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ANNUAL BANNED-SUBSTANCE REVIEW

Annual banned-substance review – Analytical approaches in human sports drug testing

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Abstract

Within the complex construct of today's antidoping work, continuously updated routine doping controls, as well as advancements in sampling and analysis have been of particular relevance and importance. New analytes of existing classes of prohibited substances are frequently included into sports drug testing assays, analytical approaches are optimized to allow for better sensitivities, selectivity, and/or faster turnaround times, and research dedicated to addressing analytical issues concerning scenarios of both (potentially) inadvertent doping and new emerging doping agents is constantly conducted. By way of reviewing and summarizing, this annual banned-substance review evaluates the literature published between October 2018 and September 2019 offering an in-depth evaluation of developments in these arenas and their potential application to substances reported in WADA's 2019 Prohibited List.

KEYWORDS

alternative matrices, doping, mass spectrometry, sport

1 | INTRODUCTION

The deliberate act of doping in sport has been shown to be fueled by various different factors, appellatively summarized as a dopogenic environment by Backhouse et al.,[1] ranging from (amongst others) the enormous overall demands of the modern sporting life that might exceed an athlete's personal resources to local level factors such as the athlete's team, coach, physicians, and peers[2-6] as well as sport motivation.[7] In addition, presumably or evidently inadvertent antidoping rule violations (ADRVs) have continued to be recorded,[8,9] underlining the importance of education and information of the athletes' support personnel and health care professionals.[10-13] A recent exhibit in support of the need for constantly improving antidoping efforts in general[14,15] but also for the enormous potential and the complementary nature of continuously enhanced analytical methods and police investigations was Operation Aderlass in 2019.[16] This investigation distinctly illustrated the evolution or adaptation of doping practices in sport to currently enforced (whilst debated[17-20]) antidoping regulations[21] and testing capabilities.

The annually updated World Anti-Doping Agency (WADA) edited Prohibited List represents the central document detailing substances and methods of doping in sport.[21] Its content has been the subject of continued discussions, particularly with regard to the extent or necessity of evidence for performance enhancing properties of banned substances[22,23] as well as the consideration of new substances or methods for future inclusion.[24-26] As in 2018, the 2019 Prohibited List is composed of 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3) (Table 1), with main modifications concerning the rearrangement, deletion, and addition of doping agents in the categories S1–S4.[27] The modification and reorganization of the category S1 (Anabolic agents) focused on the clarification that also metabolites of endogenous anabolic androgenic steroids (AAS) are prohibited when administered. With the exemption of selected metabolites that are known to be sold as nutritional supplements or may affect the interpretation of an athlete's steroid profile, metabolites

TABLE 1 Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2019

	Class	Sub-group		Examples
S0	Non-approved substances[a]			Rycals (ARM036), sirtuins (SRT2104), AdipoRon
S1	Anabolic agents[a]	1	Anabolic androgenic steroids	
			(a) Exogenous	1-Androstenediol, clostebol, danazol, metandienone, methyltestosterone, methyltrienolone, stanozolol, tetrahydrogestrinone
			(b) Endogenous	Androstenediol, testosterone, dehydroepiandrosterone, nandrolone
		2	Other anabolic agents	Clenbuterol, selective androgen receptor modulators (SARMs), tibolone, zeranol, zilpaterol
S2	Peptide hormones, growth factors, related substances and mimetics[a]	1.1	Erythropoietin-receptor agonists	Darbepoietin (dEPO), erythropoietin (EPO), EPO based constructs (EPO-fc, methoxy polyethylene glycol-epoetin beta (CERA)), peginesatide, EPO-mimetic agents and their constructs (CNTO-530, peginesatide)
		1.2	Hypoxia-inducible factor (HIF) activating agents	Cobalt, molidustat, roxadustat, vadadustat, xenon
		1.3	GATA inhibitors	K-11706
		1.4	TGF-beta (TGF-β) inhibitors	Luspatercept, sotatercept
		1.5	Innate repair receptor agonists	Asialo EPO, carbamylated EPO
		2.1	Chorionic gonadotrophin (CG) and luteinizing hormone (LH), and releasing factors (males only)	Buserelin, deslorelin, gonadorelin, leuprorelin
		2.2	Corticotrophins and their releasing factors	Tetracosactide-hexaacetate (Synacthen®), adrenocorticotrophic hormone (ACTH), corticorelin
		2.3	Growth hormone (GH), its fragments and releasing factors	AOD-9604, hGH 176–191, GHRH and its analogs (CJC-1293, CJC-1295, sermorelin, tesamorelin) GHS (ghrelin, anamorelin, ipamorelin, macimorelin, tabimorelin) GHRPs (alexamorelin, GHRP-1, GHRP-2, etc.)
		3	Growth factors and growth factor modulators	Fibroblast growth factors (FGFs) Hepatocyte growth factor (HGF) Insulin-like growth factors (e.g. IGF-I), Mechano growth factors (MGFs), Platelet-derived growth factor (PDGF), Thymosin-β4 and its derivatives (TB-500) Vascular-endothelial growth factor (VEGF),
S3	Beta-2-agonists[a]			Fenoterol, reproterol, bambuterol
S4	Hormone and metabolic modulator[a]	1	Aromatase inhibitors	Anastrozole, letrozole, exemestane, formestane, testolactone
		2	Selective estrogen receptor modulators (SERMs)	Raloxifene, tamoxifen, toremifene
		3	Other anti-estrogenic substances	Clomiphene, cyclophenil, fulvestrant
		4	Agents preventing activin receptor IIB activation	Domagrozumab, stamulumab, bimagrumab

(Continues)

TABLE 1 (Continued)

	Class	Sub-group		Examples
		5	Metabolic modulators	AICAR, GW1516, insulins, meldonium, SR9009, trimetazidine,
S5	Diuretics and masking agents[a]		Masking agents	Probenecid, hydroxyethyl starch, desmopressin
			Diuretics	Acetazolamide, bumetanide, furosemide, triamterene
S6	Stimulants[b]		Non-specified stimulants	Adrafinil, amfetamine, benfluorex, cocaine, modafinil
			Specified stimulants	Cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane
S7	Narcotics[b]			Buprenorphine, fentanyl, morphine
S8	Cannabinoids[b]			Hashish, marijuana, JWH-018, HU-210
S9	Glucocorticoids[b]			Betamethasone, dexamethasone, prednisolone
M1	Manipulation of blood and blood components[a]	1	Administration or reintroduction of any quantity of blood	Autologous, homologous and heterologous blood, red blood cell products
		2	Artificially enhancing the uptake, transport or delivery of oxygen	Perfluorocarbons (PFCs), efaproxiral, hemoglobin-based blood substitutes
		3	Intravascular manipulation of blood or blood components by physical or chemical means	
M2	Chemical and physical manipulation[a]	1	Tampering	Urine substitution, proteases
		2	Intravenous infusion	
M3	Gene and cell doping[a]	1	The use of polymers of nucleic acids or nucleic acid analogues	DNA, RNA, siRNA
		2	The use of gene editing agents designed to alter genome sequences and/or the transcriptional or epigenetic regulation of gene expression	
		3	Use of normal or genetically modified cells	
P1	Beta-blockers[c]			Acebutolol, atenolol, bisopropol, metoprolol

[a]Prohibited at-all-times.
[b]Prohibited in-competition only.
[c]Depending on the rules of the international sport federations.

are no longer detailed under S1. Further to this, 2-androstenone, 3-androstenone, 2-androstenol, and 3-androstenol were shifted to S4.1 (Aromatase inhibitors), in accordance with their predominant mechanism of action. Additional examples of hypoxia-inducible factor (HIF) activating agents were included under S2.1.2 with daprodustat and vadadustat, and macimorelin was added as a new example for growth hormone (GH), its fragments and releasing factors (S2.2.3).

The monitoring program of 2019 continued identically to the 2018 edition, i.e. following-up on the in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotic analgesics codeine, hydrocodone, and tramadol.[28] Of note, prevalence data on caffeine[29] and tramadol[30] as generated in the context of the monitoring program, were reviewed and discussed. Both substances are expected to remain in future WADA monitoring programs,[31] albeit for tramadol an in-competition ban was issued by the Union Cycliste Internationale (UCI) due to medical concerns in March 2019.[32] For nicotine, dissenting study results concerning the drug's ergogenic properties were reported, with one investigation showing an increase in exercise performance in time-to-exhaustion tests,[33] while another study did not confirm effects on the participating athletes' performance in a 1 hour time trial experiment,[34] presumably attributable to study design differences. Also, analyses concerning potential patterns of misuse regarding corticosteroids and any combination of beta-2 agonists were pursued, and the monitoring of 2-ethylsulfanyl-1H-benzimidazole (bemitil) was extended for at least another year, taking into account the recommendation of

TABLE 2 References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2018/2019

	Class	Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	Complementary methods & general
S1	Anabolic agents	1	Anabolic androgenic steroids				
			(a) Exogenous	47, 48, 51			39–45, 50, 52, 53
			(b) Endogenous	56, 57	58, 59	56, 57, 64–68	60, 61
		2	Other anabolic agents	71	72, 74		73
S2	Peptide hormones, growth factors, Related substances and mimetics	1.1	Erythropoietin-receptor agonists				75–77
		1.2	Hypoxia-inducible factor (HIF) activating agents				84–88, 145
		1.4	TGF-beta (TGF-β) inhibitors		82, 83		78, 79, 81, 82
		2.1	Chorionic gonadotrophin (CG) and luteinizing hormone (LH), and releasing factors (males only)				98, 100
		2.3	Growth hormone (GH), its fragments and releasing factors		93, 94, 97		89–92, 95, 96
S3	Beta-2-agonists				106		101–108
S4	Hormone and metabolic modulators	1	Aromatase inhibitors	109			110, 111
		4	Agents modifying myostatin function(s)		83		
		5	Metabolic modulators		113, 117, 122, 124, 125		113, 115, 116, 119–122
S5	Diuretics and masking agents				127, 128		130, 131
S6	Stimulants				127, 133		132
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products				134–145
M3	Gene and cell doping						146, 147

including bemitil glucuronide as a target analyte,[35] determined in samples collected in the events of both in- and out-of-competition.

