetastarch and glycerol [45].

When a longitudinal SP of an athlete shows 'abnormal' values for the T/E ratio marker, as assessed by the adaptive model, then an 'Atypical Passport Finding Confirmation Procedure Request' is created and both the testing authority and the laboratory are notified about this. Then the laboratory has to confirm the T/E ratio value for the sample that triggered the follow-up and consequently perform the IRMS analysis, which will prove if an AAF really exists or not. When a longitudinal SP is not available for an athletes' urine to be compared with, then the SP of this sample is assessed – compared with population-based ranges. If a value of the SP is out of these ranges then a Suspicious Steroid Profile Confirmation Procedure Request is generated and the laboratory, unless the testing authority justifies that a follow-up is not necessary, has to perform the SP confirmation and the IRMS analysis [34].

In any case, either for an Atypical Passport Finding Confirmation Procedure Request or a Suspicious Steroid Profile Confirmation Procedure Request, the positivity of a case can be determined – at least till now – only by the IRMS analysis. The 2014 released from WADA TD for the IRMS analysis TD2014IRMS [47], together with the technical notes and its update in 2016 [48], provides a strict framework for the accredited laboratories but also depict the difficulties of the applied method. Every laboratory determined its own reference $\Delta\delta$ ^{13}C ranges, while these ranges should be less than the WADA thresholds set for every pair of endogenous reference compound–target compound. Furthermore, since 2014, IRMS analysis is mandatory in order to prove the exogenous origin of formestane

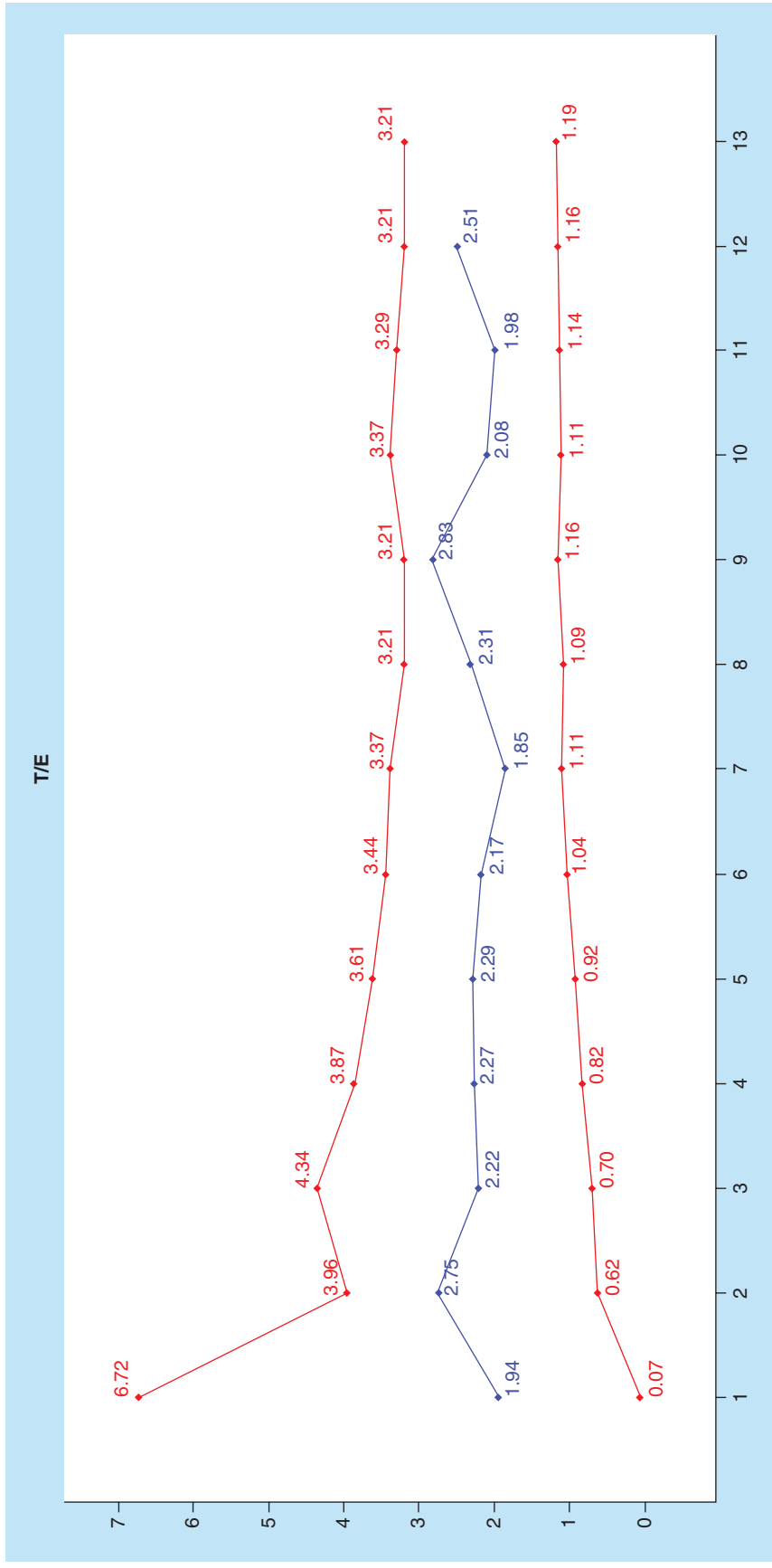

Figure 2. Example of steroid profile generated by the Bayesian model of the athlete's biological passport for the testosterone/epitestosterone ratio parameter. The blue line represents the measured T/E values, whereas the individual limits are shown by the red lines. The figure was created using WADA's athlete biological passport software Version 2.4.2.
E: Epitestosterone; T: Testosterone.
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at concentrations between 50 and 150 ng/ml [49,50], and boldenone and/or its metabolites at concentrations between 5 and 30 ng/ml [51].

