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Annual banned-substance review 16th edition—Analytical approaches in human sports drug testing 2022/2023

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Abstract

In this 16th edition of the annual banned-substance review on analytical approaches in human sports drug testing, literature on recent developments in this particular section of global anti-doping efforts that was published between October 2022 and September 2023 is summarized and discussed. Most recent additions to the continuously growing portfolio of doping control analytical approaches and investigations into analytical challenges in the context of adverse analytical findings are presented, taking into account existing as well as emerging challenges in anti-doping, with specific focus on substances and methods of doping recognized in the World Anti-Doping Agency's 2023 Prohibited List. As in previous years, focus is put particularly on new or enhanced analytical options in human doping controls, appreciating the exigence and core mission of anti-doping and, equally, the conflict arising from the opposingly trending extent of the athlete's exposome and the sensitivity of instruments nowadays commonly available in anti-doping laboratories.

KEYWORDS
anabolics, contamination, doping, exposure, sport

1 | INTRODUCTION

Minimizing or even preventing doping in sport has been shown to represent a massive undertaking, which necessitates a holistic approach, including (among other critical aspects) the continuous improvement of analytical approaches that allow for the detection of prohibited substances and methods of doping using athletes' doping control samples.[1] While historically anti-doping measures have predominantly relied on analytical chemistry, the consideration and implementation of other angles in support of an optimized anti-doping effort have received growing attention, ranging from enhanced and intelligence-based athlete identification,[2] sample collection and testing plans[3] to the exploitation of concepts applied in safety science research.[4] Here, an in-depth understanding of adverse incident causation (i.e., an adverse analytical finding [AAF] and anti-doping rule violation [ADRV]) and means to prevent future occurrences has been sought, conceptualized either in the light of average sample-to-ADRV rates or in a Systems Theoretic Accident Model and ProcesseS (STAMPS) context, designed to assist in identifying interacting systemic conditions creating the undesirable outcome of an ADRV. In other words, the influence of the system surrounding the athletes and shaping their attitude, behavior, and decisions with regard to doping should be taken into consideration rather than identifying a sole cause for failure concerning the integrity of sports in one individual.[5]

Determining the prevalence of doping in elite sport remains challenging, especially because analytical chemistry-based approaches have been discussed as underestimating the number of intentional ADRVs on the one hand while, at the same time, several AAFs have been attributed to exposure and contamination scenarios in the past.[6] Likewise, estimating doping prevalence by means of surveys proved complex as

recently shown by the comparison of a data obtained via the Unrelated Question Model (UQM) or the Single Sample Count (SSC) strategy.[7] A considerable discrepancy was found when evaluating the surveys completed by athletes participating at the World Championships in Athletics 2011 and the 2011 Pan-Arab Games, where the prevalence of past year doping was reported with 43.6% and 57.1% (UQM), respectively, 21.2% and 10.6% (SSC). Of note, a more recent randomized response technique survey applied to European recreational athletes outlined a prevalence of doping of 1.6% in the tested population.[8] A limitation however was that athletes were asked about the use of substances that they *thought* were prohibited in their sport, which essentially excludes a comparison to the previous study's outcome.

Also, the potential role of dietary supplements in the context of doping and its prevalence in sport was subject of various investigations. More than 50% of the study participants declared the use of dietary supplements,[9,10] and data suggest that such dietary supplement use is, to some extent, also a gateway to the use of prohibited performance enhancing substances.[11,12] Besides, the issue of contaminated and adulterated dietary supplements resulting in AAFs during doping controls still exists, both in manufactured and compounded products,[13,14] which substantially increases the risk of AAFs and ADRVs as well as health issues as recently outlined by Pascali et al.[15] and Tran et al.[16]

Advancing test methods for doping controls is vital for modern sports drug testing programs,[17] as demonstrated by attributable effects and impact[18] as well as their implementation into routine application at major sporting events.[19,20] Research aiming at facilitating such advances are commonly aligned with the World Anti-Doping Agency's (WADA's) Prohibited List[21] and its 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3) (Table 1). The 2023 edition of the Prohibited List featured only few modifications in comparison to the 2022 version,[22] for example, the addition of further examples of prohibited substances such as androst-4-ene-3,11,17-trione and 17α-methylepithiostanol to section S1.1 and ractopamine, S-23, and YK-11 to section S1.2, apitegromab to section S4.3, torasemide to section S5, solriamfetol to section S6b, and voxelotor to section M1.