In continuation of the 11th edition of the annual banned-substance review,[36] literature published between October 2018 and September 2019 is evaluated (Table 2), focusing on advancements in sports drug testing approaches enabled by complementary strategies, enhanced analytical instrumentation, and/or optimized target analyte selection.

2 | ANABOLIC AGENTS

2.1 | Anabolic-androgenic steroids

Today's dimensions of the illicit anabolic-androgenic steroid (AAS) market were recently delineated when a pan-European police operation led to the seizure of thousands of kilograms of AAS as well as the arrest of hundreds of individuals.[37] The extent of the presumably existing customer base is remarkable considering the ever-growing body of evidence regarding the questionable quality of distributed products[38] and the adverse effects associated with AAS (mis)use, including both health[39-43] as well as legal[44,45] issues. It was assumed that the confiscated material was predominantly intended to reach the recreational sport clientele; however, adverse analytical findings (AAFs) caused by AAS have continued to represent the most frequent reason for ADRVs also in elite sport.

2.1.1 | Initial testing procedures – Comprehensive screening and metabolism studies

While indirect indicators of AAS misuse such as blood laboratory markers (e.g. high density lipoprotein/cholesterol ratio, serum

γ-glutamyl transpeptidase levels, etc.) or physical signs have been suggested to be of added value to the antidoping toolbox,[46] evidence of doping practices with AAS necessitates a comprehensive analytical spectrum provided by initial testing procedures (ITPs) of the utmost sensitivity, followed by dedicated confirmatory analyses of diagnostic target analytes. In addition, profiling of endogenous steroids is an integral part of the AAS ITP procedure, requiring the quantitation of selected naturally occurring target compounds. Commonly, testing for steroidal analytes is accomplished by methods employing gas chromatography (GC) combined with triple quadrupole (QqQ)-based mass spectrometry (MS). More recently, the advancements in hyphenating GC to high resolution/high accuracy MS (HRMS) have allowed for moving from strictly targeted screening approaches towards a combined analyte-specific and non-targeted data acquisition, accomplished without jeopardizing assay sensitivity or quantitation performance. In that context, Polet et al. reported on a GC–HRMS-based ITP, covering a total of 294 target substances plus 14 internal standards, including 96 AAS (or corresponding metabolites) and 14 endogenous steroids for the analysis of the urinary steroid profile.[47] Following established sample preparation protocols utilizing enzymatic hydrolysis of glucuronic acid conjugates, liquid–liquid extraction (LLE), and trimethylsilylation, samples were analyzed on a GC-quadrupole/time-of-flight (Q/TOF) MS. The GC was equipped with an ultra-1 analytical column (length 15 m, inner diameter 0.2 mm, film thickness 0.11 μm), electron ionization (EI) was accomplished at low energy (18 eV), and the MS was operated in scheduled full scan or combined full scan and MS/MS mode. With a maximum mass error of less than 5 ppm, the test method proved competitive to currently employed GC-QqQ-MS approaches in terms of accomplished limits of detection (LODs) and offered further the option of including additional analytes (if compatible with the sample preparation protocol) and retrospective data reprocessing. Focusing also on assessing the utility of GC–HRMS analysis in doping controls, Abushareeda et al. compared the performances of a GC-Q/TOF- and GC-Q/Orbitrap-based MS concerning the analysis of 39 exogenous AAS (or representative metabolites) and six endogenous steroids plus four internal standards.[48] Spiked urine samples and proficiency test specimens were prepared in accordance to previously reported methods also utilizing enzymatic hydrolysis, LLE, and trimethylsilylation. Both analytical systems comprised a GC with a BPX5 analytical column (length 30 m, inner diameter 0.25 mm, film thickness 0.1 μm) and EI was conducted at 70 eV. The Q/TOF MS and the Q/Orbitrap MS allowed for mass resolutions of 12,000 (@ m/z 272) and 60,000 (@ m/z 200) and mass errors of less than 5 ppm and 2 ppm, respectively, and assay LODs matched the WADA-established minimum required performance levels (MRPLs)[49] for all analytes, facilitated by few analyte-specific MS/MS experiments. Also bias and measurement uncertainty were determined for the six endogenous steroidal analytes testosterone (T), epitestosterone (EpiT), androsterone (A), etiocholanolone (E), 5α-androstane-3α,17β-diol (5αAdiol), and 5β-androstane-3α,17β-diol (5βAdiol), and all were found acceptable between 2.1% and 15.1% (bias) and 2.6% and 17.2% (measurement uncertainty). Overall, the GC-Q/Orbitrap data were described as superior but both systems were found fit-for-purpose in anti-doping analysis, and the employed full scan HRMS measurements also allowed for data reprocessing where desirable. Proof-of-concept concerning the retroactive analysis of datasets (and corresponding long-term stored urine samples) was provided by Lommen et al., who demonstrated the practicality of data reduction and subsequent automated evaluation.[50] Employed in the context of screening ITP data, false-negative results were virtually excluded and algorithm settings tailored to individual analytes allowed for reducing the frequency of false warnings to an acceptable minimum. While the quality of a manual scientist-conducted data evaluation especially concerning false warnings was unmatched, the speed of data reprocessing by the automated process was found to be excellent. It might be relevant to clarify though whether the mass resolution of the original uncompressed data files is critical to the performance of the automated reprocessing. Moreover, the importance of adequate internal standards for optimal data evaluation was underlined by Sobolevsky et al., applicable to both currently processed specimens as well as to reprocessed datasets.[51] Especially for GC–MS-based test methods requiring sample derivatization (such as trimethylsilylation), the completeness of the analytes' chemical conversion needs verification, which was shown to be elegantly accommodated by means of stable isotope-labeled boldenone.

Another factor necessitating consideration in the interpretation of sports drug testing analytical data is the co-administration of permitted drugs potentially affecting the elimination profiles of prohibited substances. Mazzarino et al. comprehensively studied the influence of antifungal agents and antidepressants (selective serotonin reuptake inhibitors and serotonin antagonists/reuptake inhibitors) on in vitro and in vivo metabolic reactions and, consequently, the traceability of metandienone metabolites in human urine.[52] Especially the antifungals ketoconazole and miconazole were found to interfere with the phase-I and phase-II metabolism of metandienone, potentially negatively affecting the detection window for commonly targeted long-term metabolites in routine doping controls. Whether alternative metabolic pathways for AAS exist when antifungal drugs are administered remains to be elucidated.

Complementary to urine and blood analysis, hair testing was shown to allow for contributing supporting information in selected cases of AAFs with AAS. By means of five case vignettes, Salomone et al. demonstrated the capability of modern analytical methods to determine clostebol acetate in hair at the low pg/mg level and the accordingly obtained information on the duration of drug intake/exposure.[53] A total of 50 mg of hair was required, washed, pulverized, and extracted into methanol for subsequent liquid chromatography (LC)-MS/MS analysis. Using a LC system equipped with a C-18 analytical column (100 × 2.1 mm, particle size 1.8 μm) and a QqQ-based MS operated in multiple reaction monitoring (MRM) mode, LODs for clostebol and clostebol acetate were found at 0.3 and 1.1 pg/mg, respectively. While all hair samples (including head, arm, leg, and pubic hair) returned negative test results for clostebol, six specimens/segments were reported to contain clostebol acetate at 3–21 pg/mg. In the context of disciplinary hearings and case reviews, such information (where available) can

be considered as additional evidence and contribute to decision-making processes.