New entries & modifications to the WADA Prohibited List

The Prohibited List was published for the first time in 1963 by the International Olympic Committee. Since 2004, WADA is responsible for the preparation and publication of the List. The List is a cornerstone of the code identifying the substances and methods prohibited in competition, out of competition and in particular sports. A substance or method that fulfills any two of the three criteria, as mandated by the code, shall be considered for introduction to the Prohibited List. Briefly, a substance or method shall be included to the Prohibited List if there is any evidence (medical, scientific or pharmacological) that this substance or method, alone or in combination could demonstrate a performance enhancement action; or may have any actual or potential health risk to the athlete; and/or the abuse of substance or method violates the spirit of sport. Substances and methods are classified by categories (e.g., steroids, stimulants and gene doping). The List is updated annually following an extensive consultation with specialists from different fields.

Matabosch *et al.* [52] re-evaluated the budesonide metabolism resulting in the detection of 16 metabolites in total (including ten previously unreported). The main metabolite, 16α-hydroxy-prednisolone, was excreted in urine for a time period of up to 24 h after administration while 6β-hydroxy-budesonide for up to 48 h. Since 2014, metabolite 6β-hydroxy-budesonide has been the marker of budesonide abuse instead of 16-hydroxy-prednisolone.

A monitoring program is also established by WADA in consultation with governments regarding substances that are not included in the Prohibited List, but which WADA wishes to monitor in order to detect patterns of misuse in sport.

During the last few years, traditional drugs, as well as new drug compounds developed and studied in preclinical and clinical trials for the treatment of serious diseases, have been prohibited by WADA due to their potential of being abused in sports.

Xenon, a hypoxia-inducible factor activator, was included in the WADA Prohibited List in September 2014. Xenon is used as a narcotic gas at atmospheric pressure due to its high water and fat solubility compared with other gases, such as nitrogen, helium and argon. Except from its anesthetic properties, xenon increases the EPO production through its hypoxia-inducible factor activation, and therefore, it is featured as a banned substance.

Methods for xenon analysis have been developed in human plasma, blood and urine using GC–MS [53,54]. Results have shown that xenon can be detected for up to 40 h postanesthesia in urine samples while in plasma and blood samples after storage for up to 30 h.

Meldonium (Mildronate) is another example for this scenario that had drawn a lot of attention recently. It was introduced to the 2015 WADA-monitoring program [55] and based on the scientific information and collected data during its monitoring, a high prevalence of its use was revealed by athletes and teams, which led to its inclusion to the 2016 Prohibited List [2].

Meldonium [56] is a nonspecified substance prohibited at all times (in and out of competition) and it is classified as a hormone and metabolic modulator by WADA. It is an anti-ischemic drug developed at the Latvian Institute of Organic Synthesis. Its medical use is based on the inhibition of the carnitine biosynthesis. Meldonium is effective in the treatment of heart ischemia and its consequences, as well as in the treatment of CNS functions. However, Meldonium demonstrates a performance enhancement action for athletes, improved rehabilitation after exercise and protection against stress. The introduction of Meldonium in the List was announced on 16 September 2015 with the presentation of the WADA Prohibited List for 2016 [2], which came into effect on 1 January 2016. Nevertheless, since then a lot of questions have been raised by the antidoping stakeholders regarding the results management of adverse analytical finding for meldonium. The lack of previous excretion studies for meldonium made it difficult to state if an adverse analytical finding for meldonium was due to an administration before or after 1 January 2016. This forced WADA to issue two notices on 13 April and 30 June 2016 providing guidance regarding the results management and adjudication process to be followed [57,58]. Different processes are to be followed depending on the collection date and the urinary concentration of meldonium.

Desmopressin was introduced in the Prohibited List in 2011 [59] as a masking agent. It is a peptide containing nine amino acids. Due to its structure, its inclusion in the List forced the laboratories to develop new methods for the detection of small peptides [60,61]. The releasing factors of GH were introduced in the List in 2014 [62], while they were tested for the first time in Olympic Games in Sochi 2014 [63]. The detection of growth hormone releasing peptides in urine has become mandatory for all the laboratories since March 2016.

Peptide hormones & blood transfusion

Not many scientific inventions were released for peptide hormones like EPO, human chorionic gonadotropin (hCG), luteinizing hormone (LH), hGH or blood

transfusions after the Olympics in London 2012. The most relevant inventions/improvements and ongoing research activities are discussed below.

EPO

For the detection of EPO abuse, WADA published a new TD in 2014 [64], which allows the laboratories to use sarcosyl polyacrylamide gel electrophoresis as a screening and confirmation tool for all currently available EPO molecules (Figure 3). The majority of efforts since then were developing methods to increase the sample throughput, decreasing analysis time and making the method more economical. Reihlen *et al.* [65], for example, automated part of the 3-day procedure by including the BlotCycler device, which performs all washing and incubation steps after the proteins are transferred from the gel to the membrane. The device does not only help in saving human resources and thus making the method more economical but also improves standardization and reduces the possibility for human error. Another improvement presented by Garribba *et al.* [66] is a shorter blotting time by using a vacuum-driven blotting system instead of the typically used semidry blotting systems. The authors highlight the relevance for situations in which a very fast turnaround time is required like for Olympic Games.

In an application note by Schwenke [67], the use of precasted Velum SAR gels in combination with capillary blotting is described and the author highlights the easier handling of Velum gels compared with the otherwise used NuPage gels. A disadvantage is the unfortunately very long blotting time of 2 h.

