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Olympic anti-doping laboratory: the analytical technological road from 2016 Rio De Janeiro to 2021 Tokyo

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The summer Olympic Games is the major mega sports event since the first modern era Olympiad, held in Athens, Greece in 1896. International Olympic Committee (IOC) has the responsibility of the organization of the summer and winter Games ensuring the broadcast in all corners of earth. The World Anti-Doping Agency (WADA) is the responsible organization of the fight against doping in sports. IOC and WADA support the event's country WADA Accredited Laboratory to incorporate the maximum of the new analytical technologies to become applicable during the event's antidoping testing. The current study reviewed the last 5 years progresses of the antidoping system with emphasis on the laboratory field.

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The World Anti-Doping Agency (WADA) antidoping system [1] is dynamically changing due to new doping trends, new analytical tools, technologies and knowledge in sciences of sports. Adaptations are applied to WADA core document, the Code [2] and the lower level documents International Standards (IS) [3], Technical Documents (TD), Technical Letters (TL) [4] and Guidelines [5]. UNESCO, the United Nations (UN) body responsible for education, science and culture, has adopted the WADA Code through the International Convention against Doping in Sport, to facilitate governments to apply a non-governmental document, such as the Code. The UNESCO Convention against Doping in Sport has been ratified in the end of 2020 by 191 UN country members [6]. In the same governmental direction, WADA has signed memorandum of understanding with the UN Office on Drugs to establish cooperation and information-sharing of sports doping [7].

Major efforts have been dedicated by WADA the years 2016–2021 toward the application of Code compliance [8] by the antidoping stakeholders and the independence of testing from sports organizations. The Code Compliance Monitoring Program [9] has been created. Antidoping organizations independence from sport and governments aims the exclusion of involvement of anyone with conflict of interest. WADA has published the Guide [10] to strengthen National Anti-Doping Organizations' (NADO) operational independence. The importance of compliance and independence have become several times obvious, as of the cases of Russian [11], as reported in "the Independent Person 2nd Report" by Professor RH McLaren [12] and International Weightlifting Federation doping scandals [13]. The WADA Accredited Laboratory organizational independence from sports or antidoping organizations has been also become mandatory in the 2021 IS for Laboratories (ISL) [14].

The WADA Accredited Laboratories' specifications are described in the IS for Laboratories and IS Prohibited List (List) [15]. At level three WADA documents for laboratories, there are 14 TDs, 23 TLs, 4 Guidelines together with a big volume of confidential information between Laboratories, World Association of Antidoping Scientists (WAADS) [16] and WADA Science Department. The analytical technology in use by the WADA Accredited Laboratories has become more sensitive and more specific, in other words, more prohibited substances and metabolites in lower concentrations in urine and blood samples can be detected with modern mass spectrometry

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(MS). One of the major challenges of the above laboratory documents is the protection of the clean athletes from environmental exposure to prohibited substances, that may be detected in antidoping analysis in low traces, for example, by meat consumption [4].

Antidoping movement has been connected to Olympic Games (OG), since International Olympic Committee (IOC) was the initial organizer of the antidoping system to support the Olympic movement up to 2004, when WADA undertook this role. A number of articles have described the history of the IOC/WADA Accredited Laboratories in OG [17–19]. Like the ancient Greek diary, which was based on the 4-year frequency of organization of OG in Olympia, Greece, this article came as the continuation of the previous work [20] and described the antidoping laboratory related activities of the years between the OG 2016 in Rio, Brazil until the 2021 OG of Tokyo. Analytical and pharmacological progresses, which resulted in WADA regulatory adaptations related to laboratory operations were examined through the changes of the WADA documents.

The World Anti-Doping Code (Code)

Two important elements have been introduced in the antidoping system in 2018. The first is the WADA's eLearning educational platform, ADeL (Anti-Doping eLearning), which constitutes the central hub for all of WADA's educational resources [21]. ADeL focuses to professionally educate target groups such as athletes, coaches, physicians, antidoping organizations' employees, parents, the Athlete Passport Management Units (APMU). The second is the creation of the non-profit antidoping organization International Testing Agency (ITA) [22], targeting transparency and independence of testing and results management, addressing conflicts of interest among testers and tested, increasing expertise and scientific support on the antidoping organizations' side and optimizing resource availability in testing programs.

The 2021 Code amended a number of important subjects from the previous version of Code of 2015: the role of the new ITA [22], the Russian doping scandals, the positive results from IOC sample re-analysis, the prohibited substances which are also substances of abuse, detection of unconscious exposure of athletes to contamination of prohibited substances from environmental sources, flexibility in sanctions, WADA's right to take possession of samples and data, the independence of NADOs and the compliance with international human rights 'norms. In level 2 of the WADA documents, three new ISs were created in the recent years: education, compliance and results management. All ISs can be found in the WADA website [3].