With regard to the Monitoring Program, aimed at providing statistical data to support changes to the list of banned substances, the recording of the prevalence of ecdysterone at-all-times was conducted also in 2023, warranting a differentiation of the observed findings by target analyte and (estimated) concentration, considering the reportedly high urinary levels (ca. 400–700 ng/mL) as the result of dietary habits.[23,24] Likewise, the detection of the β_2-agonists salmeterol and vilanterol below the minimum reporting level (MRL)[25] was included in the program. The in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine and the narcotic analgesics codeine, hydrocodone, and tramadol remained as part of the 2023 Monitoring Program, complemented by dermorphin and its analogs. Concerning nicotine, a study by Zandonai et al. reported on an overall decline in nicotine findings in Italian doping control samples when comparing the periods 2012–2014 with 2015–2020,[26] and Bartik et al. presented data indicating no significantly altered physical performance parameters after oral administration of 8 mg of nicotine to healthy non-smokers but a lower perception of pain,[27] all of which contributing to the assessment of the drug's status in anti-doping in the future. New additions to the Monitoring Program concerning in- and out-of-competition periods were GnRH analogs in female athletes younger than 18 years and polyhydroxyphenylene thiosulfonate sodium (hypoxen) in all athletes. In the light of a recent investigation that indicated a substantial performance-enhancing effect in time-to-exhaustion studies, maybe dipeptidyl-peptidase-4 inhibitors such as saxagliptin will receive attention in the context of sports drug testing programs.[28]

Following a continued scientific debate, the status of thyroid hormones did not change in 2023, furnished also by prevalence study data suggesting minimal (if any) evidence for thyroid hormone abuse in Australian athletes.[29] However, the fact that liothyronine (synthetic derivative of thyroid hormone) administrations were shown to affect urinary steroid profile parameters was mentioned to justify consideration of thyroid hormone use in evaluations of the steroidal module of the athlete biological passport (ABP).[30]

In continuation of the 15th edition of the *annual banned-substance review*, literature published between October 2022 and September 2023 was evaluated (Table 2), focusing on advancements in sports drug testing approaches enabled by complementary strategies, improved analytical instrumentation, and/or optimized selection of target analytes.

2 | ANABOLIC AGENTS

2.1 | Anabolic-androgenic steroids (AAS)

The physiological relevance of testosterone (T) is well established and its qualification as therapeutic agent is unquestioned in cases of, for example, deficiency disorders and sex transformation.[116] The misuse of T and its synthetic derivatives (commonly referred to as AAS), mostly for the purpose of performance- and/or image-enhancement, has however been associated with severe health risks,[117–119] raising the question as to the dose-dependent effects on androgen-responsive tissues and potential saturation levels. Here, Derwand et al. provided first insights using a rat model, where up to 50 mg of testosterone per kg bodyweight was administered daily for up to 3 weeks, suggesting a ceiling of the dose–response of various organs at daily testosterone dosages of 10 mg/kg.[53] Also blood parameters such as serum erythropoietin (EPO) concentrations and percentage of reticulocytes were corroborated to be affected under supraphysiological AAS use,[54] and health risks observed in humans appear to outreach physical effects, with personality disorders and clinical psychiatric syndromes in males and females having recently been attributed to AAS misuse, too.[120–123]

In the light of the 2021 anti-doping testing figures, where anabolic agents (and in particular AAS) represented 40% of reported AAFs, the extensive efforts that resulted in various publications within the past 12 months (aiming at faster and orthogonal strategies as well as more comprehensive and extended analytical retrospectivity for AAS in sports drug testing) appear warranted. Those efforts focused further on an improved understanding of the metabolism of AAS, the combined

TABLE 1 Overview of prohibited substances and methods of doping according to the World Anti-Doping Agency (WADA) Prohibited List of 2023.

	Class	Subgroup		Examples	Prohibited	
					At all times	In-competition only
S0	Non-approved substances			BPC-157, rycals (ARM036), sirtuins (SRT2104), AdipoRon	x	
S1	Anabolic Agents	1	Anabolic androgenic steroids		x	
				Androstenediol, 1-androstenediol, clostebol, danazol, dehydroepiandrosterone, metandienone, methyltestosterone, methyltrienolone, nandrolone, stanozolol, testosterone, tetrahydrogestrinone		
		2	Other anabolic agents	Clenbuterol, osilodrostat, ractopamine, selective androgen receptor modulators (SARMs), zeranol, zilpaterol		
S2	Peptide hormones, growth factors, related substances, and mimetics	1.1	Erythropoietin-receptor agonists	Darbepoietin (dEPO), erythropoietins (EPO), EPO based constructs (EPO-Fc, methoxy polyethylene glycol-epoetin beta [CERA]), peginesatide, EPO-mimetic agents, and their constructs (CNTO-530, peginesatide)	x	
		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) signaling 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 analogs and fragments	Lonapegsomatropin, somapacitan, AOD-9604, hGH 176–191,		
		2.4	GH releasing factors	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)		