Finally, although not tested and applied in routine settings Vogel *et al.* [68] published a method in which EPO receptor-coated magnetic beads for the isolation and the enrichment of EPOs are used. The fascination about this procedure is that new molecules with erythropoietic effects but with low affinity to the EPO antibodies used for the current methodology could possibly be detected.

hCG & LH

hCG, a peptide hormone usually produced in pregnant women is also abused by male athletes to stimulate the endogenous testosterone production by targeting the same receptor as LH. Up to the year 2015, laboratories were screening for hCG using immunological methods with antibodies directed against many different hCG forms and subunits like intact hCG, β-hCG, nicked

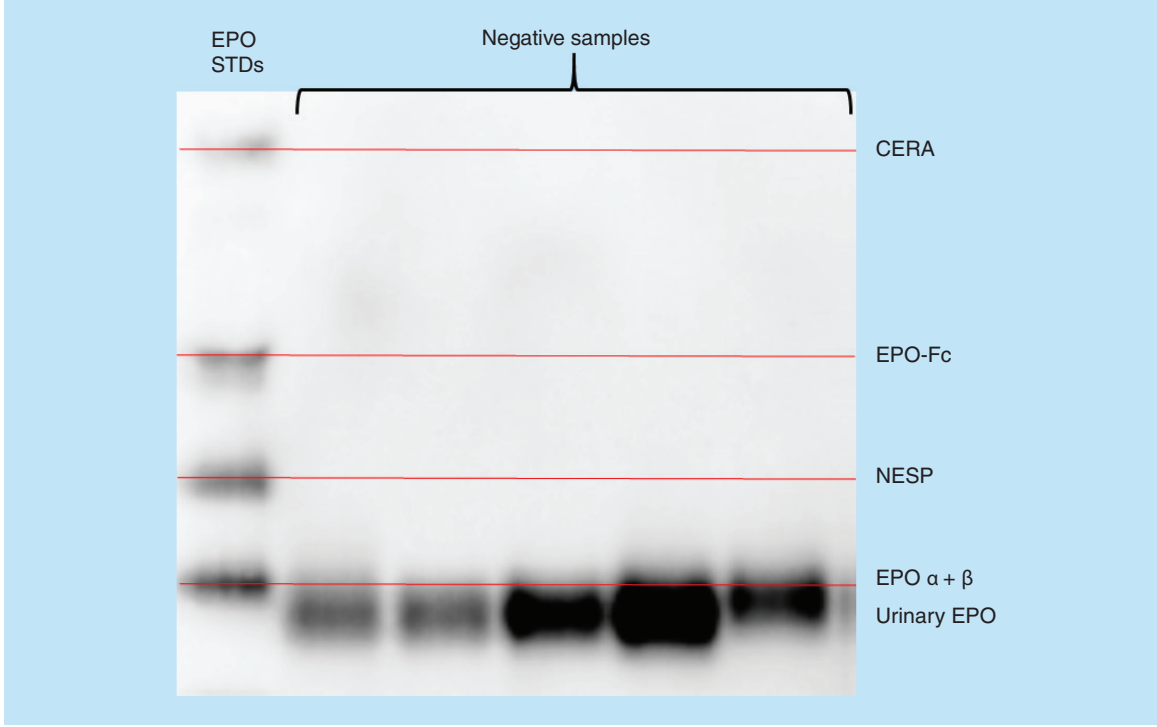

Figure 3. Sarcosyl polyacrylamide gel electrophoresis image showing the higher apparent molecular masses of continuous erythropoietin receptor activator, erythropoietin-Fc, novel erythropoiesis stimulating protein and Epoetin-α & β in comparison to endogenous urine erythopoietin.

CERA: Continuous erythropoietin receptor activator; EPO: Erythropoietin; NESP: Novel erythropoiesis stimulating protein.

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hCG and the β-core fragment, often referred to as total hCG assays. Men with familial hCG, a condition with consistently elevated β-hCG concentrations, might cause a positive finding with these total hCG assays; so in order to reduce the possibility of falsely elevated values, laboratories were asked to use methods very specific to the intact form only. The WADA Guidelines on hCG and LH findings in male athletes [69] attempt to harmonize the used methodologies and to facilitate interlaboratory comparisons, and laboratories are now obliged to use two different kits for screening and confirmation; however, both are specific for the intact hCG form only. To further increase the specificity, methodologies applying MS were developed and are fit for purpose [70,71].

While the authors believe that using MS for confirmation of a suspicious sample is of high value, they doubt that the disadvantages in analysis time, sample throughput and costs make it useful as initial screening procedure.

In addition to WADAs changes for the hCG methodology, the analysis of LH was added in 2015 to the list of substances to be screened for. Increased concentrations of urinary LH in male athletes may be an indication of the abuse of this substance for the same reason as described above for hCG. Elevated values might, however, also indicate the use of gonadotropin-releasing hormone and its synthetic analogs or estrogen blockers, which induce the release of endogenous LH [72]. Samples with high LH values are thus objected to LC–MS analysis for small peptides. Suppressed urinary concentrations of LH in male athletes may on the other hand also be an indication of the misuse of anabolic steroids – which makes LH a valuable, supplemental marker in interpreting SPs [73].

Blood transfusion

In regard of blood transfusions, which athletes use to increase their circulating hemoglobin mass and thus their oxygen carrying capacity, there are two accredited methodologies currently existing to detect this kind of doping in the athlete. The direct method uses an immunological approach (flow cytometry) to determine a variety of antigens on the red blood cell membrane. Unfortunately, this technology is limited to homologous (from a different person) blood transfusions only. Autologous blood transfusion, the technique in which the athlete donates and stores his own blood for retransfusion before major events, is only detectable by the hematological module of the ABP [74]. While the method was already used during the Olympic Games 2012 in London the number of athletes who are registered now in the ABP test pool is much higher compared with 4 years ago, so that WADA and National Anti-Doping Organizations have more information on the athletes, facilitating the detection of doping-related irregularities in their hematological profiles (Figure 4), such as after receiving a transfusion.

Research is ongoing and current investigations focus on plasticizers, miRNAs, plasma iron and hepcidin, gene expression in reticulocytes, improvements in the detection of homologous blood transfusion and on CE of red blood cells.