International Standard for Laboratories

Two versions of ISL in 2019 and 2021 [14] have been released in the last 5 years to harmonize ISL with the WADA documentation and the new ISO/IEC 17025:2017 [23]. Some important changes were for the independence and impartiality of laboratory staff, removal of samples procedure from a laboratory for retroactive analysis and quality assessment and requirements of Approved laboratories only for hematological Athlete Biological Passport (ABP). Blood and urine sample procedures have been unified in one chapter, the fifth of ISL to facilitate harmonization. Reorganization of WADA External Quality Assessment Scheme (EQAS) specifications and evaluation of Laboratory EQAS and routine analytical testing performance has been defined in two new chapters of ISL, the sixth and seventh. Procedural rules to describe laboratory disciplinary hearings has been also incorporated.

Analytical dimensions of International Standard for Prohibited List

WADA annually updates the List of prohibited substances and methods [24]. The 2021 List was modified and redesigned to improve navigation and usability. It includes 11 classes of prohibited substances (S0-S9 and P1) and three prohibited methods (M1-M3), after alcohol removal in 2017. One of main modifications of 2021 List was the introduction of the Substances of Abuse, which comprises as prohibited substances that are frequently abused in society as street drugs outside sports. Cocaine, diamorphine (heroin), methylenedioxymethamphetamine (MDMA, ecstasy) and tetrahydrocannabinol (THC) are examples of such Substances of Abuse. Moreover, other substances are being investigated to be classified as Substances of Abuse in the future [24]. List 's class M2.2, for intravenous infusion or injections, has been restricted for medical needs [25]. List's S1.1 anabolic androgenic steroids (AAS) were combined into one class and the sub-groups of AAS (S1.1a. exogenous and S1.1b. endogenous) have been removed since 2020 [26]. The technical document TD2021 for Isotope Ratio Mass Spectrometry (IRMS) is the tool to determine the substances' origin (i.e. whether they are endogenous or exogenous) [27].

Selective androgen receptor modulators (SARMs) have been included to the List since 2008 as other anabolic agents' class (S1.2). Thevis and Volmer studied fragmentation patterns of three selected SARMs drug candidates, GSK2881078, PF-06260414 and TFM-4 using both Liquid Chromatography Mass Spectrometry (LC-MS/MS) and Gas Chromatography Mass Spectrometry (GC-MS/MS), showing the mass spectral data for these substances in order to support the development of new methods and research of drug metabolism [28]. Gadaj *et al.* detected 14 SARMs in urine from different species, including humans, using semi-quantitative LC-MS/MS with Limit of Detection (LOD) range from 0.002 to 0.2 ng/ml [29]. Rading *et al.* investigated the *in vivo* metabolism of GSK2881078 after single oral administration and analyzing urine and hair matrices [30]. This study showed that intact GSK288107 could be detected in the both matrices at pg level. Geldof et al. evaluated *in vitro* metabolism of LDG-4033 and detected five metabolites by LC-MS/MS [31].

TGF-β superfamily is a multifunctional polypeptide that regulates physiological processes such as proliferation, differentiation, development of embryo and angiogenesis [32]. Sotatercept and luspatercept are examples of TGF-β signaling inhibitors and have a negative role in the regulation of physiological mechanisms such as erythropoiesis. WADA has introduced these substances in 2017 List. Additional clinical studies of sotatercept have been planned [33], while luspatercept has been granted approval and market circulation [34]. Walpurgis *et al.* characterized sotatercept and luspatercept in serum by immunoaffinity purification, tryptic digestion and LC-MS/MS [35]. In another study, the combination of antidoping detection of Erythropoietin Receptor Agonists (ERAs) and luspatercept has been achieved [36].

Hypoxia-inducible factor (HIF) stabilizers, also named HIF prolyl-hydroxylase inhibitors, play an important role in the regulations of HIF signaling. HIF stabilizers have been developed as therapeutic agents for a variety of diseases such as anemia of chronic kidney disease [37,38], ischemia and inflammation [39]. Many studies have been published related to metabolism and detection for HIF stabilizers, for example [40–48]. Recently, Mazzarino et al. developed new screening and confirmation analytical approaches to detect misuse of nine HIF inhibitors. This study's method has achieved LOD between 0.25 and 2.0 ng/ml and limits of identification (LOI) of 0.5–2.0 ng/ml. In addition, the evaluation of detectability for this method was performed by analyzing excretion studies' samples from daprodustat (GSK1278863), molidustat (BAY 85-3934) and roxadustat (FG-4592) [49].

Beta2 adrenergic agonists are a class of drugs that are commonly used to treat respiratory diseases such as bronchial asthma and chronic obstructive pulmonary disease (COPD) [50]. WADA has placed restrictions on the use of all beta2-agonists at all times, In-Competition (IC) and Out-Of-Competition (OOC) due to the potential use as anabolic agents and side effects. Based on the existing studies that are related to different routes of administration of salbutamol [50–54], formoterol, salmeterol [55] and vilanterol WADA has introduced the maximum allowed doses [24,56].