(Continues)

TABLE 1 (Continued)

	Class	Subgroup		Examples	Prohibited	
					At all times	In-competition only
				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			Fenoterol, reproterol, vilanterol	x	
S4	Hormone and metabolic	1	Aromatase inhibitors	Anastrozole, letrozole, exemestane, formestane, testolactone	x	
	Modulators	2	Anti-estrogenic substances [anti-estrogens and selective estrogen receptor modulators (SERMs)]	Bazedoxifene, raloxifene, tamoxifen, toremifene, clomiphene, cyclophenil, fulvestrant	x	
		3	Agents preventing activin receptor IIB activation	Domagrozumab, stamulumab, bimagrumab	x	
		4	Metabolic modulators	AICAR, GW1516, insulins, meldonium, SR9009, trimetazidine,	x	
S5	Diuretics and masking agents		Masking agents	Probenecid, hydroxyethyl starch, desmopressin	x	
			Diuretics	Acetazolamide, bumetanide, furosemide, triamterene		
S6	Stimulants		Non-specified stimulants	Adrafinil, amfetamine, benfluorex, cocaine, modafinil		x
			Specified stimulants	Cathine, ephedrine, etamivan, methylephedrine, methylhexaneamine, octopamine, pseudoephedrine, sibutramine, strychnine, tuaminoheptane		x
S7	Narcotics			Buprenorphine, fentanyl, morphine, pentazocine		x
S8	Cannabinoids			Hashish, marijuana, JWH-018, HU-210		x
S9	Glucocorticoids			Betamethasone, dexamethasone, prednisolone		x
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood	Autologous, homologous and heterologous blood, red blood cell products	x	
		2	Artificially enhancing the uptake, transport, or delivery of oxygen	Perfluorocarbons (PFCs), efaproxiral, hemoglobin-based blood substitutes	x	
		3	Intravascular manipulation of blood or blood components by physical or chemical means		x	
M2	Chemical and physical manipulation	1	Tampering	Urine substitution, proteases	x	
		2	Intravenous infusion	More than 100 mL per 12-h period	x	
M3	Gene and cell doping	1		DNA, RNA, siRNA	x	

TABLE 1 (Continued)

Class		Subgroup		Examples	Prohibited	
					At all times	In-competition only
			The use of nucleic acids or nucleic acid analogs that may alter genome sequences and/or alter gene expression by any mechanism. This includes but is not limited to gene editing, gene silencing, and gene transfer technologies			
		2	Use of normal or genetically modified cells			
P1	Beta-blockers			Acebutolol, atenolol, bisopropol, metoprolol	x[a]	x[a]

Abbreviation: GHRH, growth hormone releasing hormone.
[a]Depending on the rules of the international sport federations.

use of targeted testing approaches and -omics-based marker monitoring, as well as new or advanced means that facilitate the differentiation of natural and endogenously produced steroids from synthetic analogs.

2.1.1 | Initial testing procedures (ITPs)—Comprehensive screening and metabolism studies

Exploiting the continuously growing instrumental quality and analytical experience in applying ion mobility (IM) in concert with liquid chromatography (LC) and high resolution/high accurate mass (HRAM) mass spectrometry (MS) to doping control analytical challenges, Wedge et al. reported on an optimized method targeting 40 steroidal agents relevant in sports drug testing.[39] By means of ultrahigh performance LC (UHPLC) hyphenated via electrospray ionization (ESI) to an IM unit and a quadrupole/time-of-flight (Q/TOF) mass analyzer, spiked urine samples were assayed after concentration by solid-phase extraction (SPE). The robustness of the determination and utility of collision cross-section (CCS) data was convincingly demonstrated, and a chromatographic run of as little as 2 min (using a C-18 analytical column, 2.1 × 50 mm, 1.8 µm) was shown to suffice for separating various isomeric/isobaric substances. Also, adequate sensitivity was reported by means of examples such as testosterone (175 pg/mL), epitestosterone (183 pg/mL), and stanozolol (84 pg/mL), but the full potential of the approach will require further proof-of-concept as the majority of the measured analytes represent intact and unconjugated species while authentic post-administration samples largely contain metabolites with substantially different physicochemical properties, and the employed sample preparation omitted so far any deconjugation step.