DNA analysis by real-time PCR to test for homologous blood transfusion was proposed by Manokhina in 2013 [75] and Stampella *et al.* [76], published recently a validation study in which they describe that the DNA analysis is sensitive enough to detect DNA of a different individual even when RBC concentrates were leukocyte depleted. The risk of false-negative results as described by Krotov *et al.* [77] thus could be minimized using DNA analysis. He reported that based on the phenotyping of 535 Russian athletes, it might be statistically easy for athletes to match their blood beforehand to avoid tested positive with the flow cytometric method.

The use of plasticizers originating from the material of the bags, which are used to store blood, was published already before 2010 as an approach to detect autologous blood doping. Unfortunately, due to the uptake of such plasticizers from food packages like plastic bottles, the method has a high cutoff value and can be used only indicatively or maybe in combination with other parameters. Recent research in this field was published by Varlet-Marie *et al.* who describe the relevance of di(2-ethylhexyl)phthalate blood levels for blood transfusion detection [78]. Another publication from this year showed surprisingly that even the use of plasticizer-free bags might result in the detection of urinary di-(2-ethylhexyl) phthalate metabolites [79].

A very interesting concept was presented this year by Salamin *et al.* [80] investigating the gene expression of reticulocytes after blood transfusion, which shows some genes downregulated after transfusion; however, further studies are needed to see if these findings can be implemented in doping control.

Leuenberger *et al.* investigated iron and hepcidin in two different studies. The authors found that iron in EDTA blood was elevated up to 25-fold 6 h after transfusion and proposed a threshold of 45 µg/dl as a simple screening for autologous blood doping [81].

The authors also found hepcidin to be upregulated after transfusion, but discussed that there is still much more knowledge needed in regard to confounding factors affecting this molecule before its use as a biomarker for detection can be considered [82].

Finally, Harrison *et al.* [83] presented very promising results of a WADA project, which focused on the use of

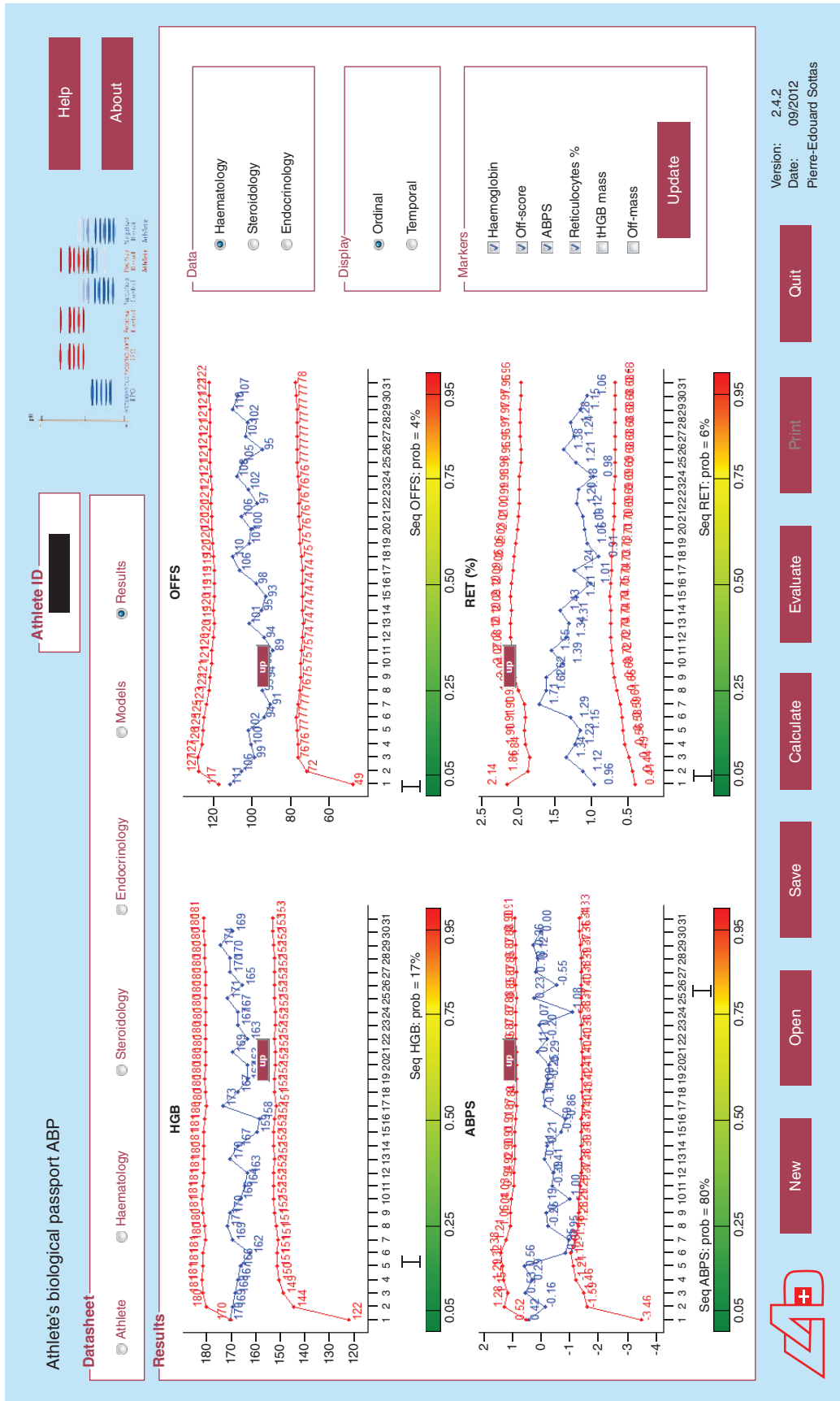

Figure 4. Example of the longitudinal data for the hematological parameters of an athlete in the athlete biological passport software. The blue line represents the results of the athletes analyzed samples over time while the red line above and below are the individualized threshold values calculated by the software. The figure was created using the World Anti-Doping Agency's ABP software Version 2.4.2.
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CE for the analysis of the red blood cell size. With the red blood cells undergoing vesiculation during storage they differ in size from native cells. Indeed, the proof-of-concept was successful and the authors are in the process to prepare an autologous blood transfusion study for validation of their results.

hGH

To detect doping with hGH, two approaches are available. The first approach [84] is based on the use of the so called 'isoform differential immunoassays' and is based on the fact that hGH in blood occurs as a mixture of different isoforms in relatively constant ratios while recombinant GH consists only of the monomeric 22-kDa isoform.

