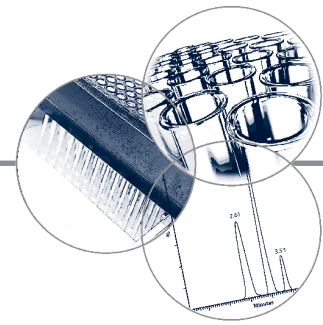

Advances in the detection of designer steroids in anti-doping

The abuse of unknown designer androgenic anabolic steroids (AAS) is considered to be an issue of significant importance, as AAS are the choice of doping preference according to World Anti-doping Agency statistics. In addition, unknown designer AAS are preferred since the World Anti-doping Agency mass spectrometric identification criteria cannot be applied to unknown molecules. Consequently, cheating athletes have a strong motive to use designer AAS in order to both achieve performance enhancement and to escape from testing positive in anti-doping tests. To face the problem, a synergy is required between the anti-doping analytical science and sports anti-doping regulations. This Review examines various aspects of the designer AAS. First, the structural modifications of the already known AAS to create new designer molecules are explained. A list of the designer synthetic and endogenous AAS is then presented. Second, we discuss progress in the detection of designer AAS using: mass spectrometry and bioassays; analytical data processing of the unknown designer AAS; metabolite synthesis; and, long-term storage of urine and blood samples. Finally, the introduction of regulations from sports authorities as preventive measures for long-term storage and reprocessing of samples, initially reported as negatives, is discussed.

The World Anti-Doping Agency (WADA) [1], which is considered to be the accepted organization by sports and governmental organizations worldwide to combat doping in sports, revises and publishes at least once per year the 'Prohibited List' as an International Standard [2]. The List identifies substances and methods that are – according to the **WADA Code** [3] – prohibited as doping, because of their potential of either enhancing performance or masking drug abuse. The substances of the List are claimed to induce pharmacological effects on the cell, the tissues and the organism. Anabolic Agents constitute Class S1 of the List and they comprise the following drug categories with anabolic action: exogenous (synthetic) and endogenous anabolic androgenic steroids (AAS), as well as other anabolic agents such as selective androgen-receptor modulators (SARMs). Examples of drugs and medicines that fall under the Class S1 are the synthetic AAS stanozolol, metandienone, oxandrolone, tetrahydrogestrinone, oral turinabol, SARMs, zeranol, and so on. However, drug interaction with cells to induce a certain pharmacological effect can be achieved by several structural features of the drug molecule, which practically creates an unlimited combination of the molecular features that could provide the particular effect. Since the List comprises prohibited pharmacological effects and respective drug categories, it is not possible to be exhaustive, hence, the following phrase has been added: *"and other substances with a similar chemical structure or similar biological effect(s)"* [2]. The meaning of the last phrase is that prohibited substances are not only those referred to as examples in the List, but also any other molecule, known, secreted or designed in the future, legally marketed or not, with or without clinical studies, having the same pharmacological effect.

The WADA accredited laboratories [4] perform the analysis mainly in urine samples, detecting small drugs contained in the List by using explicitly MS, either coupled to GC or LC. Detection and reporting of prohibited substances is based on specific criteria described in the WADA Technical Document for Identification Criteria for Qualitative Assays [5]. According to this document, in order to report for a violation of the List, laboratories must match in strict ranges chromatographic retention times and abundances of ions specific for the compounds of interest, both in the athlete's sample and in a sample originating from an excretion study or a synthetic reference material analyzed contemporaneously. Without the existence of the reference material, the reporting of a prohibited substance of the List in an anti-doping sample is impossible. As a result, there is a motive for the unethical scientists to create new molecules unknown to the anti-doping community, the designer drugs. The designer drugs are structurally modified analogs or derivatives of known substances, which were never approved

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Key Terms

WADA Code: The Code is the fundamental and universal document upon which the World Anti-Doping Program in sport is based. The purpose of the Code is to advance the anti-doping effort through universal harmonization of core anti-doping elements. It is intended to be specific enough to achieve complete harmonization on issues where uniformity is required, yet general enough in other areas to permit flexibility on how agreed-upon anti-doping principles are implemented.

Nutritional supplements: Preparations intended to provide nutrients that may otherwise not be consumed in sufficient quantities such as aminoacids, minerals, vitamins, fatty acids, fiber. They can be contaminated with steroids either marketed or unmarketed.

for human use in the past or never made it to production by pharmaceutical companies. They are used by cheating athletes in order to avoid detection by the WADA laboratories. Designer drugs induce less, similar or better pharmacological effect and usually circulate in the market without following formal regulations (labeling, approval and clinical studies) or via the Internet as **nutritional supplements**. Another motive for illegal laboratories to produce designer drugs is to avoid legislative limitations imposed on known molecules because of public health issues.

The current Review presents several aspects of the designer AAS in sports doping. The idea of designer AAS has been around for quite a while and elements of their history, as well as the current situation, are of great importance for both the anti-doping science and public health in general. Since the financial interest to produce new designer AAS is substantial, the rationale behind the molecular changes of the already existing AAS to create new designer molecules is explained later on in the article. The problem of the production and circulation of illegal molecules is known to the sports and public authorities and certain measures have been taken against illegal laboratories. A list of the designer synthetic AAS is presented in 'The chemistry' section. The use of designer AAS does not only appear in human sports, but also in animal racing samples as well. The anti-doping laboratories have made progress for the detection of designer AAS using MS and bioassays. Anti-doping laboratories, guided by the need of elucidating the metabolism of the designer AAS, have adopted sample preparation techniques and performed synthesis of designer AAS metabolites. However, *in silico* predicted analytical data related to designer AAS have also been used. In addition, the sports authorities have introduced the element of time in the fight against cheating athletes: *"I cannot catch you now; I'll catch you later, when I know more about the designer drugs you are using"*. As a result, accredited laboratories have made relevant adaptations in their procedures such as long-term storage of samples and data reprocessing of already analyzed samples that were initially reported as negatives.