Hormone and metabolic modulators class (S4) is divided into four subclasses after merging of subclasses 4.2 'selective estrogen receptor modulators' and 4.3 'other anti-estrogenic substances' [24]. Myostatin and activin A are cytokines that belong to the TGF-β superfamily and are important regulators of skeletal muscle mass. Myostatin inhibitors are molecules like myostatin-binding proteins, myostatin-neutralizing antibodies and agents for reducing myostatin expression. Walpurgis *et al.* investigated detection of monoclonal human anti-ActRII antibody bimagrumab in serum by using three steps of approach: affinity purification, tryptic digestion and LC-High Resolution (HR) MS [57]. In a study, the treatment with bimagrumab was confirmed to be safe and effective for adiposity and metabolic disturbances of adult patients with obesity and Type 2 diabetes [58]. Domagrozumab (PF-06252616) is an example of anti-myostatin antibody that was developed for the treatment of Duchenne muscular dystrophy (DMD) by Pfizer pharmaceutical company. Walpurgis *et al.* established two complementary methods to detect domagrozumab and other related antibodies in serum using western blot and LC-HRMS [59]. However, Pfizer has announced that it is terminating two clinical studies evaluating domagrozumab for the treatment of DMD because it did not meet its primary efficacy end point [60]. The doping control laboratory in Cologne developed the detection of follistatin-based inhibitors of TGF-β metabolic reactions [61]. A thorough review has been published also by Cologne laboratory on this subject [62].

Monitoring Program is updated annually by WADA for substances to detect patterns of misuse in sport [63]. The recent application of MetAlign software for selected substances on the WADA Monitoring List, tramadol (TRA) and ecdysterone (ECDY) by using fast-retroactive processing of reduced LC HR full scan (FS) MS datafiles to reveal the prevalence of doping substances [64] has been developed.

Technical documents

Below the Code and the level two ISs, level three of the WADA documents incorporates the group of laboratory mandatory TDs [4]. TDs are purely technical, as their name indicates, and their revision is frequent often multiple times within a year.

Minimum required performance levels TD

The minimum required performance levels (MRPL) [54] define the minimum concentrations of LODs and LOIs the laboratories are obliged to achieve for the substances of the List. Those parameters are influenced by the pharmacological actions of the respective substances and the sport performance enhancement. The tolerance in the substances' detection divides the MRPL of substances into two categories: substances under zero tolerance, which should be detected in as low as possible concentration and substances with a minimum reporting limit of concentration below which the sample is reported negative even if the substance is detected. The specifications become more complex by time as it is reflected in the MRPL document and new classes of substances, like HIF stabilizers, have been added following the List updates.

The minimum reporting limits allow the athletes to use prohibited substances for therapeutic reasons without therapeutic use exemption (TUE), as the glucocorticoids. The novel approach by WADA under way to the 2022 List is the permission or prohibition of glucocorticoids in sport based on the pharmacological influence on performance enhancement and the risk of adverse effects on health. Substance-specific reporting limits are under introduction to the new version of MRPL TD to better distinguish prohibited and permitted use in sport [65].

EPO TD

The new TD2021EPO [66] has introduced many new specifications of ERAs analysis. Those new specifications comprise the use of sensitivity quality controls to ensure that the initial testing procedure (ITP) and confirmation procedure (CP) analytical performance is the same as of method validation experiments. The introduction of MRPL for ERAs in urine and blood together with relevant LOD and the use of the biotinylated monoclonal mouse anti-human EPO clone AE7A5 [67,68] are also new additions. A new immunoaffinity chromatography of anti-EPO antibody-coated sepharose gel beads immobilized in cartridges was developed for facilitating sample preparation by omitting the commonly employed urine pre-filtration step [69]. The introduction of internal standards (ISTDs) [70] made by rat EPO into the ERAs method was presented for quality control of the entire protocol. The practice of the athletes to apply low doses of recombinant EPO daily or every second day, instead of larger doses administrated once or twice or more per week, is an example of the difficulties faced by the antidoping Laboratories in ERAs analysis. In the study published by the laboratories in Seibersdorf (Austria) and Cologne (Germany) [71], the epoetin zeta biosimilar was detected after it was administered intravenously and subcutaneously to volunteers. Urine and serum samples were analyzed by applying the biotinylated clone AE7A5 EPO antibody with optimized sarcosyl polyacrylamide gel electrophoresis (SAR-PAGE) protocol.

Athlete biological passport

The idea of athlete biological passport (ABP) [72] was generated in the early 2000s to monitor hematological parameters for an athlete hematological profile. Even from the beginning of 80s, Manfred Donike in the doping control laboratory in Cologne established the ratio of testosterone to epitestosterone (T/E) as an ancestor ABP of exogenous testosterone administration when the ratio was exceeding 6. However, it was known that some male athletes have for all their tests measured normal ratio around or higher than 6. This information was being taken into consideration from testing authorities to not impose sanctions to those athletes. Even today, the T/E is the most important marker of the steroidal ABP module. Since ABP concentrates human, testing and analytical variations, the technical variability of testing must be minimized by harmonization of sample collection, transportation and analysis. For the harmonization of testing, WADA has created and maintains the ABP Operating Guidelines [73]. The objectives of ABP are to create doping scenarios and to provide information for target testing. For the hematological ABP module, further testing can be directed to Erythropoiesis-Stimulating Agents 1 (ESAs) or homologous blood transfusion (HBT). For the steroidal ABP module, further testing can be, for example, the use of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) to detect testosterone-like steroids administered exogenously. The human variability is attributed to single or combination of conditions such as physiological, doping, medical and confounding factors like training, use of nonprohibited substances that influence ABP [73].