2.1.2 | Steroid profiling in urine and serum

The so-called steroid profile and the thereof created steroidal module of the athlete biological passport (ABP) consisting of urinary concentrations and selected ratios of testosterone, epitestosterone, androsterone, etiocholanolone, 5αAdiol, and 5βAdiol, has been shown to be of particular value in identifying atypical alterations in an individual's urinary steroid pattern. Suspicious test results commonly referred to as atypical passport findings (ATPFs)[54,55] are routinely followed-up by isotope ratio mass spectrometry (IRMS, vide infra) and, where indicated, also by DNA testing to probe for potential sample swapping. The ABP's advantage over population-based reference ranges has been the superior sensitivity of the longitudinal and athlete-specific monitoring of the steroid profile towards testosterone and testosterone prohormone misuse, particular in consideration of different UGT2B17 enzyme genotypes amongst athletes. Martin-Escudero et al. systematically revisited the influence of a testosterone cypionate injection on the steroid profile and urinary LH concentrations of individuals with different (ins/ins, ins/del, and del/del) UGT2B17 polymorphisms.[56] In accordance to previous studies, urine samples of wild type (*ins/ins*) homozygotes yielded ATPFs in all specimens collected 10 days post-administration; urinary steroid profiles of heterozygotes (*ins/del*) were suspicious in 93% of all samples, and only 21% of specimens collected from mutant homozygotes (*del/del*) returned ATPFs. However, all specimens of the 10-day post-administration period of all study groups fulfilled the criteria for AAFs when analyzed by IRMS, which outlines the importance of efficient initial testing procedures and strategies (such as the ABP) supporting the identification of samples necessitating further investigations and analyses. While numerous studies exist concerning the impact of endogenous steroid misuse on the steroid profile of male athletes, little information is available concerning the effects of T or T prohormone administration on the steroidal module of the ABP of females. Buisson et al. therefore investigated the traceability of dehydroepiandrosterone use (DHEA, orally administered to 11 healthy individuals) by means of established strategies of steroid profiling and subsequent GC-combustion-IRMS (GC/C/IRMS) analysis.[57] In 10 out of 11 ABPs, ATPFs were recorded and in particular T/EpiT as well as 5αAdiol/EpiT were found to be indicative of DHEA use for up to 36 h post-administration. Of note, the carbon isotope signature of EpiT was shown to be affected by DHEA administrations in women; a phenomenon that was not observed in concomitantly studied male participants of the same drug intervention. In addition to urine samples, saliva specimens were collected and analyzed for DHEA content. A significantly elevated concentration of saliva DHEA compared with baseline was recorded for 24 h post-administration, indicating another complementary option, the viability of which for sports drug testing purposes is to be explored in future studies.

The added value of additional target analytes contributing to routine steroid profiling was demonstrated by Esquivel et al., who reported on the improved retrospectivity for testosterone misuse when employing urinary sulfo-conjugated testosterone metabolites in doping controls.[58,59] Following single dose testosterone administrations either via the oral (testosterone undecanoate, 120 mg) or intramuscular (testosterone cypionate, 100 mg) route, urine samples were collected up to 16 days and analyzed by an LC–MS/MS-based method that allowed for quantifying 14 steroid sulfates. The test method employed a mixed-mode weak anion exchange SPE for urine extraction, and extracted phase-II metabolites were quantified using a QqQ-based LC–MS/MS instrument. The analytes were separated on a C-18 analytical column (2.1 × 100 mm, 1.8 μm particle size) and 5 mM ammonium formate (solvent A) and methanol (solvent B) as eluents, both containing 0.01% formic acid. The mass spectrometer was operated with scan-to-scan polarity switching in MRM mode, enabling the quantitation of 14 sulfoconjugated steroids and computing of corresponding concentration ratios. In general, the intra-individual monitoring of steroid sulfates and marker ratios was recommended over using population-based reference ranges. In the context of oral T undecanoate administration, particularly epiandrosterone (EpiA) sulfate was found to be significantly influenced, enabling extended detection windows up to 144 h. Following intramuscular T cypionate injection, the A sulfate/T sulfate ratio provided superior detection windows when compared with the commonly employed T/EpiT ratio, and the analytical benefit was observed for urine samples collected from persons with Caucasian (basal T/EpiT ratio 1–4) as well as Asian (basal T/EpiT ratio < 0.3) urinary steroid profiles. To date, most routine doping control test methods targeting natural/endogenous urinary steroids focus on glucuronide-conjugated analytes in agreement with respective WADA technical documents.[54] Accordingly, sample preparation and analysis strategies are optimized for this subset of analytes; nevertheless, with the continuously growing dataset and knowledge about sulfo-conjugated steroidal substances, obligatory complementary and/or modified routine test methods might be justified in the future.

The continuously improving instrumental sensitivity and selectivity exploited for urinary steroid conjugate analysis has been shown also to allow for pursuing new avenues with regards to serum steroid testing. Ponzetto et al. continued profiling serum steroidal substances by SPE followed by LC-HRMS,[60] and assessed the utility of established markers such as T and dihydrotestosterone (DHT) in addition to glucuronides of A, E, 5αAdiol, and 5βAdiol, as well as the above mentioned EpiA sulfate as an antidoping analytical tool. Chromatography was conducted using a C-18 analytical column (2.1 × 150 mm, particle size 1.7 μm) operated with 0.1% aqueous formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B), and the effluent was directed via electrospray ionization (ESI) into a Q Orbitrap mass analyzer recording full scan and MS/MS data. The longitudinal monitoring of the serum-derived free and conjugated steroids allowed for detecting repeated oral administrations of 80 mg of T undecanoate up to 48 h after drug intake, indicating the potential of serum tests in complementing the to-date exclusively urinalysis-based

steroidal module of the ABP. Exploiting the steroidal module of the ABP expanded towards serum testing recently allowed for a precedent, where the intra-individual variation of serum T was found evidentiary for an ADRV of two female athletes,[61] suggesting the analysis of serum (or plasma) steroids be intensified in future routine doping controls. Whilst being analytically identical, such tests are not conducted in the context of female athletes' eligibility to compete in selected athletics disciplines.[62,63]

2.1.3 | Confirmatory testing procedures – Isotope ratio mass spectrometry

A variety of naturally occurring endogenous steroidal analytes are monitored in routine doping controls including the aforementioned steroid profile, respectively, the steroidal module of the ABP as well as other endogenous markers of AAS misuse. In accordance to WADA's regulations,[55] the carbon isotope signatures of target compounds (TCs) and endogenous reference compounds (ERCs) are determined in the case of suspicious initial testing results, and several ERCs have been suggested including e.g. pregnanediol (PD), pregnanetriol (PT), 11-OH-A, and 11-oxo-E. The individual variability of these ERCs was the subject of a study by de la Torre et al., aimed at assessing the possibility and/or need for identifying potential outliers of ERC $\delta^{13}C$ values, affecting the subsequently computed and utilized $\Delta^{13}C$ value of the ERC and respective TC(s).[64] A total of 56 non-athletes' urine samples and 1094 routine doping control samples were analyzed and evaluated, demonstrating well conserved carbon isotope signatures along the underlying biosynthetic pathways towards these ERCs in the absence of interfering drug administrations. Consequently, when monitoring multiple ERCs in routine doping controls, outliers resulting from e.g. cortisol, pregnenolone, or progesterone administrations or procedural artefacts can be eliminated by allowing for a maximum standard deviation of 0.54‰ and a range of 1.2‰. Traditional Asian medicine composed of deer musk extracts was reported to contain a variety of steroidal substances that evidently influence an athlete's steroid profile. In a comprehensive analysis of six wild and 14 domestic deer musk preparations, He et al. did not observe relevant amounts of substances potentially affecting the carbon isotope signature of ERCs. However, a broad range of $\delta^{13}C$ values of TCs was found, with significantly more depleted values in products prepared from wild animals (from −27.1 to −29.6‰) than from domesticized deer (from −20.5 to 24.9‰).[65] Consequently, the administration of some preparations might exhibit a significant impact on an athlete's ABP with GC/C/IRMS analyses yielding inconclusive results. A similar issue was identified with regard to the detection of 19-norandrosterone, (19-NA), the main metabolite of the AAS nandrolone. In the case of urinary 19-NA concentrations between 2.5 and 15 ng/mL, IRMS analyses are mandatory to support differentiating between an endogenous formation and an exogenous source of the analyte, and $\Delta^{13}C$ values greater 3‰ constitute an AAF.[66] Brailsford et al. recently reported on the detection of four nandrolone ester formulations, which were found to yield $\delta^{13}C$ values for nandrolone between −21.5 and − 22.5‰,[67] thus precluding the corroboration of an ADVR by means of IRMS in various instances. The situation has gained further complexity by the fact that nandrolone, ingested by athletes via offal or meat obtained from uncastrated pigs, can exhibit a broad range of carbon isotope signatures as presented by Hülsemann et al.[68] In the course of follow-up studies concerning ATF/AAF case investigations, the seasonal variability of $\delta^{13}C$ values of wild boar-derived edible tissue and, consequently, also the contained nandrolone, was reported. Hence, depending on the athlete's ERC $\delta^{13}C$ values, a doping control urine sample test result can either be atypical/inconclusive (i.e. the animal's $\delta^{13}C$ values coincidentally match those of the athlete) or lead to an AAF as demonstrated in an elimination study with wild boar testicles and retrospective carbon isotope signature determination through bristle $\delta^{13}C$ determinations.