The past & present of the designer AAS

Since the 1970s, sports authorities have banned the use of AAS and other performance-enhancing drugs. Nonetheless, since 1966, in East Germany, the German Democratic Republic (DDR) government and its state security 'Stasi' coordinated the development of new synthetic AAS to enhance sports performance [6]. No further anti-doping regulations from official authorities had been established until then, thus, no doping rules' violation existed. A typical example DDR synthetic AAS is the famous oral turinabol (or dehydrochormethyltestosterone) [7].

After 1982, the DDR regime also created endogenous designer AAS to escape the anti-doping tests for testosterone abuse, which were organized by the International Olympic Committee (IOC), the International Association Athletics Federation (IAAF) and the anti-doping Laboratory of Cologne, West Germany [8]. Epitestosterone and androstenedione were also included in the synthesized endogenous steroids of that time period. The rationale behind the creation of designer endogenous AAS takes into consideration the fact that athletes trying to avoid the detection of synthetic AAS were interrupting the relevant therapy close to the competition periods, changing to taking testosterone esters. Exogenous testosterone was mixed with endogenous, making its direct urinary detection impossible, due to the fact that the mass spectra of the endogenous and the exogenous preparation are identical. Its indirect detection is based on the measurement of the ratio testosterone to epitestosterone (T/E) [8]. Epitestosterone is the inactive isomeric molecule of testosterone and its biosynthesis is inhibited after testosterone intake. The mean human population statistic for the urinary T/E is close to unity and the threshold ratio chosen to be the limit for doping purposes was set to 6:1 by both the IOC and the IAAF. To circumvent the anti-doping controls after the abuse of testosterone esters, DDR sports medicine administered athletes with testosterone and epitestosterone esters produced by the state pharmaceutical manufacturer Jenapharm [6]. Since 2005, the WADA has changed the reporting threshold for T/E from 6:1 to 4:1 in order to improve the sensitivity for the detection of T misuse [8] (see also the 'Endogenous designer AAS' section).

Nowadays, two trends for the circulation of designer AAS exist: the first trend comprises the creation of novel molecules in order to be used by cheating athletes without failing doping tests. Since the 1980s, MS detection of synthetic AAS has improved, together with improvements in anti-doping system regulations after WADA's activation in 2004. As a result, cheating athletes switched to the abuse of designer AAS. The most

striking example was the Bay Area Laboratory Co-operative (BALCO) case [9]. BALCO was a San Francisco Bay laboratory that was supplying steroids to athletes. BALCO was initially known as a vitamin and mineral shop, which was later transformed to a laboratory that illegally produced black market steroids sold to elite athletes of baseball, American football and athletics. The 'products' of BALCO comprised the designer AAS norbolethone [10], the tetrahydrogestrinone (THG) [11] and the 'cream', – a salve containing mixture of testosterone and epitestosterone. Norbolethone is a synthetic AAS that was available as a pharmaceutical in the 1960s; however, it was never marketed due to its toxicity. THG is also a designer AAS. The 'cream' was widely used by athletes because it gave normal T/E ratios following administration. Another famous synthetic AAS, seized by Canadian customs in 2004, is desoxymethyltestosterone (MADOL or DMT) [12,13] that was initially detected by the US Accredited Laboratory of University of California, Los Angeles (UCLA; CA, USA) [12]. It is worth mentioning that no Adverse Analytical Finding (AAF) for elite athletes is related to MADOL. It is probable that the UCLA and the Canadian Accredited Laboratories [12,13] timely communicated the detection data to all WADA accredited laboratories and in this way MADOL was no longer a tempting substance for cheating athletes. The cream is another illegal preparation for avoiding detection of testosterone abuse, although it is less effective than testosterone injections. In 2008 the Cologne Accredited Laboratory (Germany) revealed an important case of the abuse of the designer synthetic AAS methyltrienolone, involving 11 Greek weightlifters [14]. The origin of the methyltrienolone synthesis is unknown, but sanctioned athletes claimed the use of Chinese nutritional supplements.

The second trend for the circulation of designer AAS is the nutritional supplement market. Several countries, such as the USA, have introduced legislations to restrict the production and circulation of nutritional supplements based on AAS, such as the US Anabolic Steroids Control Act, 2004 [15]. Nutritional supplements circulate through the Internet, in shops, in gyms, and so on. Nonhormonal supplements such as vitamins and amino acids may contain designer AAS not declared on the labels of the products [16]. Unfortunately, several reports have been published relating these nutritional supplements with AAF cases in doping controls [17,18]. A thorough review was recently published by Teale *et al.* describing the phenomenon of designer drugs for the entire spectrum of the prohibited drug classes for doping control [19].

Authorities against illegal laboratories

In May 2011, WADA circulated guidelines with the title: *Coordinating Investigations and Sharing Anti-Doping Information and Evidence* [20]. In this document, WADA recognizes the crucial role of the National Anti-doping Organizations (NADOs) to expand the fight against doping, apart from their existing anti-doping programs, with further measures to be taken against illegal laboratories and illegal substances trafficking networks. As expected, the BALCO case is referred to in the document. BALCO's activities were revealed with the involvement of the United States Anti-doping Agency (USADA) [9]. Another important investigation against illegal laboratories held in the USA in 2007 – the Operation Raw Deal – is mentioned [20]. New elements of the fight against doping are described in this report [20]:

- The concept of 'non-analytical' anti-doping rule violations;
- Perpetrators falling outside sport's authorization;
- Activating the public authorities in the fight against doping in sport; and, finally;
- Strengthening relationships between NADOs and public authorities.