The scientific knowledge for ABP evaluation is a subject for many research projects. For the steroidal module, Mullen *et al.* have examined the influence of menstrual cycle and emergency contraceptive administration [74]. Schulze *et al.* have evaluated the concentration of urinary steroids during different phases of the menstrual cycle and found that the median concentration of E was 133% higher in the ovulation phase compared with the follicle phase. In addition, serum T is a more significant biomarker for female T doping than E [75]. Pregnancy has a significant effect on the steroidal profile, which may lead to atypical results of ABP [76]. Mullen *et al.* have evaluated the hematological and steroidal modules of ABP, along with hormonal and micro ribonucleic acid (miRNA) biomarkers in men after administration of a single micro-dose of T gel [77]. Börjesson *et al.* have studied and measured hematological parameters, lipid profile and endocrine status in relation to the pervious anabolic steroids and other doping agents abuses in women and men [78]. In a recent study by Schulze and his colleagues presented that the combined ratio using testosterone sulfate (TS) and epitestosterone sulfate (ES) is more sensitive than the formal ratio T/E only after testosterone intramuscular administration in men. Furthermore, this combined ratio is not affected by the deletion polymorphism of the *UGT2B17* gene [79]. The steroid ABP influence by the administration of prohibited aromatase inhibitors aminoglutethimide, letrozole and anastrozole was studied in male and female volunteers [80]. The effect of intense training in steroidal ABP was examined and its stability was confirmed, as the ABP steroidal parameters are ratios of concentrations of steroids [81]. The influence of the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen on the steroidal ABP was investigated and it was proven that the daily administration of therapeutic dose affected steroid ABP parameters. Further studies are needed to clarify whereas the variation due to the NSAID administration is within or out of the normal variation of the ABP [82].

The continuous generation of new research data optimized the specificity and detection of hematological ABP for doping practices, like autologous and homologous blood transfusions, administration of ESAs, HIF stabilizers, etc. The harmonization of the blood collection and transportation conditions and the analytical quality control are fundamentals for the specificity and sensitivity of hematological ABP. In 2018–2019, ABP was completed with the change of the hematological analyzers of the WADA laboratories from Sysmex XT/XE to Sysmex XN series, with the simultaneous activation of the external quality control of XN CHECK and the networking of the Internet-based external quality control service Sysmex Network Communication System (SNCS).

For the hematological ABP module, the focus was oriented to the significance of plasma volume (PV) in the profile evaluation. The influence of endurance exercise on PV and ABP parameters was investigated in triathletes and it was proven that, because of hemodilution, hemoglobin (Hb) and hematocrit (Hct) decreased significantly in PV depended manner, followed by an increase of reticulocyte percentage (ret%) [83]. PV variations in cyclists participating in racing, with analogous Hb variations were recorded by another study, suggesting that the hemodilution parameter improved ABP evaluation [84]. Additional information from a model of PV estimation based on plasma transferrin, albumin, creatinine, total protein and low-density lipoprotein improved specificity of ABP evaluation related to PV fluctuations [85]. The iron supplementation and the athletes' exposure to high altitude and hypoxia conditions was investigated proving the ABP atypical findings could result from oral or intravenous iron supplementation [86]. The seated body position for 5–10 min before blood sample collection has been recommended to reduce variation in Hb and Hct [87]. The effect in blood ABP of a 14-day living/training under controlled hypoxia conditions has been studied[88].

The influence of the two extremes of athletes' hydration condition, such as dehydration [87] and hyperhydration [73] during the blood ABP sample collection have been investigated. In similar studies for the steroidal ABP module, the urine specific gravity (SG) correction has to be applied to the correct steroids' concentrations for diluted samples due to hyperhydration [89]. The series of studies [89–93] confirmed that the hyperhydration of athletes during sample collection do not have a masking effect through the urine dilution with respect of ABP parameters, since urine SG concentration correction is always applied.

Autologous (ABT) and homologous (HBT) blood transfusions continue to be one of the most challenging practices [94]. The operation Aderlass [95] run by police is a proof why analytical detection of ABT and HBT is limited. In analytical field, an innovative study incorporated a hemoglobin profile index computed using different hemoglobin variants to identify post-withdrawal and post-transfusion blood samples of volunteers [96]. In another study for ABT detection, the detection of red blood cell microparticles after blood bag storage conditions was examined [97]. For the same purpose of ABT detection, the idea of plasticizers detection from the blood bags used for storage was examined in an untargeted urine metabolomics study, which revealed several plasticizers' metabolites as pattern of detection [98].