The validity of this test was legally challenged but studies from 2013 and 2014 confirmed the existing thresholds and could show that the test was not affected by exercise, enabling utmost confidence in its results [85,86].

The drawback of the isoform test is the short detection time of approximately 2 days, which makes the combination with the second approach the 'hGH biomarkers test' very valuable [87]. The biomarker test estimates the concentrations of IGF-1 and N-terminal propeptide of type III collagen, which are present in serum and increase substantially after hGH abuse [88].

Recent studies on the decision limits and exercise as confounding factor supported the validity of the test results and enabled the use of a bigger variety of assays [89,90].

WADA introduced this year the second version of the guidelines regarding the hGH biomarkers test, which includes now two immunoassays, as well as one LC–MS method for IGF-1 and two immunoassays for N-terminal propeptide of type III collagen [91].

Research for new biomarkers to detect hGH abuse is currently ongoing including genes and proteins, but some research are also looking into improvements of logistical issues. Ferro *et al*. recently published an article in which RAB31, FN1 genes and FN1 protein are discussed as potential biomarkers. The results of their study, which included the assessment of some possible confounding factors, showed a high sensitivity and a longer detection window for recombinant hGH (rhGH) detection than IGF-1 itself [92].

Dried blood spots instead of serum were also tested for performing the hGH isoform analysis and proofed valid. The use of dried blood spots could have huge potential due to its minimal invasiveness (finger-prick capillary blood instead of venous blood), low transport costs and high sample stability [93].

Conclusion & future perspective

Since the 2012 Olympic Games of London, new analytical advancements and amended WADA regulations have been applied in the antidoping testing analysis. New long-term metabolites of exogenous AAS were identified and introduced to the routine screening and confirmation analysis expanding the detection window of their misuse. The introduction of the steroidal module of ABP based on individual reference values combined with population-based reference limits was considered as a more effective tool. Furthermore, sarcosyl polyacrylamide gel electrophoresis technique was introduced both as a screening and confirmation tool allowing the laboratories to detect all the available EPO molecules.

Olympic Games is a major sports event governed by the values of excellence, friendship and respect. The antidoping system has undertaken the role to protect the Olympic ideal to be not influenced and clean from doping. Toward to this goal, antidoping laboratories face the challenge to continuously improve their analytical methodologies by introducing the spearhead of analytical technology into their routine practice.

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Executive summary

- Retrospective analysis of negative samples implementing new technologies is a powerful tool in the fight against doping.
- The prolongation of the detection window of exogenous anabolic androgenic steroids through the identification of new long-term metabolites is of utmost importance in the context of retrospectivity of urine analysis.
- The implementation of the steroidal module of the athlete's biological passport is an effective tool for the detection of the administration of synthetic forms of endogenous steroids.
- In 2014, sarcosyl polyacrylamide gel electrophoresis introduced both as a screening and confirmation method for all currently available erythropoietin molecules.

References

Papers of special note have been highlighted as:
•• of considerable interest

1 World Anti-Doping Code (2015). https://wada-main-prod.s3.amazonaws.com

2 World Anti-Doping Agency (WADA) Prohibited List (2016). https://wada-main-prod.s3.amazonaws.com

3 Catlin DH, Sekera MH, Ahrens BD, Starcevic B, Chang YC, Hatton CK. Tetrahydrogestrinone: discovery, synthesis, and detection in urine. *Rapid Comm. Mass Spectr.* 18(12), 1245–1249 (2004).

4 Sekera MH, Ahrens BD, Chang YC, Starcevic B, Georgakopoulos C, Catlin DH. Another designer steroid: discovery, synthesis, and detection of 'madol' in urine. *Rapid Comm. Mass Spectr.* 19(6), 781–784 (2005).

5 Esposito S, Deventer K, Goeman J, Van der Eycken J, Van Eenoo P. Synthesis and characterization of the N-terminal acetylated 17–23 fragment of thymosin beta 4 identified in TB-500, a product suspected to possess doping potential. *Drug Test. Analysis* 4(9), 733–738 (2012).

6 World Anti-Doping Agency (WADA). www.wada-ama.org

7 Ben Bloom. IOC confirm 45 more positive doping cases in retests from Beijing 2008 and London 2012 Olympics. *The Telegraph,* 22 July (2016).

8 World Anti-Doping Agency (WADA) 2014 Anti-Doping Testing Figures Report. https://wada-main-prod.s3.amazonaws.com

9 J.E.F Reynolds, *Martindale – the Extra Pharmacopoeia* (33rd Edition). Pharmaceutical Press, London, UK, (2002).

10 Thevis M, Schänzer W. Analytical approaches for the detection of emerging therapeutics and non-approved drugs in human doping controls. *J. Chromatogr. A* 101, 66–83 (2014).

11 de la Torre X, Curcio D, Colamonici C, Molaioni F, Ciliaa M, Botrè F. Development and validation of a GC–C-IRMS method for the confirmation analysis of pseudo-endogenous glucocorticoids in doping control. *Drug Test. Analysis* 7(11–12), 1071–1078 (2015).

12 Geyer H, Adulterated nutritional supplements and unapproved pharmaceuticals as new sources of doping substances for fitness and recreational sports. In: *Doping and Public Health.* Routledge, Taylor & Francis Group LLC, FL, USA (2016).