2.1.4 | Other anabolic agents

Selective androgen receptor modulators (SARMs) have been undergoing extensive research programs due to their reported tissue-selectivity and, consequently, promising therapeutic utility.[69] No drug candidate of the class of SARMs, however, has yet received clinical approval; nevertheless, the number of AAFs as the result of the presence of SARMs (or respective metabolites) in routine doping controls has continued to increase. The basis of the sensitive and specific detection of a subset of the enormous variety of SARM-like drug candidates has been supported by sophisticated ITPs such as those reported by Ventura et al.[70] but also by identifying most adequate urinary metabolites.[71,72] A comprehensive analytical assay covering a total of 15 SARMs was reported, validated for the determination of the target analytes in animal and human urine.[70] The sample preparation included LLE only, and compounds of interest (including those most frequently reported in AAFs) were analyzed by LC-QqQ-MS/MS. The instrument was composed of a LC system equipped with a C-18 analytical column (2.1 × 100 mm, particle size 1.6 μm) employing a gradient elution using 0.1% acetic acid (solvent A) and methanol (containing 0.1% acetic acid, solvent B), interfaced via ESI to a QqQ-based mass analyzer operated with polarity switching. Accomplished LODs ranged from 0.05–0.75 ng/mL (in equine urine) and, in consideration of the fewer ion suppression effects observed for human urine, similar LODS are expected. Of note, only intact and unconjugated drug candidates were used as target compounds; here, future adjustments might be required to ensure adequate detection windows, as earlier studies demonstrated substantial metabolic conversions of some of the SARMs included in the presented test method, which are currently not taken into consideration. One of these SARMs is LGD-4033, which was recently shown to be most efficiently detected by means of its glucuronic acid conjugate or an unconjugatedly excreted bishydroxylated metabolite.[72] In that study, following the oral administration of 10 mg of LGD-4033, detection windows of up to 21 days were accomplished for metabolic products of LGD-4033 in urine using LC-Q/TOF-MS. The LC was equipped with a C-18 analytical column (2.1 × 100 mm, 1.8 μm particle size), and ammonium formate (5 mM, containing 0.01% formic acid) and

acetonitrile/water (90/10, v/v, also containing 5 mM ammonium formate and 0.01% formic acid) were used as solvents A and B, respectively. The MS was operated in full scan and targeted MS/MS mode, and separate analytical runs in positive and negative ionization mode were conducted.

A distinct challenge in sports drug testing that, despite various studies, has still not been analytically addressed, is the differentiation of clenbuterol originating from an inadvertent ingestion via meat contamination from a deliberate doping offence scenario. In order to support the option of follow-up investigations prior to asserting an ADRV due to a clenbuterol-related AAF, WADA amended the World Anti-Doping Code in May 2019. Before the amendment, the reporting of ATFs was limited to endogenous substances; from 1 June 2019 onwards, laboratories shall report ATFs for urinary clenbuterol concentrations below 5 ng/mL, enabling in-depth investigations by antidoping organizations concerning potential meat contamination scenarios.[73] If such a scenario plausibly matches the analytical finding, the tested athlete will not be confronted with an ADRV. Further analytical insights and means remain however desirable, and the idea of factoring enantiomeric compositions into the decision-making process was pursued by Dolores et al.[74] A fast LC-QqQ-MS-based test method enabling the separation of *R*- and *S*-clenbuterol enantiomers was developed, supporting the analysis of clenbuterol-containing matrices in less than 7 min. Chromatography was conducted using an analytical column (4 × 100 mm) with a chiral stationary phase composed of α1-acid glycoprotein immobilized to spherical silica particles (5 μm) and isocratic elution of analytes with 10 mM ammonium formate (solvent A) and acetonitrile (solvent B) at 97/3 (v/v). Diagnostic precursor/product ion pairs for clenbuterol were monitored in SRM mode allowing for LODs of 12.5 pg/mL. Interestingly, no internal standard was employed. Elimination studies simulating doping scenarios and meat (food) contamination were conducted using Wistar rats, and urine samples were prepared by alkaline LLE for LC–MS/MS analysis. The obtained results demonstrated that a significant difference in enantiomer abundance existed in urine samples collected after drug administration, while contaminated meat ingestion did not present significantly different signal intensities of the two clenbuterol enantiomers. Whether this phenomenon also translates to humans is yet unclear and the analytical approach would benefit from implementing stable isotope-labeled clenbuterol as an internal standard; nevertheless, the presented method demonstrates the feasibility of routinely separating and detecting clenbuterol isomers at relevant concentrations.

3 | PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES, AND MIMETICS

3.1 | Erythropoietin-receptor agonists and transforming growth factor-beta (TGF-β) inhibitors

Most analytical assays enabling the detection of erythropoietin (EPO)-receptor agonists in doping control blood or urine samples utilize gel electrophoretic approaches, optimized to accommodate the diverse physico-chemical properties of recombinant human erythropoietin, its analogs and/or EPO-mimetic agents.[75] The established methods have been shown to provide robust detection windows unaffected by hyperhydration,[76] yet faster and less laborious ITP alternatives have been desirable and the applicability of an integrated capillary electrophoresis (CE)/Western blotting system was assessed by Desharnais et al.[77] Following established protocols, 15 mL of urine are concentrated by ultrafiltration and, subsequently, ESAs are immunopurified before loading into autosampler plates for automated CE-supported analysis. The CE system was equipped with a 12–230 kDa nano-capillary (5 cm × 100 μm) based separation module, which allowed for immobilizing proteins to the capillary wall after electrophoresis followed by incubation with primary and secondary antibodies for chemiluminescent detection. Pilot study data demonstrated LODs for human urinary erythropoietin and recombinant human EPO biological reference preparation (BRP) between 1 and 3 mIU/μL, and EPO-Fc, NESP, and CERA were detected at concentrations ranging from 1.5 to 6 pg/mL. While routinely employed Sarcosyl (SAR)-polyacrylamide gel electrophoresis (PAGE)-based methods are reportedly more sensitive, the herein presented approach offered competitive features especially with regard to sample throughput and workload, warranting the monitoring of future developments.

The successful implementation of additional target analytes such as the transforming growth factor-beta (TGF-β) inhibitors sotatercept and luspatercept[78] into different conventional ESA-specific ITPs was reported by various research units. For instance, Martin et al. described a strategy employing magnetic nanoparticles coated with monoclonal anti-EPO-, anti-ActRIIA-, and anti-ActRIIB antibodies used to immunoextract 0.3–1.0 mL of serum or plasma.[79] By means of a pH gradient ranging from pH 2–10, isoelectric focusing (IEF) and double blotting, sotatercept, luspatercept, various ActRII-Fc fusion proteins and the commonly tested erythropoietins were jointly analyzed, enabling LODs of approximately 5 ng/mL for sotatercept and luspatercept and 15–100 pg/mL for erythropoietin and its derivatives, meeting the requirements of corresponding WADA technical documents.[80] Reichel et al. demonstrated the capability of a SAR-PAGE-based method to detect both analytes in spiked human serum at LODs of 1 ng/mL.[81] Similar to earlier approaches, only 50 μL of serum was required, which was subjected to magnetic nanoparticle-supported immunoprecipitation, and resulting extracts were applied to 10% BisTris gels. A semi-dry Western blot was conducted, and target analytes were detected by means of biotinylated primary antibodies directed against sotatercept and luspatercept in combination with a streptavidin-horseradish peroxidase complex, yielding an overall accelerated and cost-effective test method for TGF-β inhibitors. Complementary to these approaches, Walpurgis et al. presented a test method for the detection of sotatercept and luspatercept in human serum by mass spectrometry-based strategies.[82] A volume of 200 μL of serum was subjected to ammonium sulfate precipitation, and target analytes were extracted from the obtained particulate by means of magnetic nanoparticles coated with anti-ActRIIA and -ActRIIB antibodies. The

extract was subsequently trypsinized and signature peptides of both the Fc- and/or the receptor domain of the TGF-β signaling inhibitors were used as target analytes in LC-HRMS(/MS). The LC system utilized a C-18 analytical column (3 × 50 mm, 2.7 μm particle size) operated with 0.2% formic acid (solvent A) and acetonitrile (containing 2% DMSO, solvent B). Mass spectrometric detection was accomplished in targeted SIM and data-dependent MS/MS mode at a resolution of 30,000 (FWHM), and the overall assay LODs was reported with 50 ng/mL for the intact decoy receptors. While gel electrophoretic approaches proved superior concerning LODs, confirmatory amino acid sequence information is provided using the presented methodology. The applicability of MS-based analytical approaches to the detection of TGF-β signaling inhibitors in dried blood spots (DBS) samples was shown by Lange et al., who successfully determined sotatercept and luspatercept as well as the ActRIIA/B-antibody bimagrumab.[83] In an initial testing procedure, dried blood spots were extracted into phosphate-buffered saline and target antibodies were enriched by isolation using protein-G-coated magnetic nanoparticles followed by trypsin digestion and LC-HRMS(/MS) analysis of proteotypical peptides. Compound-dedicated confirmatory analyses of the same analytes from dried blood spots consisted of the extraction of the spots into ammonium bicarbonate solution with subsequent ammonium sulfate precipitation of the IgG content and immunoaffinity purification of the particulate by activin A-coated magnetic nanoparticles. After tryptic digestion, diagnostic peptides were determined by LC-HRMS(/MS) analysis using similar instrumental conditions as presented by Walpurgis et al.[82] The LOD of the assays were found to be 250 ng/mL, and the application of the test method to clinical trial post-administration samples containing bimagrumab provided proof-of-concept with the unequivocal detection of the drug candidate in specimens sampled after subcutaneous as well as intravenous administration.

3.2 | Hypoxia-inducible factor (HIF) activating agents

Orally available prolyl hydroxylase inhibitors such as roxadustat,[84] vadadustat, molidustat, desidustat,[85] etc. have been under development for several years as an alternative therapeutic option to erythropoietin, and in 2018, roxadustat received clinical approval in China as a first representative of this drug class.[86] In the light of recent blood doping scandals[16] where, amongst others, the use of hypoxia-inducible factor activating agents was mentioned as a means to manipulate the athletes' blood parameters affecting the hematological module of the ABP,[87] intensifying the consideration of cobalt as a doping agent appears warranted. Hoffmeister et al. demonstrated in a double-blind and placebo-controlled study that cobalt supplementation at 10 mg/day over a period of 5 days is required to significantly stimulate erythropoiesis.[88] Urinary cobalt concentrations increased from baseline values up to ca. 600 ng/mL within the first 3 h post-administration, providing further insights for potential future urinary cobalt thresholds.