APMU [99] network has been organized by WADA since 2014, however the network was harmonized by the relevant WADA TDs in 2019 and 2021 [73]. The APMU(s), currently hosted by 16 worldwide WADA Accredited Laboratories. APMU evaluate the ABP on behalf of testing authorities for athletes under testing authorities' testing plans. An external group of steroidal and hematological modules experts complement the internal APMU scientists to provide independent assessments. Scientific knowledge is used for ABP reviews based on athlete's anonymity. The entire APMU activity is conducted within specific ADAMS [100] module. ABP reviews can range from a normal ABP to an ABP of an athlete who 'very likely' used a prohibited substance or method and 'very unlikely' that it is the result of any confounding factor or medical cause. In the latter cases, extensive investigation is conducted, which may result to an antidoping rule violation and penalty to the relevant athlete. The APMU(s) are active to review passports during the Tokyo OG in a 24 h reviewing time period submitting recommendations for further actions to IOC and ITA, which has the antidoping responsibility on behalf IOC, while athletes are still present in Japan.

The current chapter discussed only a small part of wealth and diversity of the generated new scientific knowledge related to ABP during the last 5 years. The application of Artificial Intelligence in the antidoping detection and particularly in the ABP and APMU(s) fields was the theme of a recent WADA call for research [101]. The existing ABPs can become more specific and more sensitive with the inclusion of additional biomarkers. In this direction, a study from the doping control laboratory in Doha (Qatar) proposed the creation of additional endogenous steroid ratios from a combination of current endogenous steroids and sulfate-conjugated steroids fraction detected in ITP [102]. The significance of quantitation of endogenous steroids in blood has been proved through a recent Court of Arbitration for Sport (CAS) decision as a complementary approach to the urinary ABP for the detection of T doping [103]. Actually, CAS has issued the decision in which Ukrainian athletes were found to have administered exogenous T detected in blood samples [104]. In blood doping, research on RNA has provided promising results to reveal EPO doping such as the measurement of *ALAS2* gene RNA in blood after EPO administration can become a useful complementary parameter to hematological ABP [105]. The combination of cell proteins CD71 and Band3 with RNA-based biomarkers has been suggested for the detection of autologous blood transfusion [106]. Finally, in another study the effect of blood storage in citrate phosphate dextrose adenine (CPDA1) on the red blood cell (RBC) membrane proteome was investigated [107].

IRMS

IRMS is the main analytical methodology to report the exogenous origin of endogenous steroids in urine samples. The last 5 years, a number of changes have been incorporated to the TD2021IRMS [27]. For analytes outside the testosterone metabolism, 6α-hydroxy-androstenedione (6α-OH-AD) and prednisone and prednisolone have been added to already existed boldenone metabolites and formestane. Another change referred to the use of an additional second Endogenous Reference Compound (ERC) when reporting adverse analytical findings (AAFs), where the preference of the first reported ERC remains to be pregnanediol. The doping control laboratory in Ghent (Belgium) presented a validated method unifying the sample preparation of all IRMS analytes [108]. In relation to new potential analytes for IRMS, epiandrosterone sulfate (EpiA-S) has been studied as a target analyte for the determination of T misuse. EpiA-S was found to be a potential biomarker for exogenous origin that can be detected for longer time than the other urinary metabolites of T [109]. Detection of 7-oxo-dehydroepiandrosterone (7-oxo-DHEA) administration by IRMS was performed by the novel metabolite 5α-androstane-3β, 7β-diol-17-one [110].

Decision Limits, hCG, LH, 19-NA, LDPs TDs

The TD2021DL [56] specifies the quantitative method parameters for the substances with a reporting threshold and the measurement uncertainty applications. The increase of reporting concentrations significant figures from 2 to 3 and the introduction of urine SG measurement uncertainty to the SG calculations, were the main changes. The main changes in the TD for human Chorionic Gonadotrophin and Luteinizing Hormone (hCG/LH) in the last 5 years was the introduction of total hCG measurement in ITP only, where CP has to be conducted only for the intact hCG molecule [111]. The main change conducted in the TD2021NA [112] for the analysis and reporting of nandrolone metabolite 19-norandrosterone (19-NA), was that the CP of 19-NA does not require accurate quantification. The TD for the creation of Laboratory Documentation Packages (LDPs) to report details of the sample chain of custody and analysis related to AAF and atypical finding (ATF) reports was substantially upgraded in analytical details, from the respective in force in 2016 [113].

GH TD

The interest to combat the use of recombinant human growth hormone (rhGH) in sports remains very intense. The methods involved in the rhGH detection include the isoform detection method [114] and the biomarker monitoring [115]. The main technological progress conducted after the 2016 OG was the development of the LC/MS top-down detection of intact IGF-Imethod, supported by data and validation among five laboratories [116].

Dried blood spot

The application of whole blood dried on a piece of filter papers is known as dried blood spot (DBS) collection technique. Samples are obtained from finger pricking [117] and from upper arm [118]. DBS has been demonstrated as an important complimentary matrix for sports drug testing, which do not replace urine and blood analysis [119]. Numerous detection methods for doping agents using DBS matrix have been developed highlighting the advantages of this technique. These advantages include the minimization of invasiveness of DBS in blood collection. DBS facilitates the work of doping control agencies by its logistical advantage and its cost–effectiveness [120]. In March 2019, WADA and IOC organized a meeting to set the DBS as a valuable addition to the approved testing methods. Recently, WADA approved the use of DBS to test for banned substances in Tokyo Olympics for the first time. Also, it is planned to implement this new method for the 2022 Winter Olympic and Paralympic Games in Beijing, China [121].