13 Thevis M, Thomas A, Beuck S, Butch A, Dvorak J, Schänzer W. Does the analysis of the enantiomeric composition of clenbuterol in human urine enable the differentiation of illicit clenbuterol administration from food contamination in sports drug testing? *Rapid Comm. Mass Spectrom.* 27(4), 507–512 (2013).

14 Musenga A, Cowan DA. Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control. *J. Chromatogr. A* 1288, 82–95 (2013).

15 World Anti-Doping Agency (WADA) TD2013MRPL. https://wada-main-prod.s3.amazonaws.com

16 Delgadillo MA, Garrostas L, Pozo OJ *et al.* Sensitive and robust method for anabolic agents in human urine by gas chromatography-triple quadrupole mass spectrometry. *J. Chromatogr. B* 897, 85–89 (2012).

17 De Brabanter N, Gansbeke WV, Geldof L, Van Eenoo P. An improved gas chromatography screening method for doping substances using triple quadrupole mass spectrometry, with an emphasis on quality assurance. *Biomed. Chromatogr.* 26, 1416–1435 (2012).

18 Jeong ES, Kim SH, Cha EJ *et al.* Simultaneous analysis of 210 prohibited substances in human urine by ultrafast liquid chromatography/tandem mass spectrometry in doping control. *Rapid Comm. Mass Spectr.* 29(4), 367–384 (2015).

19 Sobolevsky T, Rodchenkov G. Mass spectrometric description of novel oxymetholone and desoxymethyltestosterone metabolites identified in human urine and their importance for doping control. *Drug Test Anal.* 4, 682–691 (2012).

20 Sobolevsky T, Rodchenkov G. Detection and mass spectrometric characterization of novel long-term dehydrochloromethyltestosterone metabolites in human urine. *J. Steroid Biochem. Mol. Biol.* 128, 121–127 (2012).

•• **Due to this work hundreds of positive cases have been reported for samples initially reported as 'negative'.**

21 Geyer H, Schänzer W, Thevis M. Anabolic agents: recent strategies for their detection and protection from inadvertent doping. *Br. J. Sports Med.* 48, 820–826 (2014).

22 Guddat S, Fußhöller G, Beuck S *et al.* Synthesis, characterization, and detection of new oxandrolone metabolites as long-term markers in sports drug testing. *Anal. Bioanal. Chem.* 405, 8285–8294 (2013).

•• **Due to this work a lot of positive cases have been reported for samples initially reported as 'negative'.**

23 Tudela E, Deventer K, Van Eenoo P. Sensitive detection of 3'-hydroxy-stanozolol glucu-ronide by liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A* 1292, 195–200 (2013).

•• **Due to this work a lot of positive cases have been reported for samples initially reported as 'negative'.**

24 Schänzer W, Guddat S, Thomas A, Opfermann G, Geyer H, Thevis M. Expanding possibilities concerning the detection of stanozolol misuse by means of high resolution/high accuracy mass spectrometric detection of stanozolol glucuronides in human sports drug testing. *Drug Test Anal.* 5, 810–818 (2013).

•• **Due to this work a lot of positive cases have been reported for samples initially reported as 'negative'.**

25 Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-term markers for the detection of methyltestosterone misuse. *Steroids* 78(1), 44–52 (2013).

26 Fragkaki AG, Angelis YS, Kiousi P, Georgakopoulos CG, Lyris E. Comparison of sulfo-conjugated and gluco-conjugated urinary metabolites for detection of methenolone misuse in doping control by LC–HRMS, GC–MS and GC–HRMS. *J. Mass Spectrom.* 50(5), 740–748 (2015).

27 Kiousi P, Angelis YS, Fragkaki AG *et al.* Markers of mesterolone abuse in sulfate fraction for doping control in human urine. *J. Mass Spectrom.* 50(12), 1409–1419 (2015).

28 Rzeppa S, Viet L. Analysis of sulfate metabolites of the doping agents oxandrolone and danazol using high performance liquid chromatography coupled to tandem mass spectrometry. *J. Chromatogr. B* 1029–1030, 1–9 (2016).

29 Balcells G, Pozo OJ, Garrostas L *et al.* Detection and characterization of clostebol sulfate metabolites in Caucasian population. *J. Chromatogr. B* 1022, 54–63 (2016).

30 Lu J, Fernández-Álvarez M, Yang S, He G, Xu Y, Aguilera R. New clostebol metabolites in human urine by liquid chromatography time-of-flight tandem mass spectrometry and their application for doping control. *J. Mass Spectrom.* 50(1), 191–197 (2015).

31 Rzeppa S, Heinrich G, Hemmersbach P. Analysis of anabolic androgenic steroids as sulfate conjugates using high performance liquid chromatography coupled to tandem mass spectrometry. *Drug Test. Analysis* 7(11–12), 1030–1039 (2015).

32 Tudela E, Deventer K, Geldof L, Van Eenoo P. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography–high resolution mass spectrometry. *Drug Test Anal.* 7(2), 95–108 (2015).

33 Esquivel A, Pozo OJ, Garrostas L *et al.* LC–MS/MS detection of unaltered glucuronoconjugated metabolites of metandienone. *Drug Test Anal.* doi:10.1002/dta.1996 (2016) (Epub ahead of print).

34 World Anti-Doping Agency. Endogenous anabolic androgenic steroids: measurement and reporting. TD2016EAAS, ver. 1.0. www.wada-ama.org

35 Ouellet A, LeBerre N, Ayotte C. A simplified and accurate method for the analysis of urinary metabolites of testosterone-related steroids using gas chromatography/combustion/isotope ratio mass spectrometry. *Rapid Comm. Mass Spectr.* 27(15), 1739–1750 (2013).