The Memorandum of Understanding between WADA and Interpol is also published, showing the importance of police involvement in the fight against doping [20]. Three other reports [21–23] also associate the fight against doping with the reinforcement of national legislations. The first report [21] deals with the illegal drugs trafficking in various countries. Another report studies the Italian situation of doping in sports [22]. This report, which can be considered as indicative for many other countries, examines Italy's anti-doping criminal law experience with a twofold purpose: to analyze the production and distribution of doping products; and, to give evidence of how anti-doping criminal provisions and their enforcement can contribute to improve the fight against doping, both within and outside the sports community. The multilateral use of legislation to control the production, movement, importation, distribution and supply of performance-enhancing drugs in sport (PEDS) by several countries is the subject of a report written by Houlihan and García in 2012 [23]. Furthermore,

Key Term

Designer steroids: Steroids synthesized to closely resemble existing known compounds but with sufficient chemical diversity to evade doping control tests.

The Australian Crime Commission conducted an investigation and published a report in 2013 examining the extent to which organized crime is related to illicit drug markets [24].

The aforementioned reports make several references to the role of the Chinese pharmaceuticals industries in the production of raw materials for prohibited drugs. Aligned to these references, WADA's General Director made a statement in February 2013: *"99% of the raw materials that are used through the Internet to make up in your kitchen or your backyard laboratory are emanating from China"* [25]. However, J Zhixue, the head of Chinese NADO, replied immediately [26] asking for evidence concerning the alleged "99%", albeit admitting anti-doping problems in China.

The International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) and WADA have collaborated to combat the latest doping techniques [27] announcing the following declaration: *"The Joint Declaration on Cooperation in the Fight against Doping in Sports facilitates voluntary cooperation between IFPMA member companies and WADA to identify medicinal compounds with doping potential, minimize misuse of medicines still in development, improve the flow of relevant information, and facilitate development of detection methods."* The WADA report on the *Lack of (In) Effectiveness of Testing Programs* published in May 2013 [28] completes a thorough description of the problem of illegal drugs' circulation in sports.

Designer synthetic AAS: the chemistry

Designer AAS are substances with sufficient chemical diversity from known AAS, developed either in the past for clinical practice, or to evade doping control from official doping authorities. These designer AAS pose a serious health risk to consumers due to limited available pharmacological and toxicological data. The male hormone testosterone (Figure 1) is the basic steroidal structure upon which a considerable number of modifications can be applied in order to achieve the design of novel molecules with enhanced anabolic potency and reduced androgenic effect.

Androgens mediate their action through their binding to the androgen receptor (AR) [29,30]. Besides natural androgens, AR binds a variety of synthetic molecules with different affinities. AR ligands are classified as steroidal or nonsteroidal based on their structure, or as agonist or antagonist based on their ability to activate or inhibit transcription of AR target genes. The strength of the interaction between a ligand and a receptor is difficult to predict, since AAS with similar structures can possess different affinities for a given receptor, while structurally different ligands may show similar affinities [31]. Relative binding affinity (RBA) has been used as a term for the quantitative estimation of the receptor–ligand interaction. Methyltrienolone binds AR so strongly that it is used in studies as a reference substance to estimate the RBAs of other steroids, which are characterized as strong (19-nortestosterone and methenolone) or weak ligands (stanozolol and methandienone). Other compounds show RBAs that are too low to be determined (oxymetholone and ethylestrenol). A possible explanation for steroids with anabolic–androgenic activity *in vivo,* but that do not bind to AR, is the existence of an indirect mechanism of action, for example, via biotransformation to active compounds [32,33]. Structure–activity studies have revealed that the most important structural elements of a steroidal structure for effective binding to the AR are:

- The 3-keto group in the A-ring [31]. The reduction of this 3-keto group to an alcohol (either to α- or β-isomers) does not favor binding [34];
- The 17β-hydroxyl in the D-ring [31]. Any modification or elimination of the 17β-hydroxyl group reduces the AR binding affinity. A reduction in binding affinity also occurred by esterifying, for example, the 17β-hydroxyl in testosterone [34]. The 17α-hydroxyl group is not favorable to binding either;
- The 5α-steroidal framework [34];
- A small steric substitution at the 7α-position, but large substituents, reduce affinity. It has been shown that in 17β-hydroxy-4-androstenes the combined removal of the 19-methyl group and 7α-methylation can enhance binding to the AR [35].

Other studies demonstrated that key structural characteristics of a steroidal structure that affect either anabolic or androgenic activities of a given steroid are:

- The 17α-alkylation. 17α-alkylation contributes to the prolongation of the anabolic effect. The oral effectiveness of 17α-alkylated androgens is due to lower hepatic inactivation; the intracellular metabolism is limited and

transformation of this particular part of the molecule does not occur leading to liver disturbances [36,37]. 17α-alkylation also prevents aromatization of A-ring to estrogens [38];

- The 17β-hydroxyl group. Its esterification (by propionate, enanthate, cypionate, decanoate and undecanoate esters) induces enhancement of anabolic activity, and also its prolongation due to the reduction in the elimination rate as a result of the slow release of the parent non-esterified molecule. The absence of a 17-hydroxyl group induces the complete loss of androgenic activity [39], while due to the oxidation to 17-keto steroids, the androgenic activity is significantly reduced or disappears [40];

- The C-4,5 double bond. Its presence seems to cause an increase in activity;

- The 3-keto group. It is necessary for androgen activity, but has no effect on anabolic activity [40,41]. However, 3-deoxy steroids, in the presence of the C-4,5 double bond, were found to be compatible with high anabolic to androgenic activities (e.g., ethylestrenol);

- The removal of the 19-methyl group. This structural change offers, partially, dissociation of the androgenic and anabolic activities for a given molecule [42];

- The modification of ring A, either by the junction with another ring (e.g., a pyrazol ring, as in stanozolol), or by the introduction of an oxygen atom (e.g., oxandrolone), leads to a considerable increase in anabolic activity.