3.3 | Growth hormone (GH), its fragments and releasing factors, chorionic gonadotrophin (CG), and luteinizing hormone (LH)

Detecting the misuse of growth hormone (GH) by means of routine doping controls has been a particularly complex task despite the development and continuous refinement of different analytical approaches,[89-91] and the declarations of confessing athletes frequently included statements concerning their GH use regimens (and strategies to avoid detection). Instead of employing population-based reference ranges as currently utilized for the GH-2000 score and its main parameters, i.e. serum concentrations of the insulin-like growth factor-I, IGF-I, and procollagen type III N-terminal peptide (P-III-NP), the use of individual reference ranges for these markers as obtained by longitudinal monitoring has been suggested in the past. In that context, the capability of an ABP-like approach to detect a 2-week drug administration regimen of low- and high-dose GH administrations was assessed by Lehtihet et al.[92] IGF-I, P-III-NP, and different miRNA markers were analyzed from pre- and post-administration serum samples collected from healthy male volunteers up to 4 weeks after the cessation of drug use, and individual maximum permissible values were arbitrarily established at three standard deviations above the persons' baseline values for each parameter (including the individual GH-2000 score). While the miRNA did not provide profitable results, the longitudinal monitoring of IGF-I, P-III-NP, and the GH-2000 score yielded promising test results that potentially allow for significantly extending the detection window for GH administrations. Hence, creating a routine workflow for an endocrine module of the ABP appears particularly useful and desirable, which necessitates, however, robust analytical procedures ensuring the comparability of quantitative test results. Most analyses concerning IGF-I and P-III-NP are currently conducted by immunological methods; more recently, several protocols for LC–MS(/MS)-based assays were published and, especially when using stable isotope-labeled internal standards, precise and accurate test results were obtained. This strategy was confirmed by Bronsema et al., who determined IGF-I from human plasma by bottom-up analytical approaches as well as conventional immunoassay analysis, and the comparison of both platforms demonstrated good correlation of test results.[93] For LC–MS/MS analysis, 50 μL of plasma was diluted with 8 M aqueous urea solution, and cysteine bonds were reduced and alkylated prior to trypsin digestion. The obtained peptides were solid-phase extracted and the eluate was analyzed on an LC equipped with a C-18 charged surface hybrid column (2.1 × 150 mm, 1.7 μm particle size) using 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B). The effluent was directed via ESI to a QqQ-based analyzer measuring diagnostic precursor/product ion pairs for two signature peptides (T-1 and T-4) in MRM mode, allowing for a limit of quantification (LOQ) of 10 ng/mL.

Comprehensiveness is critical to routine sports drug testing programs, and besides the utmost sensitivity and robustness, the compatibility of assays with new target analytes is especially helpful for effective workflows. The best possible analyte recovery is one major

aspect of initial testing procedures, and improving the enrichment of peptidic substances from biological matrices by using ion-pair SPE was demonstrated by Judak et al.[94] Acidifying urine with formic acid or trifluoroacetic acid was shown to enhance the recovery of nine model peptides, allowing for lowering the test methods' LODs in doping controls as is frequently required to cope with new additions to the illicit drug market such as the recently detected analogs of growth hormone releasing peptides (GHRPs). On various occasions, the detection of GHRPs and a synthetic version of the GH releasing hormone (GHRH) modified at the N-terminus by attaching an additional glycine residue was reported.[95,96] Whether this modification is eliminated by in vivo metabolic reactions yielding established target analytes of GHRPs and GHRH remains to be shown; if the modification is sufficiently resistant to proteolytic activities in vivo, test methods will potentially necessitate adaptation. A flexible strategy employing a dilute-and-inject approach combined with online-trapping and LC-HRMS(/MS) was presented by Görgens et al., covering a total of 35 peptidic drugs or respective urinary metabolites including (amongst others) 11 growth hormone releasing factors and 12 luteinizing hormone (LH) releasing hormones.[97] Online trapping was accomplished by means of isocratic loading of 40 μL of urine onto a phenyl-hexyl trapping column (3 × 10 mm, 2.6 μm particle size), and the analytes were subsequently backflushed for separation onto a C-18 analytical column (2 × 50 mm, 2.7 μm particle size). Sample loading was accomplished by using 99% A (1% formic acid containing 1% DMSO)/1% B (acetonitrile) within 3 min, and gradient elution was conducted within 14 min thereafter. Using positive ESI, all analytes were detected by full scan MS (resolution 35,000), targeted SIM (resolution 70,000), and data-dependent MS/MS (resolution 17,500), which allowed for estimated LODs between 50 and 200 pg/mL for all substances. Due to the generic instrumental setup and minimal sample preparation, the inclusion of additional analytes is facilitated and also retrospective data mining is readily accommodated.

Only a few analytes are tested almost exclusively by immunological methods in routine sports drug testing laboratories, and two of these substances are human chorionic gonadotropin (hCH) and luteinizing hormone (LH). An ultra micro analytical system technology for assaying hCG was assessed by Martinez-Brito et al., demonstrating the platform's fitness-for-purpose for doping control purposes as an initial screening tool.[98] Due to the target epitope of the employed monoclonal antibody being located at the hCG β-chain, total hCG rather than intact hCG levels are determined, but assay precision, linearity, accuracy, and specificity were shown to comply with the requirements outlined in the WADA technical document.[99] The questions as to whether hyperhydration affects urinary LH determinations and whether a dilution effect can be compensated for by specific gravity adjustment were addressed by Athanasiadou et al.[100] Hyperhydration was induced by means of water or commercial sports drink administration at 20 mL of liquid/kg body weight ingested within 30 min, and immunologically determined urinary LH values were significantly lowered by excessive fluid intake. While low urinary LH concentrations do not constitute an ATF per se, they contribute to steroid profile interpretations. Hence, as specific gravity adjustment adequately corrected for the dilution effect, this strategy was introduced in routine doping controls.

4 | β_2-AGONISTS

The occurrence of exercise-induced respiratory symptoms such as acute contraction of the bronchial passage is frequently treated with β_2-agonists in the normal population as well as in elite athletes. The latter group needs to consider applicable antidoping rules, which are multifaceted and, depending on the substance in question and the route of administration, stipulate different regulations concerning the necessity of a therapeutic use exemption or the allowed maximum daily dosages. Adequate therapies for athletes have to be guaranteed, and several groups have identified the need for guidance concerning appropriate diagnostics and subsequent treatment whilst respecting boundaries resulting from antidoping rules, arising from the suspected, purported, and/or proven misuse of β_2-agonists in sports.[101-103] The fact that β_2-agonists such as terbutaline can significantly affect the athlete's body composition was demonstrated by Jessen et al., who conducted a placebo-controlled study with a "sedentary lifestyle" cohort, a resistance-training cohort, and an endurance-training cohort.[104] Over a period of 4 weeks, terbutaline (or placebo) was administered via inhalation at 8 × 0.5 mg/day, and an increase in lean body mass of ca. 1 kg was determined for the habitual and the resistance training group, suggesting a considerable skeletal muscle growth. Conversely, no effect was reported concerning the endurance-trained cohort, which matches the observations of Molphy et al., who investigated the effect of inhaled terbutaline (2 mg and 4 mg) on athletes' 3 km time trial runs.[105] Significantly enhanced values for the forced expiratory volume were recorded, which however did not translate into an ergogenic advantage.

Considerably lower dosages of 54 μg in 24 h are permitted for inhalative use concerning formoterol, and a urinary threshold exists at 40 ng/mL, above which an AAF is reported by the antidoping laboratories. Jacobson et al. raised concerns regarding the fact that (most) commercial formulations are composed of a racemic mixture of formoterol, containing approximately 50% of the (*S*,*S*)-enantiomer of the drug that can be considered as pharmacologically inert. Due to the fact that routine doping controls do not differentiate between the different enantiomers of β_2-agonists, switching to enantiopure products (e.g. arformoterol) would effectively double the amount of active formoterol without being tangential to the established urinary threshold, and enantioselective analytical approaches were suggested for sports drug testing purposes.[106] One option to test for formoterol enantiomers was presented, consisting of an enzymatic hydrolysis of urine followed by LLE for subsequent LC–MS/MS analysis. The LC was equipped with a chirobiotic T2 chiral column (4.6 × 250 mm, 5 μm particle size) which was isocratically operated using methanol containing 0.2% acetic acid and 0.025% ammonium hydroxide. Formoterol and its stable isotope-labeled internal standard was monitored by MRM, enabling a LOQ of 22 pg/mL. While such analyses necessitate dedicated instrumentation and assays, future confirmatory

procedures in doping controls might need to consider enantiomeric separation and quantification of substances.