36 World Anti-Doping Agency. Reporting and evaluation guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids. https://wada-main-prod.s3.amazonaws.com

37 Donike M, Rauth S, Wolansky A. Reference ranges of urinary endogenous steroids determined by gas chromatography/mass spectrometry. *Proceedings of the 10th Cologne Workshop on Dope Analysis.* Verlag Sport and Buch Strauβ, Cologne, Germany, 69–80 (1993).

38 Jakobsson J, Ekstrom L, Inotsume N *et al.* Large difference in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. *J. Chin. Endocrinol. Metab.* 91, 687–693 (2006).

39 Donike M, Ueki M, Kuroda Y *et al.* Detection of dihydrotestosterone (DHT) doping: alterations in the steroid profile and reference ranges for DHT and 5 alpha-metabolites. *J. Sport Med. Phys. Fitness* 35, 235–250 (1995).

40 Sottas PE, Saudan C, Schweizer C *et al.* From population- to subject-based limits of T/E ratio to detect testosterone abuse in elite sports. *Forensic Sci. Int.* 174, 166–172 (2008).

41 Zorzoli M, Rossi F. Implementation of the biological passport. The experience of the International Cycling Union. *Drug Test. Analysis* 2, 542–547 (2010).

42 Sottas PE, Robinson N, Saugy M. The athlete's biological passport and indirect markers of blood doping. *Hand Exp. Pharmacol.* 195, 305–326 (2010).

43 World Anti-Doping Agency. Endogenous anabolic androgenic steroids: measurement and reporting. TD2014EAAS, ver. 1.0. https://wada-main-prod.s3.amazonaws.com

44 Kuuranne T, Saugy M, Baume N. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. *Br. J. Sports Med.* 48, 848–855 (2014).

45 Goergens C, Guddat S, Orlovius A-K *et al.* 'Dilute-and-inject' multi-target screening assay for highly polar doping agents using hydrophilic interaction liquid chromatography high resolution/high accuracy mass spectrometry for sports drug testing. *Anal. Bioanal. Chem.* 407, 5365–5379 (2015).

46 World Anti-Doping Agency. www.wada-ama.org

47 World Anti-Doping Agency WADA Technical Document TD2014IRMS. https://wada-main-prod.s3.amazonaws.com

48 World Anti-Doping Agency. Detection of synthetic forms of endogenous anabolic androgenic steroids by GC/C/IRMS. TD2016IRMS, ver. 1.0. https://wada-main-prod.s3.amazonaws.com

49 Polet P, Van Renterghem P, Van Gansbeke W, Van Eenoo P. Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry. *Steroids* 78(11), 1103–1109 (2013).

50 Piper T, Fusshöller G, Emery C, Schänzer W, Saugy M. Investigations on carbon isotope ratios and concentrations of urinary formestane. *Drug Test Anal.* 4(12), 942–950 (2012).

51 Piper T, Geyer H, Gougoulidis V, Flenker U, Schänzer W. Determination of 13C/12C ratios of urinary excreted boldenone and its main metabolite 5β-androst-1-en-17β-ol-3-one. *Drug Test. Anal.* 2(5), 217–224 (2010).

52 Matabosch X, Pozo OJ, Pérez-Mañá C *et al.* Identification of budesonide metabolites in human urine after oral administration. *Anal. Bioanal. Chem.* 404(2), 325–340 (2012).

53 Thevis M, Piper T, Geyer H *et al.* Measuring xenon in human plasma and blood by gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* 28(13), 1501–1506 (2014).

54 Thevis M, Piper T, Geyer H *et al.* Urine analysis concerning xenon for doping control purposes. *Rapid Commun. Mass Spectrom.* 29(1), 61–66 (2015).

55 World Anti-Doping Agency Monitoring Program 2015. https://wada-main-prod.s3.amazonaws.com

56 Sjakste N, Gutcaits A, Kalvinsh I. Mildronate: an antiischemic drug for neurological indications. *CNS Drug Rev.* 11(2), 151–168 (2005).

57 World Anti-Doping Agency. https://wada-main-prod.s3.amazonaws.com

58 World Anti-Doping Agency. https://wada-main-prod.s3.amazonaws.com

59 World Anti-Doping Agency. The 2012 Prohibited List International Standard. https://wada-main-prod.s3.amazonaws.com

60 Esposito S, Deventer K, T'Sjoenb G, Vantilborgh A, Van Eenoo P. Doping control analysis of desmopressin in human urine by LC–ESI–MS/MS after urine delipidation. *Biomed. Chromatogr.* 27, 240–245 (2013).

61 Thomas T, Höppner S, Geyer H *et al.* Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry. *Anal. Bioanal. Chem.* 401, 507–516 (2011).

62 World Anti-Doping Agency. The 2014 Prohibited List International Standard. https://wada-main-prod.s3.amazonaws.com

63 Sobolevsky T, Krotov G, Dikunets M *et al.* Anti-doping analyses at the Sochi Olympic and Paralympic Games 2014. *Drug Test. Anal.* 6, 1087–1101 (2014).

64 World Anti-Doping Agency. Harmonization-of-analysis-and-reporting-of-ESAs-by-electrophoretic-techniques. TD2014EPO, ver. 1.0. https://wada-main-prod.s3.amazonaws.com

65 Reihlen P, Blobel M, Kempkes R *et al.* Optimizing SAR-PAGE. *Drug Test Anal.* 7(11–12), 1014–1016 (2015).

66 Garribba F, Turi S, Corpetti G, Khiabani A, De la Torre X, Botrè F. A modified procedure based on a vacuum-driven blotting system for the detection of erythropoietin and its analogs. *Bioanalysis* 6(12), 1605–1615 (2014).

67 Schwenke D. Improved detection of EPO in blood and urine based on novel Velum SAR precast horizontal gels optimized for routine analysis. www.dyeagnostics.com

68 Vogel M, Blobel M, Thomas A *et al.* Isolation, enrichment, and analysis of erythropoietins in anti-doping analysis by receptor-coated magnetic beads and liquid chromatography-mass spectrometry. *Anal. Chem.* 86(24), 12014–12021 (2014).