Obtaining indication for AAS misuse by means of biomarker monitoring approaches has been revisited by Kolliarti-Turner et al., who assessed the option of using RNA sequencing in blood and muscle tissue to differentiate AAS users from non-users in a pilot study setting.[55] While investigating mechanisms potentially contributing to the muscle memory phenomenon regarding AAS,[124] gene expression profiles of a control group were compared with trained athletes without AAS consumption and those with AAS use with a minimum period of drug abstinence of 2 weeks. While significant changes in muscle gene expression profiles associated with anabolic processes allowed for clustering AAS users via analyses of muscle tissue samples, the whole blood transcriptional signature was not found to provide the information that enables the identification of previous AAS, thus preventing an implementation of this strategy for the time being. Whether or not downstream biosynthetic products at the proteome level can provide further or longer-lasting indicators was discussed by Sansoucy and Naud.[56] Based on earlier studies where plasma proteins such as thyroxine-binding globulin and high density lipoprotein (HDL) were shown to be affected by AAS use (especially at supratherapeutic amounts), as well as apolipoprotein 1, alpha-2-Heremans-Schmid glycoprotein, and vitamin D binding protein being up/downregulated by growth hormone injections, further research into proteome alterations caused by misuse of anabolic agents was considered warranted.

TABLE 2 References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2022/2023.

				References			
	Class	Subgroup		GC/MS (/MS)	LC/MS (/MS)	GC/C/IRMS	Complementary methods & general
S1	Anabolic agents	1	Anabolic androgenic steroids	31–38	39–49	38,50–52	53–57
		2	Other anabolic agents		58–63		
S2	Peptide hormones, growth factors, related substances and mimetics						
	1.1	Erythropoietin-receptor agonists		64–69		70–72	
		2.3	Growth hormone (GH), its fragments, and releasing factors		73		74,75
		2.4	GH releasing factors		76,77		
		3	Growth factors and growth factor modulators		78–80		
S3	Beta-2-Agonists				81–83		84–86
S4	Hormone and metabolic modulators	2	Anti-estrogenic substances (anti-estrogens and selective estrogen receptor modulators [SERMs])		87		88
		3	Agents preventing activin receptor IIB activation		89		90
		4	Metabolic modulators		91–93		
S5	Diuretics and masking agents				67,94		95
S6	Stimulants				67,94,96		95,97,98
S7	Narcotics						99
S8	Cannabinoids						100
S9	Glucocorticoids						101,102
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products		103		104–110
M2	Chemical and physical manipulation		Tampering		111		
M3	Gene and cell doping						112–114
P1	Beta-Blocker						115

Abbreviations: GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; GC/MS, gas chromatography/mass spectrometry; LC/MS, liquid chromatography/mass spectrometry.

Eventually, such (pre)screening tools might provide means to effectively complement targeted test methods that unequivocally verify or disprove the presence of a defined prohibited substance in doping control samples. Those targeted test methods benefit from continued research into drug metabolism processes and new/improved instrumental approaches, generating and contributing information that has been essential, for example, for creating test methods of superior detection windows and for providing datasets supporting result interpretations and decision-making processes at the result management level.

For instance, Gronert et al. assessed the utility of an in vitro model, exploiting a human hepatocellular carcinoma cell line (Hep G2) cultivated into spheroids, in mimicking the in vivo metabolism of AAS.[125] In a proof-of-principle study, the viability of spheroids and their potential to biotransform an AAS into known (long-term) metabolites was demonstrated by means of metandienone, with the

experimental conditions showing best results when scaffold-based 3D cultivation of spheroids was employed. In a more conventional setting, Muresan et al. compared the metabolite patterns of bolasterone (Figure 1, **1**) obtained from incubations with rat liver microsomal preparations or rat urine samples after oral administration of bolasterone at 40 mg/kg bodyweight.[40] Bolasterone has been predominantly monitored in human doping control urine samples by means of its major metabolite 7α,17α-dimethyl-5β-androstane-3α,17β-diol and gas chromatographic-mass spectrometric methods. Here, in vitro incubation samples as well as post-administration urine samples were subjected to LC-ESI-HRAM MS analyses, and a total of 18 metabolites were reported, five of which were found in both experiments, eight only in vitro, and six only in vivo, complementing the knowledge on this AAS biotransformation process. Analyses were conducted using a C-18 analytical column (2.1 × 100 mm, 5 µm) and elution solvents A (0.1% formic acid) and B (methanol, containing 0.1% formic acid) to introduce the analytes via ESI into a Q/Orbitrap instrument. The longest-lasting metabolites in vivo were four bishydroxylated species of bolasterone, where hydroxylations were tentatively located at carbon 6 and the steroidal C- or D-ring, allowing for the detection of metabolites for up to 7 days resulting from the high single dose application. To which extent these results are transferable to urinary metabolic patters of humans remains to be shown, also in the light of the fact that the urine sample preparation was apparently limited to liquid–liquid extraction (LLE) without prior enzymatic hydrolysis.