The structural characteristics mentioned above inspired research teams to synthesize a vast number of **designer steroids** (even for ethical purposes), retaining one or more of the above modifications while further modifying the structure of known anabolic steroids at positions where no significant reduction to AR binding or biological activity (either anabolic or androgenic) was induced. These further modifications (and/or their combinations) include:

- Alkylation at different positions in the steroidal structure, such as methylation at C-1 (e.g mesterolone), C-2 (e.g., drostanolone), C-6 (e.g., 6-methyltestosterone), C-7 (e.g., bolasterone), C-17 (also, ethylation or ethynylation, e.g., methandienone, norethandrolone and danazol, respectively) and C-18;

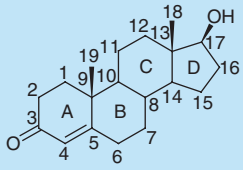

Figure 1. Testosterone, a representative steroidal structure for carbon numbering.

- Introduction of a double bond at different positions in the steroidal structure, such as at C-1,2 (e.g., 1-testosterone), C-2,3 (e.g., desoxymethyltestosterone) [12,43], C-5,6 [44] and C-5,10 (e.g., tibolone). In addition, many compounds with conjugated double bonds extending from ring A and B to C have been synthesized (e.g., methyltrienolone, methyldienolone) [41,45,46];

- Addition of heteroatoms, either to replace a carbon atom of the steroidal structure (e.g., with an oxygen atom [47,48] at C-2 as in oxandrolone, C-3, C-4, C-7, C-11 [49] or with a sulfur atom [50,51], or with a nitrogen atom [52]), or as a substituent (e.g., a chlorine at C-4 as in dehydrochlormethyltestosterone or at C-7 [53], or a fluorine at C-2, at C-6 [54], at C-7 [55] or at C-9 as in fluoxymesterone);

- Hydroxylation, such as at position C-4 (oxymesterone, oxabolone) or at C-11 (fluoxymesterone);

- Fusion of heterocyclic rings to the A-ring of the steroidal structure, such as of a pyrazole ring (stanozolol), an isoxazole ring (danazol) or a furazan ring (furazabol).

Table I summarizes literature on designer AAS circulated either on the black market or in nutritional supplements.

Endogenous designer AAS

The use of preparations containing testosterone and epitestosterone as endogenous designer AAS to escape doping tests has been described in the previous sections. Two cases of preparations have become known: the case of Jenapharm [6] and the case of BALCO [9]. In urine, a T/E ratio greater than 4.0 triggers follow-up tests to

Table 1. Designer androgenic anabolic steroids from literature.

Entry	Substance	Ref.
1	1-androstenediol	[56]
2	1-androstenedione	[56]
3	Dehydrochlormethyltestosterone	[57]
4	Desoxymethyltestosterone	[12]
5	Methasterone	[58]
6	Methylnortestosterone	[58]
7	Methyldienolone	[16]
8	Methyl-1-testosterone	[59]
9	Metribolone	[16]
10	Norboletone	[10]
11	Norclostebol	[60]
12	Prostanazol	[61]
13	1-testosterone	[62]
14	Tetrahydrogestrinone	[11]
15	Methylstenbolone	[63]
16	2α,3α epithio17α methylandrostane 17β ol	[64]
17	2β,3β epithio17α methylandrostane 17β ol	[64]
18	5β-mestanolone	[61]
19	Methylclostebol	[65]
20	Promagnon	[65]
21	17-hydroxyandrosta-3,5-diene	[66]
22	Δ6-methyltestosterone	[67]
23	17β-hydroxyandrostanol[3,2-d]isoxazole	[68]
24	17β-hydroxyandrostanol[3,2-c]isoxazole	[68]
25	6α-methylandrostenedione	[69]
26	Estra-4,9-diene-3,17-dion	[70]
27	Androsta-1,4,6-triene-3,17-dione	[71]
28	4-androstene-3,6,17 trione	[72]
29	1-adrosterone	[73]
30	Methyl drostanolone	[74]
31	7α-methyl nortestosterone	[75]
32	17α-methyl nortestosterone	[75]
33	18-methyl nortestosterone	[75]
34	Halodrol	[75]
35	4-hydroxytestosterone	[75]

investigate whether the elevated T/E ratio is of natural or exogenous origin [8]. The anti-doping analytical technology has incorporated the use of the isotope ratio MS (IRMS) to enable the differentiation between endogenously produced and exogenous testosterone. The reader is directed to a thorough review [76] for more information on this technology. Briefly, pharmaceutical preparations of testosterone are synthesized by plant extracts, whereas human endogenous testosterone originates from the endocrine system. Testosterone contains 19 carbon atoms (Figure 1). The most abundant carbon isotope is ^{12}C, approximately 99% in nature, and the less abundant carbon isotope is ^{13}C, approximately 1%. Due to the differences in the synthetic routes, endogenous testosterone contains more ^{13}C atoms among the 19 carbon atoms of the testosterone molecule, compared with the pharmaceutical preparations. This difference in ^{13}C content between endogenous and exogenous testosterone is measurable for the testosterone molecule and its urine metabolites by IRMS. Doped athletes using pharmaceutical testosterone excrete testosterone and metabolites in urine with less ^{13}C atoms compared with the endogenous testosterone, because exogenous testosterone inhibits the production of the endogenous one. Many manufacturers of reference material produce ^{13}C-labeled testosterone for the analytical and pharmaceutical industries. In these reference materials, ^{13}C atoms replace ^{12}C in the positions mainly 2, 3 and 4 (Figure 1). Unpublished data presented at the 29th Cologne Workshop on Dope Analysis (13–18 February 2011) by L Bowers and D Eichner of USADA [77], raised suspicion that athletes already use pharmaceutical testosterone preparations mixed with ^{13}C-labeled testosterone, in order to create a testosterone cocktail with a ^{13}C content similar to the endogenous, with the purpose of misleading IRMS tests.