The natural occurrence of the β_2-agonist higenamine in traditional medicinal plants such as *Nandina domestica*, *Aconitum charmichaelii*, etc. has continued to fuel discussions in the antidoping context, especially regarding the question of whether the administration of over-the-counter products or dietary supplements can result in AAFs and thus in (inadvertent) antidoping rule violations. Grucza et al. reported on the analysis of five different nutritional supplements, four of which did not declare higenamine (or its natural source) as a potential ingredient but de facto contained between 12 and 19 mg of the prohibited substance per g of supplement.[107] The presence of considerably larger amounts of higenamine in dietary supplements, whilst mostly declared on the label, was reported by Cohen et al., who determined up to 62 mg per serving,[108] which further underlines the growing issue of higenamine, as the administration of such supplements is likely to cause AAFs in routine doping controls.

5 | HORMONE AND METABOLIC MODULATORS

The class "hormone and metabolic modulators" of the Prohibited List comprises five subcategories and substances of particularly diverse physicochemical and pharmaceutical nature, amongst which aromatase inhibitors, selective estrogen receptor modulators (SERMs), and other anti-estrogens constitute three out of these five subgroups. Formestane, a steroidal aromatase inhibitor, occurs naturally in human urine, and analytical strategies allowing for the differentiation of its endogenous or exogenous origin have been implemented based on target analyte abundance, drug/metabolite ratio analysis and, where indicated, GC/C/IRMS analysis. de la Torre et al. recently suggested considering an additional marker supporting the identification of formestane administration by introducing the ratio 4-OH-epiandrosterone (4-OH-EA)/4-OH-androsterone (4-OH-A).[109] While elevated 4-OH-EA/formestane was found to be characteristic for an oral administration of formestane, the transdermal application of the drug resulted in a considerably different urinary profile of the intact drug and its metabolic products. In order to facilitate differentiation of the route of administration, elimination study urine samples collected from six male volunteers receiving a single transdermal dose of 200 mg of formestane were analyzed using GC–MS and GC–MS/MS approaches. The urine samples were prepared in accordance with routine doping control protocols including enzymatic hydrolysis, LLE, and trimethylsilylation, and monitoring formestane, 4-OH-EA, and 4-OH-A demonstrated that the combination of the concentration ratios of 4-OH-EA/formestane >2 and 4-OH-EA/4-OH-A > 1 would be indicative for an oral intake of the prohibited aromatase inhibitor. Concentration ratios of 4-OH-EA/4-OH-A ≤ 1 would suggest other routes of administration such as transdermal formestane supply. Further to this, the question also arose as to whether synthetic isoflavones also necessitate consideration in the category of aromatase inhibitors.[110] Iannone et al. investigated the inhibitory potency of methoxyisoflavone and ipriflavone on the aromatization of testosterone in vitro, and compared the obtained results with the effect of currently prohibited substances such as aminogluthetimide, anastrozole, and formestane on the aromatization of testosterone. Similar inhibition kinetic constants were recorded for the aromatase inhibitory drugs and the studied synthetic isoflavones, and closely monitoring future developments in both production and use of products containing significant amounts of isoflavones was suggested. While this proposal is plausible considering the enforced Prohibited List, an ongoing debate exists concerning the general relevance of banning aromatase inhibitors in females, which was further fomented by an AAF concerning letrozole. Differentiating the misuse of the drug from a single inadvertent and accidental intake by means of a single doping control urine sample is particularly difficult, and hence the applicability of hair testing was assessed by Favretto et al. who investigated the traceability and concentration range of letrozole in hair following single dose and chronic letrozole use.[111] In a pilot study setting, amounts of 0.62 mg, 1.25 mg, and 2.5 mg were orally administered, and urine samples were collected over a period of 9 days. Head and beard hair was collected after 3, 6, 10, and 90 days, and drug concentrations ranging from 17 to 160 pg/mg were determined. Hair collected from chronic users returned concentrations ranging between 283 and 334 pg/mg over the entire hair shaft, indicating a potential alternative matrix and test option in cases where a potential inadvertent drug intake is to be investigated.

Rumors regarding the misuse of the so-called exercise mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) resurfaced during the 2019 Tour de France.[112] Due to its natural occurrence, methods enabling the differentiation of endogenously produced from synthetic AICAR was required and was addressed by means of GC/C/IRMS in the past. However, in the light of the substantial variability of intra- and inter-individual abundance and urinary concentration, trigger values for follow-up studies were desirable and, based on the analysis of over 12,000 doping control urine samples, Sobolevsky and Ahrens suggested that urinary AICAR levels in excess of 2500 ng/mL warrant GC/C/IRMS analysis.[113] A mean urinary AICAR concentration of 647 (±365) ng/mL was determined by LC–MS/MS using a dilute-and-inject approach, with a maximum concentration of 4461 ng/mL. The fact that also considerably higher urinary AICAR levels do not necessarily indicate misuse of the drug candidate was corroborated by a finding of approximately 12,000 ng/mL, the carbon isotope signature of which presented a natural endogenous δ-value.[114] Other drug candidates, however, might influence the endogenous production and/or accumulation of AICAR such as the "mitochondrial open reading frame of 12S rRNA type-c" (MOTS-c) peptide,[115,116] which consequently necessitate consideration in proactive antidoping research. Knoop et al. focused on targeting MOTS-c and *in vitro*-generated metabolic products in human plasma and serum by means of LC–MS/MS.[117] A volume of 100 μL of plasma (or serum) was enriched with stable isotope-labeled MOTS-c as internal standard, and plasma proteins were precipitated by the addition of acetonitrile. MOTS-c and its metabolites remained in solution and after concentration of the volume under reduced pressure, all target

analytes were separated on a C-8 analytical column (3 × 50 mm, 2.7 μm particle size) using 0.1% formic acid (containing 1% DMSO) and acetonitrile (also containing 0.1% formic acid and 1% DMSO) as solvents A and B, respectively. By means of unispray ionization and recording of diagnostic precursor/product ion pairs measured on a QqQ-MS in positive MRM mode, an LOD of 0.1 ng/mL was accomplished for the target peptide MOTS-c. The assay was applied to 20 fresh human plasma samples, but despite providing an adequate sensitivity and specificity, all specimens returned negative test results although reference ranges for MOTS-c were reported between 46 and 219 ng/mL. Hence, further studies will be necessary to demonstrate whether MOTS-c (or future derivatives thereof)[118] can be appropriately detected using the presented LC–MS/MS approach.[119]

Another class of exercise mimetic agents are agonists of the nuclear Rev-erb receptor with SR9009 as a prominent drug candidate. SR9009 was the subject of comprehensive in vitro metabolism studies, investigating the potential effects of sex, genetic polymorphism, and the influence of drug–drug interactions on the excretion profile of diagnostic metabolites.[120] A total of 13 metabolites was identified when incubating the drug candidate SR9009 with human liver microsomal preparations, most prominently yielding *N*-dealkylated degradation products, which presented a considerable overlap with urinary metabolites determined from elimination study samples. While sex did not appear to affect the metabolism of SR9009, a significant influence of genetic polymorphism (especially concerning CYP2D6 and CYP2C19) as well as the presence of antifungal therapeutics or antidepressants was recorded. Overall, the targeting of three metabolites resulting from dealkylation reactions (Figure 1A) was recommended, two of which were recently synthesized and commercialized as phase-I metabolites for sports drug testing purposes.[121] Of note, in vivo metabolism data indicated a significant share of glucuronic acid conjugation of these metabolites, and hence hydrolysis and/or inclusion of intact phase-II metabolites into routine doping controls is recommended.

Further insights into the elimination characteristics of the metabolic modulator meldonium were presented by Rabin et al., who investigated the excretion profile of the prohibited drug following repeated oral administrations at different dosages (daily administrations of 1 g and 2 g, respectively, over a period of 3 weeks) and potential effects of physical exercise and administration of L-carnitine.[122] Urine and blood samples were collected and analyzed by isotope-dilution LC–MS/MS, employing a reversed-phase C-18 analytical column (1 × 100 mm, 1.9 μm particle size) with 0.01% formic acid and acetonitrile used as solvents A and B, respectively, and an ESI-Q/Orbitrap-based detection employing the parallel reaction monitoring (PRM) mode. With an assay LOQ of 10 ng/mL, meldonium was determined in the participants' samples, and meldonium was detected up to 259 days after receiving the last dose (study day 280). Of note, L-carnitine supplementation provoked an increased meldonium excretion in women at the beginning of the L-carnitine intervention, but the effect was not observed at later time points and was also not consistently observed among male participants. Conversely, exercise led to temporarily decreased urinary meldonium concentrations across all groups.

Another example for the expanding utility of DBS for routine doping controls[123] was presented by Thomas et al., who demonstrated the applicability of the minimally invasive sampling technique to the analysis of insulin and insulin analogs.[124] Employing citric acid-pretreated DBS collection cards, spots of 20 μL volume were collected and subjected to an extraction protocol consisting of excising the entire spot, which was then extracted twice into 200 μL of a mixture of acetonitrile/3% acetic acid (60/40, v/v) and both volumes were combined and diluted with water prior to SPE. Insulins retained in the obtained extract were further purified by immunoaffinity enrichment and subsequently analyzed by LC-HRMS. The LC system consisted of a C-18 analytical column (3 × 50 mm, 2.7 μm particle size) and gradient elution using 0.1% formic acid (containing 1% DMSO, solvent A) and acetonitrile (containing 1% DMSO, solvent B) was conducted. Mass spectrometric detection was accomplished using either an ion mobility/Q/TOF or a Q/Orbitrap-equipped instrument, in enhanced full MS and MS/MS mode or targeted SIM and data-dependent MS/MS mode, respectively. With an LOD of 0.5 ng/mL (1.5 ng/mL for insulin detemir), the established method allowed for detecting normal non-fasting levels of human insulin and eight synthetic analogs in DBS, and authentic DBS collected from a type-I diabetic individual using insulin aspart were successfully analyzed providing proof-of-concept data.