Several analytical methodologies have been published for antidoping DBS application. The development of a fully automated DBS sample preparation for lower molecular mass peptides and other nonpeptide agonists has been reported [122]. The use of DBS to monitor ABP serum steroid profile including T concentrations was presented and comparted with direct blood analysis [123]. Recombinant human erythropoietin (rhEPO) administration and detection of *ALAS2* RNA were investigated in DBS [124]. IGF-I was successfully quantified in DBS blood collected by volumetric absorptive microsampling (VAMS) device using LC-MS/MS [125] as part of the GH biomarker screening method [115]. Analysis of insulin and insulin analogs by LC-MS/MS analysis from DBS collection cards has been described [126]. DBS sampling and analysis for detection of EPO, biosimilars and synthetic derivatives was studied by the doping control laboratory in Barcelona (Spain) [127]. Small molecule narcotic oxycodone and metabolites detection was developed after blood/plasma and urine microsampling using DBS, dried plasma spot (DPS), dried urine spot (DUS) and VAMS approaches [128]. Finally, the DBS sampling for monitoring hGH isoform method [114] has been investigated [129].

Gene doping

WADA circulated for first time the Laboratory Guidelines for the gene doping detection method using real-time polymerase chain reaction (PCR) [130]. The method aims to become Accredited by a number of WADA laboratories for routine use. The method principle is focused on the analysis of complementary DNA (cDNA) derived from the gene's messenger RNA (mRNA) sequence. In endogenous genes of human genomic DNA, the protein-coding exons are separated by noncoding introns. However, in cDNA-based transgene there are no introns but only adjacent exon/exon junctions [131]. WADA has created an analytical method to support the quality control of the analysis and the subsequent accreditation of the gene doping detection method focusing on method validation specifications, positive and negative template controls, PCR sensitive controls, primers, etc.

Technical letters

Technical letters (TLs) were initially letters sent by WADA confidentially in 2000s to WADA Accredited laboratories network to create attention to analytical subjects outside the TDs. Since then TLs became public documents [4]. All WADA TLs have been updated to reflect the 2021 Code. Some TLs have influenced the laboratories operation more than others. The selection of ISTD is an essential step for the analysis of steroids. When microbial activity is present in urine samples, the use of 17-methyltestosterone (MT) as ISTD may result in the synthesis as artifacts of 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α, 17β-diol, MT metabolites. In TL08, laboratories are strongly advised to use deuterated or C^{13} carbon labeled ISTD of endogenous steroids instead of MT or any other exogenous AAS [132]. In the same concept of artifacts, TL10, TL19, TL20 and TL21 highlighted the biotransformation of some endogenous and/or exogenous steroids that may have been produced by microbial activity. Examples of biotransformation are: the formation of boldenone, boldione and their metabolites, androst-1-ene-3,17-diol from testosterone or androsta-4-diene-3,17-dione [133], the endogenous glucocorticoids cortisol and cortisone conversion by microbial activity into prednisolone and prednisone, respectively [134], the detection

of prohibited steroids as androstenediol and aromatase inhibitor arimistane at low concentration [135] and the detection of 4-androstene-3,6,17-trione (6-OXO) produced by transformation of DHEA [136].

TL14 incorporated the procedures to be followed by laboratories in collaboration with testing authorities if there are differences in color and turbidity of urine characteristics between the A and B samples [137]. The use of zilpaterol, zeranol, clenbuterol and ractopamine as growth promoters for animal farming in some countries can be the source of a low concentration of these prohibited substances in the urine after consumption of the contaminated meat. WADA set an estimated concentration of 5 ng/ml in urine as reporting limit in TL23 [138]. The detection of one or more of six diuretics, namely acetazolamide, bumetanide, furosemide, hydrochlorothiazide, torasemide and triamterene, at low concentration in the urine samples due to contamination of pharmaceutical drugs is the subject of TL24 [139]. WADA accredited laboratories should report the result as negative if the urinary estimated concentration is equal or below to 20 ng/ml for those diuretics with the exception that the sample had been collected from an athlete participating in a sport or discipline that uses weight classes.

Long-term sample storage & retroactive data reprocessing & analysis

The idea of long-term storage of negative reported samples and their retroactive analysis has been introduced by IOC for the 2000 Sydney OG. The same concept was followed by IOC for the Athens 2004 OG samples, where retroactive analysis in 2012 [140] revealed four additional positive cases. The retroactive analysis of Beijing 2008 and London 2012 samples was conducted, and the IOC 2017 reanalysis program report announced 101 new AAFs [141]. Retroactive analysis is based on the advantage of the improved analytical technologies, knowledge of designer drugs and new metabolites, which prolong the detection of substances with zero-tolerance, especially the anabolic steroids. For the Tokyo games, the IOC appointed ITA [22] managed the global long-term sample storage program. ITA has established a centralized long-term storage facility for the samples to be collected during the Tokyo OG but also samples collected in 2020 from Olympic athletes.