Detection of designer AAS

Chromatographic techniques combined with MS, GC–MS or LC–MS are the first approach of the anti-doping laboratories for the detection of AAS. Commonly used instrumentation such as the mass selective detector (MSD) with a single quadrupole mass analyzer or the magnetic sector HRMS, operating in selected ion monitoring (SIM) mode, combine high sensitivity and specificity. These analytical instruments have been used for years for the detection of targeted anabolic steroids and their metabolites in the required low concentrations in urine. As an alternative to GC–MS, the combination of LC–MS instrumentation with electrospray ionization (ESI) has been introduced in the last decade for the detection of known steroids operating in multiple reaction monitoring (MRM) mode (for triple quadrupole analysers) or product ion scan mode (for ion-trap mass analysers). All of the above detection techniques allow efficient detection of known anabolic steroids that are included in the list of screened substances. Unknown designer AAS can be detected only by coincidence in cases when they share the same precursor and product ions with the targeted compounds and they are eluted in a

close chromatographic time inside the defined time window that is selected for the printout of the chromatograms. The preventive detection of unknown designer AAS requires a generic screening protocol, which combines a generic sample preparation with a sensitive high-resolution full-scan MS analysis [78–82]. Regarding sample preparation, the unification of different extraction/derivatization procedures applied for different classes of substances to a single extraction step, which will be able to isolate the unconjugated and conjugated (after enzymatic hydrolysis) low molecular weight substances, has been an important issue for the anti-doping laboratories. The analysis of this extract is performed by GC–MS (following a generic derivatization procedure) and/or LC–MS analytical systems that can acquire high-resolution, full-scan, accurate mass, spectrometric data, which allows for the detection of an unlimited number of known and unknown substances. Such analytical systems include GC–time-of-flight (TOF)-MS and GC–QTOF-MS and the combination of mass spectrometers with TOF, QTOF or Orbitrap® mass analysers with HPLC or UHPLC systems. In addition, with the use of mass analysers that can perform fast scan to scan polarity switching, such as the recently introduced benchtop Orbitrap mass spectrometer, the intact sulfoconjugated molecules of the designer steroids can also be detected as deprotonated molecules. The generic screening approach described above contributes to the enhancement of the preventive role of the anti-doping system against the use of designer drugs, especially if combined with the long-term storage of the samples. The acquisition of full-scan data enables the retrospective analysis of samples for the presence of designer drugs or new metabolites, without the need to reanalyze the samples, by simply reprocessing already acquired LC–MS and/or GC–MS data files. Important information, such as the molecular weight of the unknown and the elemental composition, can be obtained by accurate mass full-scan mode analysis, while the appearance of a combination of adduct ions can provide additional valuable information about the steroid structure.

Another approach for the detection and identification of unknown steroids, is the development of methods based on precursor ion scan and neutral loss scan using triple quadrupole or QTOF LC–MS/MS instruments, since steroids with common structural features under collision-induced dissociation (CID) or collisionally activated dissociation (CAD) can share common fragmentation patterns. The common characteristic product ions or neutral losses can be used as markers to identify unknown compounds. Published research describes protocols that can be used as complementary approaches to the existing analytical screening procedures of the laboratory [83–89], especially in cases of suppressed steroid profile as measured by GC–MS. In these protocols, product ion scan LC–MS analyses of known steroids were conducted and with the use of deuterium derivatives or modified structurally related synthetic analogues, characteristic fragmentation pathways are proposed that provide classification of the steroids by the characteristic product ions generated. For example, precursor ion scans of ions at *m/z* 97 and 109, indicate steroids with a 3-keto-4-ene structure and the detection of abundant product ions at *m/z* 241 and 199 or 227 and 199 indicate a 4,6,11-triene steroid with ethyl or methyl group at C-13. In a similar way, neutral loss scan can be used for the detection of unknown steroids with a particular structure. Some of the common losses observed in steroids are lacking in specificity (e.g., loss of water [18 amu] or acetone [58 amu], while others are considered more specific (e.g., 84 and 30 amu) and they can be used as a diagnostic tool for the detection and characterization of unknown steroids. As suggested by Pozo *et al.* [90], the integrated use of the four different types of scan modes (neutral loss and precursor ion scan followed by full scan and product ion scans) can be the most powerful tool for the detection and characterization of a designer steroid.

MS-based techniques are used as the standard highly sensitive routine screening methods for the known AAS. However, they depend on the known chemical structures. This led to the development of *in vitro* androgen bioassays, for the screening of designer AAS based on androgen-receptor activation instead of knowing the chemical structure. Androgen bioassays are not depended on the chemical structures. An approach based on the combination of LC separation – androgen bioactivity testing and QTOF-MS identification – was developed by Nielen *et al.* [91,92]. According to this protocol, urine samples after enzymatic hydrolysis and generic SPE are analysed using gradient LC and a dual 96-well fraction collector, where one plate is used for androgenic bioactivity detection by yeast-based reporter gene bioassay. If a well is found suspect, the duplicate plate is subjected to high-resolution LC–QTOF-MS

analysis, leading to elemental composition calculations of the designer steroids, search of electronic databases and structural elucidation. This approach was recently applied to detect and identify unknown androgens in herbal samples and sport supplements. Radioimmunoassays and ELISAs have been used in the past, showing good sensitivity for the screening of AAS, but with the disadvantage of limited specificity due to antibody crossreactivity profiles [93]. Recently, a multianalyte ELISA protocol based on a site-encoded ELISA microplate has been reported, which allows the simultaneous detection of up to 11 AAS in human serum samples in concentrations below minimum required performance levels (MRPL). This protocol enables the development of multiplexed immunoassays performed in a microarray format [94].