Trimetazidine is classified under S4.5.4 of WADA's Prohibited List but is also a metabolite of the anti-migraine calcium channel blocker lomerizine. Differentiating the source of urinary trimetazidine, i.e. the legitimate use of lomerizine or the prohibited use of trimetazidine, was accomplished by monitoring a characteristic urinary metabolite of lomerizine (Figure 1B), which was shown to be abundantly present in post-administration urine samples collected after oral application of therapeutic amounts (15 mg) of lomerizine to 10 healthy male volunteers.[125] A dilute-and-inject test method was established, utilizing a C-18 analytical column (2.1 × 50 mm, 1.7 μm particle size) operated with 0.1% formic acid (solvent A), and methanol (solvent B), and a Q/Orbitrap-based MS using targeted MS/MS experiments. The assay's LOD was reported to be 0.02 ng/mL for lomerizine, trimetazidine, and the lomerizine metabolite M6, with M6 being found at the end of the study period (276 h) at concentrations of up to 38 ng/mL, the diagnostic value of M6 and especially the abundance ratio M6/trimetazidine was corroborated. This strategy was applied in the case of 14 routine doping control samples, where the use of the anti-migraine drug lomerizine was confirmed based on the presence of both analytes in expected concentration ranges and abundance ratios.

6 | DIURETICS AND OTHER MASKING AGENTS, STIMULANTS, NARCOTICS, AND BETA-BLOCKERS

Modern analytical instruments as routinely applied in doping controls provide the necessary sensitivity and selectivity to unequivocally determine classes of prohibited substances including diuretics and other masking agents, stimulants, narcotics, and beta-blockers readily

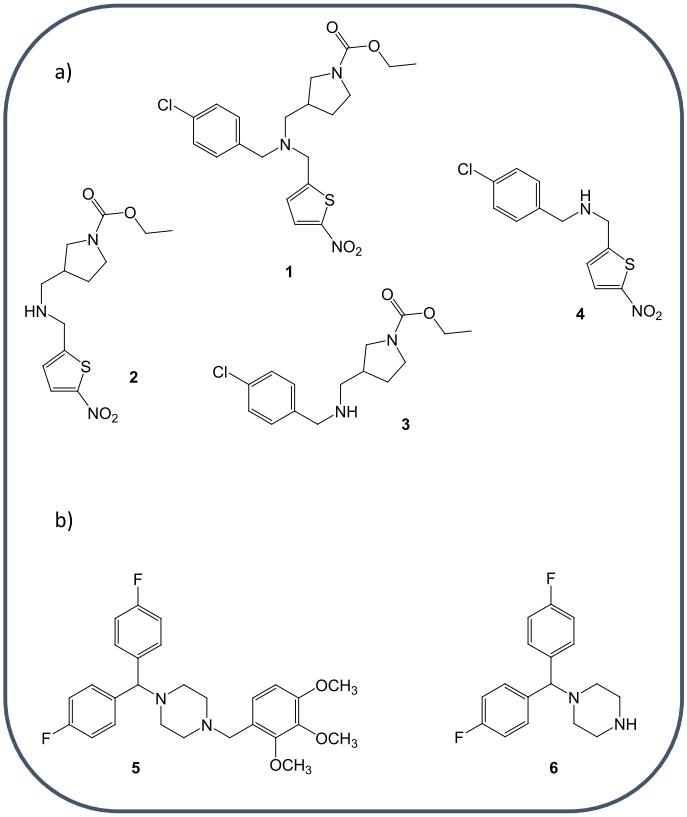

FIGURE 1 Structures of (a) SR9009 (**1**, Mol wt = 437.12 u) and urinary target analytes (**2**, Mol wt = 313.11 u; **3**, Mol wt = 296.13 u; **4**, Mol wt = 282.02 u), and (B) lomerizine (**5**, Mol wt = 468.22 u) and its characteristic metabolite bis-(4-fluorophenyl)-methylpiperazine (**6**, Mol wt = 288.14 u)

meeting WADA's MRPL. Yet, optimization of multi-analyte test methods is ongoing, particularly concerning throughput and robustness, supporting faster turnaround times, the implementation of new/additional analytes and metabolites,[126] and reduced manual workload. A comprehensive and rapid ITP was presented by Han et al., who reported on the detection of a total of 111 substances and corresponding metabolites, belonging (amongst others) to the classes of diuretics and masking agents, stimulants, beta-blockers, and narcotics on the WADA's Prohibited List.[127] Urine samples were enzymatically hydrolyzed prior to mixed-mode weak cation exchange SPE, and extracts were concentrated and injected into a LC-HRMS(/MS) employing a Q/Orbitrap mass analyzer. Liquid chromatography was conducted using a C-18 analytical column (2.1 × 100 mm, 2.6 μm particle size) and 0.1% formic acid (solvent A) respectively methanol containing 0.1% formic acid (solvent B) with a total run time of 10 min per sample. The MS was operated in two modes, full scan (at a resolution of 35000) and variable data-independent acquisition (at a resolution of 17500) divided into a total of five events, allowing for sufficient numbers of data points per chromatographic peak and adequate LODs in consideration of the mandatory MRPLs. The option of avoiding enzymatic hydrolysis and incorporating the SPE into the LC–MS/MS analysis was demonstrated for a total of 50 diuretics and masking agents by De Wilde et al.[128] Employing a two-dimensional LC setup consisting of a turbulent flow SPE column (2.1 × 20 mm, 25 μm particle size) and a C-8 analytical column (2 × 150 mm, 5 μm particle size), 20 μL of urine enriched with internal standards was loaded/online-extracted and chromatographed for subsequent QqQ-based analyte identification. The MS was operated with ESI and polarity switching, monitoring diagnostic precursor/product ion pairs in SRM mode, which allowed for LODs between 1 and 20 ng/mL. The use of the turbulent flow online-SPE approach resulted in significantly fewer matrix effects and contributed to substantially more stable retention times of target substances when compared with conventional dilute-and-inject methods, and the relatively long analytical run time of 18 min was effectively compensated for by the minimal effort required for sample preparation. The accomplished analytical sensitivities in routine doping controls are of particular importance when considering the desirable retrospectivity of sample analyses. Detection limits as reported above can, however, also result in AAFs when contaminated products are consumed as reported in the past, especially in the case of diuretics such as hydrochlorothiazide.[129] In that context, Favretto et al. discussed a case of contaminated dietary supplements that were obtained from a compounding pharmacy and used by an athlete, whose doping control samples returned two AAFs for hydrochlorothiazide at concentrations below 10 ng/mL.[130] Reproducing the scenario with sealed dietary supplement products of the same provider under controlled conditions with volunteers confirmed the possibility of an

inadvertent drug intake, calling into question whether diuretics should still be considered non-threshold substances in the future or if reporting levels should be established. The option of obtaining additional information through hair testing was assessed by Gheddar et al., who analyzed hair samples from volunteers who received a single oral dose of 25 mg of hydrochlorothiazide and specimens from patients receiving daily dosages of hydrochlorothiazide between 6.25 and 25 mg.[131] It was shown that single therapeutic dosages did not result in detectable concentrations of the drug in hair, while chronic use caused hydrochlorothiazide incorporation into hair at levels between 12 and 1845 pg/mg.

Threshold levels do exist for selected stimulants included in the WADA's Prohibited List, and AAFs are only reported when respective urinary threshold levels are exceeded as for example when pseudoephedrine was detected at concentrations higher than 150 μg/mL.[21] Although the extent of performance-enhancing effects was reported as small as outlined in a recent meta-analysis that included studies with pseudoephedrine dosages up to 2.8 mg/kg bodyweight, the misuse of even larger doses cannot be excluded and adequate test methods are required.[132] The separation of pseudoephedrine enantiomers is unlikely to be necessary in routine doping controls; nevertheless, an excellent separation employing an analytical column with an i-amylose stationary phase (4.6 × 150 mm, 5 μm particle size) and n-hexane containing 0.05% formic acid (solvent A) and 2-propanol containing 0.05% formic acid (solvent B) was accomplished.[133] Despite the unconventional composition of the mobile phases, compatibility with mass spectrometry was shown, enabling detection limits of 20 ng/mL.