69 World Anti-Doping Agency. Reporting & management of urinary human chorionic gonadotrophin (hCG) and luteinizing hormone (LH) findings in male athletes. Ver. 2.0. https://wada-main-prod.s3.amazonaws.com

70 Lund H, Snilsberg AH, Halvorsen TG, Hemmersbach P, Reubsaet L. Comparison of newly developed immuno-MS method with existing DELFIA(®) immunoassay for human chorionic gonadotropin determination in doping analysis. *Bioanalysis* 5(5), 623–630 (2013).

71 Woldemariam GA, Butch AW. Immunoextraction-tandem mass spectrometry method for measuring intact human chorionic gonadotropin, free β-subunit, and β-subunit core fragment in urine. *Clin. Chem.* 60(8), 1089–1097 (2014).

72 Handelsman DJ, Idan A, Grainger J, Goebel C, Turner L, Conway AJ. Detection and effects on serum and urine steroid and LH of repeated GnRH analog (leuprolide) stimulation. *J. Steroid Biochem. Mol. Biol.* 141, 113–120 (2014).

73 Hengevoss J, Piechotta M, Müller D *et al.* Combined effects of androgen anabolic steroids and physical activity on the hypothalamic–pituitary–gonadal axis. *J. Steroid Biochem. Mol. Biol.* 150, 86–96 (2015).

74 Pottgiesser T, Schumacher YO. Current strategies of blood doping detection. *Anal. Bioanal. Chem.* 405(30), 9625–9639 (2013).

75 Manokhina I, Rupert JL. A DNA-based method for detecting homologous blood doping. *Anal. Bioanal. Chem.* 405(30), 9693–9701 (2013).

76 Stampella A, Di Marco S, Pirri D, de la Torre X, Botrè F, Donati F. Application of DNA-based forensic analysis for the detection of homologous transfusion of whole blood and of red blood cell concentrates in doping control. *Forensic Sci. Int.* 265, 204–210 (2016).

77 Krotov G, Nikitina M, Rodchenkov G. Possible cause of lack of positive samples on homologous blood transfusion. *Drug Test Anal.* 6(11–12), 1160–1162 (2014).

78 Varlet-Marie E, J Ashenden M, Moerkeberg J, Vela C, Audran M. Relevance of di(2-ethylhexyl)phthalate blood levels for blood transfusion detection. *Bioanalysis* 6(12), 1591–1595 (2014).

79 Leuenberger N, Barras L, Nicoli R *et al.* Urinary di-(2-ethylhexyl) phthalate metabolites for detecting transfusion of autologous blood stored in plasticizer-free bags. *Transfusion* 56(3), 571–578 (2016).

80 Salamin O, Barras L, Robinson N *et al.* Impact of blood transfusion on gene expression in human reticulocytes. *Am. J. Hematol.* doi:10.1002/ajh.24470 (2016) (Epub ahead of print).

81 Leuenberger N, Ansermet C, Pottgiesser T *et al.* A fast automated screening method for the detection of blood transfusion in sports. *Drug Test Anal.* 6(11–12), 1141–1143 (2014).

82 Leuenberger N, Barras L, Nicoli R *et al.* Hepcidin as a new biomarker for detecting autologous blood transfusion. *Am. J. Hematol.* 91(5), 467–472 (2016).

83 Harrison CR, Fang JC, Walthall KJ, Green CC, Porobic V. Towards the identification of autologous blood transfusions through capillary electrophoresis. *Anal. Bioanal. Chem.* 406(3), 679–686 (2014).

84 Wu Z, Bidlingmaier M, Dall R, Strasburger CJ. Detection of doping with human growth hormone. *Lancet* 353(9156), 895 (1999).

85 Hanley JA, Saarela O, Stephens DA, Thalabard JC. hGH isoform differential immunoassays applied to blood samples from athletes: decision limits for anti-doping testing. *Growth Horm IGF Res.* 24(5), 205–215 (2014).

86 Voss SC, Giraud S, Alsayrafi M *et al.* The effect of a period of intensive exercise on the isoform test to detect growth hormone doping in sports. *Growth Horm IGF Res.* 23(4), 105–108 (2013).

87 Barroso O, Schamasch P, Rabin O. Detection of GH abuse in sport: past, present and future. *Growth Horm IGF Res.* 19(4), 369–374 (2009).

88 Kniess A, Ziegler E, Thieme D, Müller RK. Intra-individual variation of GH-dependent markers in athletes: comparison of population based and individual thresholds for detection of GH abuse in sports. *J. Pharm. Biomed. Anal.* 84, 201–208 (2013).

89 Voss SC, Robinson N, Alsayrafi M *et al.* The effect of a period of intense exercise on the marker approach to detect

growth hormone doping in sports. *Drug Test Anal.* 6(6), 582–586 (2014).

90 Holt RI, Böhning W, Guha N *et al.* The development of decision limits for the GH-2000 detection methodology using additional insulin-like growth factor-I and amino-terminal pro-peptide of type III collagen assays. *Drug Test Anal.* 7(9), 745–755 (2015).

91 World Anti-Doping Agency. Guidelines human growth hormone (hGH) biomarkers test for doping control analyses. ver. 2.0. https://wada-main-prod.s3.amazonaws.com

92 Ferro P, Ventura R, Pérez-Mañá C, Farré M, Segura J. Genetic and protein biomarkers in blood for the improved detection of GH abuse. *J. Pharm. Biomed. Anal.* 128, 111–118 (2016).

93 Reverter-Branchat G, Bosch J, Vall J *et al.* Determination of recent growth hormone abuse using a single dried blood spot. *Clin. Chem.* pii:clinchem.2016.257592 (2016) (Epub ahead of print).