A thorough review on the androgen bioassays has recently been published [95], where the various types of this approach have been described. In the next lines, some studies on bioassays of AAS in biological matrices and nutritional supplements are presented. Nielen *et al.* had developed a simple yeast-based reporter gene bioassay for trace analysis of estrogens, characterized by direct measurement of yeast-enhanced green fluorescent protein for the detection of estrogen activity in nutritional supplements [96]. It was shown that bioassays play a valuable role in the fight against doping as compared to a LC–MS/MS screening method alone. As a test to examine its efficiency, 18 nutritional supplements were analyzed and shown negative in LC–MS/MS, while two of them screened positive by androgen yeast bioassay. The applicability of a yeast androgen and estrogen bioassay, in the detection of steroid esters in hair samples of animals treated with a hormone ester cocktail, was also shown [97]. Another approach for the advantage of a yeast androgen screening was studied by Wolf *et al.* [98]. The long-term detection of methyltestosterone abuse by a yeast transactivation system has been successfully validated. For the purpose of that study, a human volunteer was orally administered a single dose of 5 mg methyltestosterone and urine samples were collected after different time periods (0–307 h). The samples were analyzed in the yeast androgen screen and in parallel GC–MS. The results demonstrate that the yeast androgen receptor was able to detect methyltestosterone abuse for a longer period of time in comparison with classical GC–MS. It was found that bioassay was able to trace methyltestosterone in urine samples for at least 14 days while the GC–MS method was able to detect it up to the sixth day from the intake. The result of this study demonstrated that the yeast reporter gene system could detect the activity of anabolic steroids such as methyltestosterone with high sensitivity even in urine, providing further evidence for the high potential of yeast androgen screening as a prescreening tool for doping analysis. Although promising, this approach has been criticised at the following points: metabolites of many AAS may be inactive and do not show androgenic activity; the background activity from endogenous sources reduces specificity; and, its applicability is limited due to reduced sensitivity, mostly in out-of-competition collected anti-doping samples, where the AAS analytes would be more easily detected due to higher concentrations in urine.

In addition, a promising strategy of screening methods for the misuse of designer steroids by their physiological effects is the use of omics technologies [99–102]:

- Transcriptomics for finding gene expression biomarkers, with *in vivo* studies in showing alteration of gene expression in human blood cells caused by steroid hormones;

- Proteomics for investigating changes in protein expression or excretion caused by AAS, with a few publications available showing that different lipoproteins or apolipoproteins, propeptide of type III procollagen, apoptotic factors, pro- and anti-inflammatory factors can be promising biomarker candidates;

- Metabolomics for detecting perturbations in the metabolic profile after administration of AAS, with creatine, creatine kinase and plasma urea levels being potential biomarkers. Recently, Dervilly-Pinel *et al.* published two protocols based on LC–HRMS fingerprinting and multivariate data analysis, to investigate metabolome modifications upon steroid administration in calves, showing urine profiles discrimination of the treated animals from the control ones [100]; the results showed that the protocols need to be applied to a larger population of treated and control animals in order to describe generic, reliable and robust biomarkers. An untargeted steroidomic approach was proposed for the discovery of

new biomarkers for the detection of testosterone intake, by applying UHPLC–QTOF-MS urine sample analysis and chemometric tools, showing the pertinence to monitor both glucuronide and sulphate conjugates, as well as a number of promising biomarkers that can be also related to the administration of other AAS.

Recently, in 2009, WADA introduced the term 'athlete biological passport' (ABP) in the WADA Code [103] as an additional indirect tool to detect athletes manipulating their physiological steroid and hematological variables, without detecting a particular prohibited substance or method. The ABP does not replace the routine methods, but rather complements analytical methods. Although there has already been some longitudinal profiling of markers of steroid doping [8], the ABP now introduces a standardized approach to determine steroid abuse through urine sampling. The ABP regulations are based on the innovative approach developed by the Swiss WADA-accredited laboratory of Lausanne [104, 105].

Data processing

Methods based on MS produce data for known, unknown, targeted, untargeted and endogenous substances of biological samples. Specific software extracts MS information from analyzed urine samples, eliminates interferences and identifies metabolites in a series of samples from excretion studies, using data from MS libraries of known substances, spectra and accurate masses databases. A number of tools for processing MS data have been proposed in the literature and are available; for example, MetaboLynx® of Waters, Sieve® of Thermo Fischer Scientific and MetAlign® [79] of RIKILT. The MetAlign is an interface-driven tool for full-scan MS-data processing. The main purpose of this software is the automated processing of MS-based metabolomics data with baseline correction, accurate mass calculation, smoothing and noise reduction and alignment between chromatograms. By comparing data after pre-processing with MetAlign, it was noted that besides the chromatogram baseline line correction, there were better defined peaks that improved peak picking for the identification of targeted and untargeted compounds [79]. For identification of untargeted peaks, an inaccurate-mass database was constructed containing approximately 40,000 pharmacologically relevant and existing compounds extracted from the Internet-accessible database PubChem [106]. Calculation of the exact mass of each protonated and deprotonated molecules, the isotope ratio and an estimate of the retention time was also performed.