7 | MANIPULATION OF BLOOD AND BLOOD COMPONENTS

Various strategies exist to artificially increase an athlete's red blood cell mass, several of which are prohibited in sports and, consequently, are commonly referred to as blood doping. Especially the enhanced arterial oxygen concentration resulting from an increased number of erythrocytes was identified as a key factor positively affecting endurance performance,[134,135] and also chronically altitude-adapted endurance athletes were reported to respond to drug-induced erythropoiesis with an enhanced hematocrit, hemoglobin concentration, reticulocyte percentage, and improved time trial performances.[136]

The hematological module of the ABP has substantially contributed to the efficacy of antidoping testing strategies in the past, and the continuous refinement enabled by a constant influx of new research data and analytical tools[137] contributes to optimized detection capabilities concerning different routes manipulating an individual's blood composition[138] such as autologous and homologous blood transfusions, the use of ESAs such as EPO, HIF stabilizers, and activators, etc. The informative value obtained from ABP readings depends on several critical factors including the standardization of sample collection procedures, the comparability of analytical data across multiple laboratories,[139] and the consideration of relevant ABP parameters and their confounding factors.[140] Fluid balance or hydration status-related plasma volume shifts were identified as relevant to the interpretation of ABP results and corresponding abnormalities, and Miller et al. suggested the longitudinal monitoring of albumin (and relative changes thereof) in combination with routine ABP parameters for an improved assessment of unusual ABP profiles.[141] In different studies with exercise-settings aimed at provoking depleted plasma volumes in athletes, changes in albumin abundance were correlated with alterations of the hemoglobin concentration and the computed plasma volume shifts. Although routine ABP analyses did not trigger atypical findings in any of those study samples, the additional albumin-derived information on plasma volume shifts could assist ABP expert reviews in authentic sports drug testing programs.

Additional, complementary information contributing to current efforts concerning the detection especially of (autologous) blood transfusions could result from alterations of the ex vivo-stored erythrocytes on the membrane proteome and/or the lipidome level. Al-Thani et al. subjected red blood cells stored for up to 35 days in conventional CPDA1 blood bags to standard proteomics analyses, indicating the quantitative change of a total of 33 proteins during storage, attributed to either storage-induced translocations of cytoplasmic proteins to the membrane or oxidation reactions.[142] In particular, spectrin, band 3 protein, and ankyrin-1 were found to be affected and could represent additional target analytes in future doping control panels; however, whether the proteins' stability, abundance, and survival after re-infusion allows for effectively identifying different volumes of blood transfusions remains to be proven. Similarly, data presented in a different study on alterations concerning the erythrocyte's phospholipid profiles upon cryogenic storage must be considered as preliminary, and proof-of-concept data are required to demonstrate the true extent of the observed potential markers' utility in doping controls.[143] Cho et al. stored red blood cells at −80°C for 72 days, and substantial changes concerning the relative abundance of six phospholipids were recorded, especially regarding the pre- and post-storage concentration of *N*-nervonoyl-D-*erythro*-sphingosylphorphorylcholine. The abundance of the storage-induced changes was, however, largely caused by omitting cryoprotectants in the process, facilitating the detection of affected lipids on the one hand but representing unrealistic and/or unwarrantable blood preservation conditions on the other.

Gasparello et al. followed up on the option of targeting the organism's response to blood transfusion at the miRNA level as a potential means to uncover blood transfusion practices in sport.[144] Six volunteers underwent blood transfusions with one unit (450 mL) of either refrigerated or cryopreserved origin, and blood samples were collected on five occasions prior to and on four occasions after blood transfusion. These samples were subjected to miRNA microarray profiling, yielding seven potential target miRNAs associated with fetal hemoglobin, erythroid differentiation, and the regulation of transcriptional repressors. The combined interpretation of up-regulations yielded significant differences between the blood samples collected 40 days prior to the blood transfusion and samples obtained 15 days post-transfusion in four out of six individuals, indicating a

considerable potential for contributing to the detection of blood doping. Nevertheless, further studies probing for potential confounding factors influencing the herein presented miRNAs or the option to combine the test method with other miRNA analytical approaches (e.g. for HIF stabilizers and activators)[145] are desirable.

8 | GENE DOPING

In accordance to recent advances in genetic engineering and gene therapy approaches, methods of gene doping will, in all probability, exploit strategies involving the injection of transgenes into (muscle) tissue in the form of viral constructs. Doping controls currently aim at detecting exon–exon junctions of the commonly intron-less transgenes, and Sugasawa et al. recently reported on a mouse model serving as an experimental surrogate for the assessment of test method's sensitivity for gene transfers accomplished via recombinant adenoviruses (rAdV) or adeno-associated viruses (rAAV).[146] Exemplified by means of a rAdV vector containing the mCherry gene, mice were transfected intravenously or intramuscularly, and transgene fragments were traceable in the blood cell fraction, in plasma, and in stool using established polymerase chain reaction (PCR)-based analytical approaches. Especially the use of droplet digital PCR applied to blood cell fraction-derived DNA yielded promising test results, and cell sorting is expected to further improve the obtained analytical information. In the light of the multitude of transgenes available as potential doping agents and the presence of multiple exon–exon junctions, de Boer et al. suggested a next-generation testing approach in order to offer a broader gene doping test spectrum but also to minimize the option of undermining PCR-based analytical approaches aimed at single exon–exon junctions only.[147] In a comprehensive approach, a panel covering all plasmid- and virus-derived copyDNA exon–exon junctions of the EPO-, IGF1-, IGF2-, GH1-, and GH2-genes was established. In brief, genomic DNA (and trace amounts of gene doping copyDNA) is isolated from blood samples, fragmented, and modified by adapter ligation prior to hybridization of the copyDNA fragments by exon–exon junction-specific biotin-labeled lockdown probes. The captured copyDNA fragments are isolated, PCR-amplified, and finally sequenced for unequivocal identification, providing a sensitivity of 1296 copies of the copyDNA in 1000 ng of genomic DNA. While this

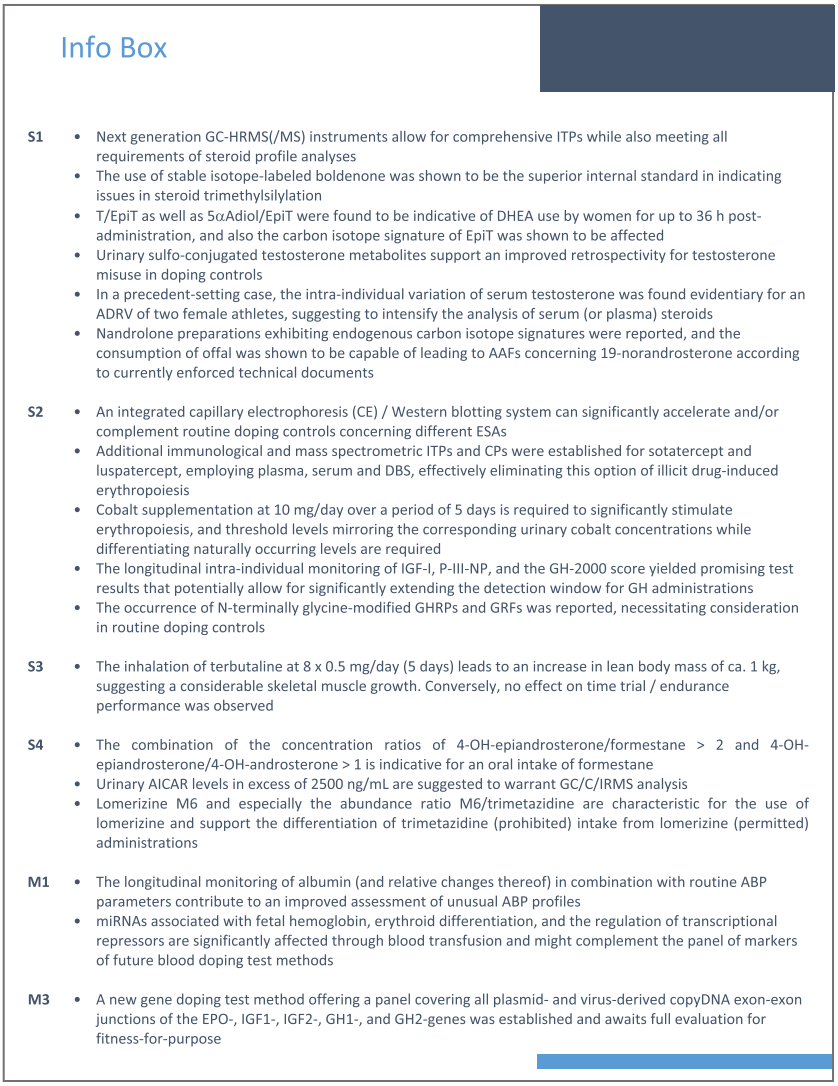

FIGURE 2 Info box on particularly relevant observations

parameter is inferior to earlier test methods, the assay's sensitivity is expected to allow for an increased sensitivity when using cell free plasma instead of whole blood and offers a particularly flexible spectrum of target analytes for the inclusion of further copyDNA sequences.

9 | CONCLUSION

Detecting the misuse of substances (and cells) that are naturally produced in humans is particularly challenging and, consequently, necessitates extra effort in both routine processes and antidoping research activities. Therefore, in 2018/2019, various studies were dedicated to improving antidoping testing strategies especially concerning compounds belonging to the classes of endogenous anabolic agents and growth factors but also regarding methods of doping including autologous blood transfusion and gene doping. As an outcome, promising results were generated that potentially open up new avenues to doping controls enabling better analytical sensitivity and retrospectivity as well as coverage of different doping strategies. In addition, further in-depth investigations into situations possibly representing confounding factors in result interpretation were conducted, taking into consideration different aspects ranging from potential drug–drug interactions to external factors such as high-altitude training or chronic high-altitude adaptation. Also, the added value as well as limitations of alternative matrices, especially hair, in doping controls were discussed in the context of concrete case investigations, focusing on analytes from the classes of AAS, aromatase inhibitors, and diuretics. Key aspects of this annual banned-substance review that has considered literature published between October 2018 and September 2019 are summarized in the Info Box in Figure 2.

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