The retroactive analysis can be greatly facilitated by the application of the MetAlign software [64,142] for fast and massive reprocessing of LC-MS/MS datafiles of samples analyzed in full-scan MS acquisition mode after electrospray ionization (ESI). The application of the MetAlign software allows the selection for reanalysis only of suspicious samples from a pool of samples under long-term storage for substances suitable to be detected by the ITP LC-MS/MS method [102,143].

Expanding the window detection of exogenous AAS for ITP & retroactive analysis

The progresses in laboratory methods were thoroughly described in the annual banned-substances review [144–148]. Several studies have been published related to the identification of long-term metabolites of prohibited substances, *in vivo* and *in vitro* metabolism and synthesis of reference materials. The production of long-term metabolite reference material of oxymetholone [149] and long-term metabolite of oral-turinabol [150] has been achieved. Martinez-Brito *et al.* evaluated the *in vivo* and *in vitro* metabolism of the MT related to hydroxylated metabolites using gas chromatography-time-of-flight (GC-Q/TOF) and GC-MS/MS [151]. Polet *et al.* reported new long-term metabolites of oxymesterone and mesterolone by using gas chromatography-chemical ionization-triple quadrupole mass spectrometry (GC-CI-MS/MS) which significantly expanded the detection window for these steroids [152].

Sulfate Phase II conjugated metabolites are also considered as long-term metabolites of exogenous AAS. The doping control laboratory in Ghent analyzed steroids' nonhydrolyzed sulfate metabolites using GC-MS/MS instead of LC-MS/MS to detect the metabolites' artifacts [153]. Another study by this laboratory [154] developed a method for identification of new sulfo-conjugated metabolites for methenolone and drostanolone using GC-MS/MS. The results showed the identification of nonhydrolyzed sulfate metabolite of methenolone detected with longer detection window up to 17 days compared with hydrolyzed metabolites.

The doping control laboratory in Cologne developed a protocol for new steroids metabolite detection using stable isotope-labeled drug with H-IRMS analysis [155]. Piper *et al.* evaluated the *in-vivo* metabolism of methylstenbolone and reported two new long-term metabolites [156]. Putz *et al.* from the same laboratory used the similar metabolite identification approach for trenbolone, which included analyte acetylation and LC-HRMS/MS. The results of this study showed 20 metabolites of trenbolone, including four main metabolites identified as trenbolone diketone and trenbolone diol derivates which were excreted as glucuronide acid and sulfate metabolites with detection window up to 6 days [157].

The Zebrafish Water Tank (ZWT) model has been implemented in the antidoping area by the doping control laboratory in Rio de Janeiro (Brazil) for evaluating metabolism of prohibited substances *in vivo*, due to zebrafish metabolic similarities to humans [158,159]. Matos *et al.* studied the metabolism of stanozolol using ZWT experimental setup. The study showed the detection of ten metabolites after 8 h of experiment, four hydroxylated sulfates and six glucuronide metabolites as 3′OH-stanzolol-glucuronide, 16ß-OH-stanozolol-glucuronide, 17epi-stanozolol-N-glucuronide and stanozolol-O-glucuronide [160].

Sensitive analytical methods are essential for detection of endogenous AAS esters because of their fast hydrolysis resulting in low plasma concentrations. Subsequently, existing literatures studies have been published to detect testosterone esters in the blood using LC-MS/MS or GC-MS/MS [161,162]. Van Renterghem *et al.* developed ultra-sensitive method and identified nine testosterone esters and two nandrolone esters at very low concentration in human plasma using GC-CI-MS/MS [163]. de la Torre and his colleague reported 14 testosterone esters and two nandrolone esters in plasma by applying full validated method using LC-MS/MS based on the Girard P derivatives's formation [164].

Antidoping laboratory statistics

WADA testing figures reports have showed the constant increase of analyzed Olympic sports' samples throughout the years [165–168]; from 193,345 samples in 2016 to 227,032 in 2019 as shown at Supplementary Figure 1. Furthermore, the highest number of reported AAFs was 1927 of 2016 as illustrated on Supplementary Figure 2. A reduction in the absolute percentage of AAFs in 2019 was about 0.97% compare to 1.6% in 2016. The introduction of meldonium to the 2016 List resulted in a spike in the number of AAFs. Meldonium only, resulted in 515 positive cases, being the most popular hormonal modulator. However, its usage has decreased significantly to only 79 findings in 2019. Meanwhile, the S4.2 anti-estrogen tamoxifen has jumped from making only 8% of the total cases within its group to 20% in 2017 reaching to 22% in 2019, replacing meldonium as the most used hormonal modulator. Supplementary Table 1 represents the percentage of selected prohibited substances which were identified as AAFs in each class from 2016 to 2019. According to WADA annual reports stanozolol was the most popular AAS anabolic agent. Stanozolol makes up almost 50% of AASs AAFs followed by drostanolone. B2-agonist and anabolic agent clenbuterol continue to make up most positive cases in S1.2 class, however the proportion of using the SARM LGD-4033 as a doping agent increased sharply reaching 62 positive cases in 2019 compared with only 6 cases in 2016. For diuretics, furosemide and hydrochlorothiazide have been the most used diuretics throughout the years 2016 till 2019. An overall increase of AAFs reported for ERAs from 66 positive cases in 2016 to 92 positive cases in the year 2019 is shown at Supplementary Figure 3. This increase could be due to the respective increase in the number of tests for ERAs, together with hGH and Growth Hormone Releasing Factors (GHRFs). Moreover, the abuse of the ERA methoxy polyethylene glycol-epoetin beta (CERA) has decreased substantially with only two positive cases in 2019 compared with 20 AAFs in 2016.