Peters *et al.* modified MetaboLynx for the determination of *in silico* predicted metabolites of glucocorticosteroids and designer modifications of anabolic steroids in human urine [79]. It was successfully used for the detection of THG [107]. Synergetic methods for the prediction of AAS metabolites, retention times and MS fragmentation have been proposed by Fragkaki *et al.* [108]. A method predicts the phase I metabolites of AAS [108]. The statistical tool principle components analysis (PCA) was used to classify the parent AAS into different classes, based on their structure's similarities. Another method [109] was used for the prediction of MS fragmentation of AAS, including designers. The results derived from the previous two studies were combined with the study of Quantitative Structure Retention Relationships (QSRR) prediction of retention times [110]. xlogP molecular descriptors have also been used [79] for retention times predictions of PubChem database compounds. Finally, an LC–MS library searching method for the identification of AAS in nutritional supplements has been developed [111].

RNA-sequencing

A recent study opened new frontiers in the detection of designer AAS, even though it was applied in meat production animals (boars and calves) [112]. Changes in the molecular level caused by the administration of AAS were quantified by a new high-throughput and sensitive technology for holistic gene expression analysis, RNA sequencing. The results demonstrated the potential of the new technology for the screening of highly regulated genes that can act as biomarker candidates for the detection of the misuse of anabolic substances in farm animals. This novel approach can be evolved as an alternative indirect detection method of designer and known AAS in human sports in the future.

Synthesis of metabolites of designer AAS

The *in vivo* production of reference substances of AAS metabolites in humans suffers from ethical, as well as practical problems associated with the implementation of clinical studies and the isolation of pure metabolites from urine. To overcome these problems, several methods of synthesis of

metabolites have been developed. The enzyme-assisted synthesis catalyzed with microsomal uridine diphosphoglucuronosyl-transferase (UGT) enzymes has been developed, offering the main advantage of the stereospecificity of the enzymes, which allows synthesis of stereospecifically pure conjugates. Moreover, enzyme-assisted synthesis is used for the rapid production of small amounts of glucuronides when needed, for example, in the build-up of an analytical method. Using the enzyme-assisted synthesis, the preparation of glucuronide conjugate standards of 11 AAS and their metabolites, which can be detected in human urine after dosing of exogenous anabolic steroids (e.g., methandienone, metenolone, methyltestosterone, nandrolone and testosterone), has been described [113]. In another study, microsomal and S9 fractions of human liver preparations were used as sources of metabolizing enzymes, and the co-substrates of the synthesis mixture were selected to favor phase I metabolic reactions and phase II conjugation reactions of relatively new AAS [114,115]. Equine liver microsomes and S9 *in vitro* fractions were also found to generate all the major phase I metabolites observed, following *in vivo* administrations of stanozolol in the equine [116].

Chemical synthesis methods have been also developed as an alternative for the synthesis and identification of AAS and/or their metabolites, as occurred in a study for 4-hydroxytestosterone [117], madol [12], tetrahydrogestrinone [11] and other AAS [13,118,119]. The approach to synthesize, characterize and certify appropriate reference materials (RMs) and certified reference materials (CRMs) from the National Analytical Reference Laboratory (NARL), which are fit-for-purpose for the current requirements of sports testing laboratories, has been described [120].

The identification of AAS metabolic pathways have been also successfully conducted through either animal experiments, as for madol [8], or using cryopreserved human hepatocytes, as for drostanolone and 17-methyldrostanolone [121] and other AAS [122,123]. The results of the *in vitro* experiments carried out using homogenized horse liver for five anabolic steroids (turinabol, methenolone acetate, androst-4–3,6,17-dione, testosterone and epitestosterone) have also been presented [124] as an alternative for AAS metabolism studies.

The chimeric uPA+/+-SCID mouse model, transplanted with human hepatocytes, has also been used to study *in vivo* human steroid metabolism, as occurred for methasterone, promagnon, methylclostebol [65], 4-androstene-3,17-dione [125] and methandienone [126].

Recently, another strategy for synthesis of the methandienone long-term metabolite, 17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one, was reported [127]. According to this, 11 recombinant strains of the fission yeast *Schizosaccharomyces pombe*, expressing different human hepatic or steroidogenic cytochrome P450 enzymes, were screened for production of this metabolite in a whole-cell biotransformation reaction. 17,17-dimethyl-18-norandrosta-1,4,13-triene-3-one, chemically derived from methandienone, was used as substrate for the biotransformation reaction, as it was converted to the final product in a single hydroxylation step. The metabolism of methyl-1-testosterone has also been studied according to this strategy [128].

Animal doping with designer AAS

In several publications related to AAS screening in animal sports, designer AAS have been introduced to the protocols, such as in [129], proving that the problem has been inherited by animal sports from the human ones. Several animal racing laboratories have conducted studies in the metabolism of designer AAS [130–132]. The *in vitro* metabolism of a designer steroid – estra-4,9-diene-3,17-dione – featuring in 2010 in a large number of marketed products on the Internet, was studied in equine, canine and human species with the major metabolites identified for target testing in sports doping control [130]. In another study the equine *in vitro* metabolism of seven steroids available for purchase on the Internet, including androsta-1,4,6-triene-3,17-dione, 4-chloro,17α-methyl-androsta-1,4-diene-3,17β-diol, estra-4,9-diene-3,17-dione, 4-hydroxyandrostenedione, 20-hydroxyecdysone, 11-keto-androstenedione and 17α-methyldrostanolone was reported [131]. Initiated by the doping scandals in human sports [11], the pharmacokinetics of THG in equine [132] and its *in vitro* metabolism [131] were also studied.