COVID-19 impact

The unprecedented COVID-19 pandemia resulted in a reduction of sports activities and testing in 2020, reduction in testing and samples. The sample collection numbers were down especially in the early months of the pandemic from March to June 2020. This remains the same in early months of 2021 as the number of events taking place is still low compared with before COVID-19. However, the latest testing figures by WADA [169] revealed that the number of collected samples started to increase. To assist the worldwide testing, WADA published two important documents: the Question and Answer (Q&A) document for athletes [170] and the Guidance, which outlined how antidoping organizations can organize their testing programs as recommended in health and hygiene procedures [171].

New approaches in sample collection procedures adapted to the COVID-19 limitations to reduce spread of infections were created. United States Anti-Doping Agency (USADA) organized the project Believe [172]. The project Believe was based on the remote OOC testing of athletes without the presence of a Doping Control Officer. The sample collection comprised urine and DBS and it was based on the distribution of sample collection material to athletes, notification of sample collection within an hour, video-linked collection of athlete's matrices, a temperature monitoring strip to verify body temperature, paperless doping control documentation and shipment of collected samples by the athlete to the collection authority. Anti-Doping Denmark, in a similar remote collection approach to USADA project Believe, introduced additional DNA analysis and matching to prove the origin of the urine samples collected, together with temperature monitoring [173]. In another approach, the use of urine marker,

different polyethylene glycols (PEG) in different PEG combinations per athlete to be administered, the authenticity of the collected urine sample was tested [174]. PEGs are considered nontoxic and safe, 60 min. after ingestion they are 100% traceable in urine. The subsequent analysis and laboratory confirmation of marker code excluded of sample manipulation.

WADA Accredited Laboratories' operations were also influenced by the COVID-19 pandemic. As the number of samples was reduced in an unexpected way the first months of 2020, several administrative decisions by laboratory had to be taken related to temporary staff employment, resources and activities to redirected in COVID-19 new conditions, etc. For the analysis safety from possible SARS CoV-2 spread from samples to staff, it was considered that laboratories handle anonymous biological samples without knowing their microbiological and potentially infectious status; whether they contain hepatitis virus, HIV or any other infectious pathogen. Consequently, routine security measures should be put in place for all operations including SARS CoV-2 measures. For example, to avoid formation and exposure to aerosols, cleaning surfaces with for example alcoholic solutions has to be applied. In one of the security measures brought into the laboratories' attention was the introduction of Biosafety Level 2 hoods. In a recent study performed by doping control laboratory in Doha, the stability of the antidoping analysis substances under the UV-C light exposure was examined. Study resulted that substances remained stable and intact under the conditions of UV-C radiation [175].

Conclusion & future perspective

WADA Accredited Laboratories apply the most advanced analytical technologies to detect a wide variety of prohibited molecular structures, from polar to nonpolar, from small molecules to proteins of hundreds of thousands daltons molecular weights. All these substances have to be analyzed with the absolute reliability using ISO 17025 and WADA Accreditations, with zero tolerance of non-compliances to the mandatory ISs, TDs and TLs, sometimes at picograms level concentrations in urine, blood or the newly introduced DBS, in massive number of samples, in fast turnaround times and with minimum costs. This environment of specifications justifies the characterization of the laboratories as the cornerstone stone of the antidoping system. At all times, the OG laboratory becomes the center of the antidoping interest. This review showed the conditions that the Tokyo Accredited Laboratory implemented to ensure the successful antidoping program of the largest and most demanding sport event of the entire world.

Executive summary

- Expending detection time window of exogenous anabolic steroids and other substances by implementation of new long terms metabolites in the ITP, combined with the use of advanced analytical instrumentations such as GC-MS/MS and LC-MS/MS. This approach is one of most important challenge of WADA-accredited laboratories.
- Retroactive analysis of negative samples is a powerful tool in antidoping analysis to detect unknown substances as new metabolites of prohibited substances and illegal drugs.
- Future development and assessment of Athlete Biological Passport.
- International Testing Agency has been assigned by IOC with full cooperation from WADA to organize and manage of the global long-term sample storage and reanalysis of the athlete samples collected in Tokyo Olympic Games.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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