Anti-doping samples preservation: urine stabilization & blood spots

The designers AAS molecules and their metabolism are unknown to the anti-doping laboratories at the time of their first circulation. WADA Code [3] has introduced the dimension of time in the anti-doping system, allowing laboratories to organize their detection of the designers with knowledge in the structures, the metabolism

and the synthesis of reference materials. The dimension of time in the anti-doping system is practically applied with samples long-term storage. Two methodologies have been developed to facilitate the urine and blood stability over time: urine samples stabilization and the DBS.

Retrospective analysis can only be conducted provided that urine samples quality is not undermined over time due to reactions enhanced by the presence of microorganisms or proteolytic enzymes in urine. Doping control urine samples are collected and stored in doping control stations and WADA-accredited laboratories, in a way that protects their identity, integrity and security [106], which is of particular importance in case that the already analyzed samples are eventually submitted to retrospective analysis [3]. What if sample delivery to the WADA-accredited laboratories is not immediate? Hydrolysis of steroid conjugates followed by modifications of the steroids' structure by oxidoreductive reactions may take place due to the occurrence of microorganisms that can be found in the human body or the surrounding environment, especially during sample transportation in the warm periods of the year [133–138]. The best practice to ensure that samples' integrity is maintained for possible reanalysis would be to store samples frozen as well as stabilized. To date, no preservative is added to sport urine samples [139]. The implementation of a specially designed sample collection container, incorporating a generic sample stabilization mixture consisting mainly of antibiotic, antimycotic substances and protease inhibitors has been proposed [140]. The purpose of an ongoing project funded by WADA is the investigation of the efficiency of the in-house chemical stabilization mixture in spray-coated form with simultaneous minimization of analytical interferences. Preliminary results demonstrate that the cell growth of five representative microorganisms (*Escherichia coli, Nocardioides simplex, Aspergillus flavus, Candida albicans* and *Enterococcus faecalis*) is completely inhibited after a 7-day incubation period at 37°C in those urine samples that were stored in spray-coated stabilized containers. Moreover, the degradation of steroid glucuronides is prevented in the stabilized urine samples [141]. The implementation of specially designed plastic urine collection containers, spray-coated in their interior surface with the stabilization mixture is currently more realistic than it was a few years ago. If this preventive approach is applied in the doping control sampling procedure, it would be a major step towards the preservation of urine samples for long-term storage and eventual retrospective analysis.

In the context of long-term storage of samples for retrospective analysis, the DBS technique is gaining increasing importance in the doping control field. It involves collection of small volumes (10–30 µl) of whole blood obtained from heel or finger pricks, drying it on a piece of filter paper, extracting and subsequently analyzing it by LC–MS. DBS offers numerous advantages over conventional whole blood, plasma or serum analysis, such as ease of collection (even in remote control stations), minimal potential of sample manipulation, cost effectiveness, less invasiveness, simplified storage and transport of DBS samples – absence of refrigeration – enhanced stability described for many analytes on the cellulose sampling paper [142–145]. An apparent limitation of the DBS method is the small blood volume collected, thus representing a challenge for the sensitive determination of some analytes in elite sports such as anabolic steroids at sub-ng/ml levels. In addition, a new plasma screening method has been developed for the retrospective reanalysis of stored samples for new xenobiotic drugs at low ng/ml levels [146]. It is based on protein depletion, UHPLC-based LC separation and detection by means of high-resolution/high-accuracy MS. The use of either DBS or plasma samples cannot replace (at least for the time being) the conventional urine analysis procedure, however, they are both attractive alternatives and can enable the retrospective qualitative data evaluation for known and unknown xenobiotics.

Conclusion

Designer AAS represent a dark and dangerous side of drug abuse in sports. AAS remain the prevalent drug-class according to WADA statistics [147]. The borderline between the use of novel substances as new therapeutics or as potential doping agents is often a challenge for cheating athletes to overstep. Control laboratories and regulatory authorities are aware of analytical advancements and legislation improvements for successful detection and prevention of AAS. This Review presented the main issues concerning AAS, such as their scientific background, progresses in their analytical detection and the preventive anti-doping that is intended to reveal positive analytical findings in samples initially reported as negatives, as occurred in the reanalysis of stored anti-doping samples from the 2004

Olympic Games [148] and the 2005 Athletics World Championships [149].

Future perspective

The emergence of novel designer AAS constitutes a serious threat to drug-testing laboratories and sporting administrators. The successful detection of new chemical structures of AAS is mainly based on the selection and design of improved analytical protocols, from sample preparation through to advanced instrumental analysis, which will give rise to enhanced sensitivity and specificity of the methods and fulfill the stringent performance limits suggested by WADA. Alternative methods for the detection of designer AAS, such as receptor-based assays (even combined with MS) and advancements in software technology concerning MS libraries, spectra and accurate mass databases will play a major role in future detection of designer AAS.

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Executive summary

Introduction: the chemistry of androgenic anabolic steroids

- Chemically modified steroids, which are not used in clinical practice and have either been synthesized in the past or have been specifically developed to circumvent doping control, are commonly known as designer androgenic anabolic steroids (AAS) and are considered an issue of major importance in the fight against doping.

Authorities against illegal laboratories

- Anti-doping laboratories and drug-testing authorities make continuous efforts to limit the extent of the illegal circulation of designer AAS through numerous doping controls and legislation of strict analytical and regulatory guidelines.

Detection of designer AAS

- Improvements of analytical protocols, as well as advancements in preparative and instrumental techniques, are promising for successful detection of designer AAS.

Data processing: synthesis of metabolites of designer AAS

- Various different methods for synthesis or *in silico* prediction of metabolites, to overcome the problem of their *in vivo* production due to ethical and practical restrictions, contribute to the successful detection of designer AAS.

Data processing: sample preservation

- The dimension of time for future detection of designer AAS has been applied, either through reprocessing of already analyzed doping control samples with up-to-date analytical data or through facilitating samples stabilization over time.

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