The fact that, eventually, confirmation of in vitro metabolism study data with authentic elimination study specimens is vital for routine doping controls was demonstrated in a case report by Thieme et al.[31] In the context of a forensic investigation, the biotransformation of the AAS methoxydienone (Figure 1, **2**) was studied employing the aforementioned HepG2-based in vitro metabolism approach as well as urine samples collected after oral administration to a human volunteer, and the urinary metabolite observed by means of routine doping control analytical procedures, presumably 18-methylnorandrosterone, was not generated in vitro but abundantly observed in post-administration urine specimens.

A protocol aiming at comprehensively screening metabolic reactions concerning AAS by means of metabolomics workflows was presented Leogrande et al.[41] Here, pre- and post-administration urine samples, collected prior to and after application of a single oral dose of 100 mg of 3-acetyl-7-keto-dehydroepiandrosterone (DHEA), were grouped into defined post-administration time intervals and subjected to LC-HRAM full scan MS analyses for subsequent principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Samples were prepared to allow for selectively targeting glucurono- or sulfo-conjugated species by appropriate enzymatic hydrolyses and LLE before injection into the LC-HRAM instrument. The LC was equipped with a C-18 analytical column (2.1 × 100 mm, 1.8 µm) and the Q/TOF mass spectrometer operated in either positive or negative mode. Obtained features facilitated a clear clustering of samples for the three time intervals (before, early [0–10 h], and long [10–45 h]), and 15, respectively, 5 steroidal biomarkers were found up- and downregulated, 11 of which were tentatively identified. The workflow proved robust, and the results obtained were in accordance with previous publications, indicating a more generic applicability of the approach for metabolism studies in anti-doping.

With the inquiry into the effect of compatibilities of sulfo-conjugated analytes in particular and analytical instrumentation on their detection windows, Albertsdottir et al. provided evidence for the fact that not only pharmacokinetics but also test method principles are important determinants concerning analytical retrospectivity.[32] Elimination study urine samples collected before and after administrations of metenolone, mesterolone, and methyltestosterone were prepared for analysis using two different approaches, one including and one excluding trimethylsilylation of the analytes extracted by LLE. The underivatized extracts were screened by LC-ESI-QqQ MS. Samples that underwent trimethylsilylation were subjected to gas chromatography (GC)-MS using GC-electron ionization (EI)-Q/TOF MS, GC-EI-triple quadrupole (QqQ) MS, or GC-chemical ionization (CI)-QqQ MS and exploiting mass spectrometric characteristics of trimethylsilylated and sulfo-conjugated steroidal analytes under GC–MS conditions as investigated previously by the same group.[33] The obtained data demonstrated that considerably different detection times per target analyte are accomplished using the different instrumental platforms, and optimizing detection windows and test methods should also include the individual consideration of analyte-and-instrument pairs.

Metenolone and mesterolone were also used as model AAS in a study focusing on improving and simplifying options of ITPs. Angelis et al. pursued two new sample preparation protocols where urine was either directly exposed to a solution of Girard's Reagent T (GRT) or first liquid–liquid-extracted and the obtained organic phase evaporated and then GRT-derivatized.[42] Both methods allowed for the combined detection of unconjugated as well as glucurono- and sulfo-conjugated metabolites, complementing existing GC–MS(/MS)-based assays at competitive analytical sensitivity. Using an instrumental setup composed of an LC (with a C-18 analytical column [2.1 × 100 mm, 1.8 µm], 5-mM ammonium formate [in 0.02% formic acid, solvent A], and acetonitrile/water [90/10, v/v] containing 5-mM ammonium formate and 0.02% formic acid), ESI source, and a Q/Orbitrap mass analyzer, the proposed test method outperformed the authors' routinely applied GC–MS/MS approach and increased the number of markers proving the steroid intake. Single oral doses of 25 and 50 mg of metenolone acetate and mesterolone were traceable for 18–19 days, with three metabolites each and all representing sulfo-conjugated species.

2.2 | Other anabolic agents

Other anabolic agents accounted for a total of 106 AAFs in 2021,[126] including 44 occurrences of clenbuterol and 53 findings of selective androgen receptor modulators (SARMs) such as enobosarm (26 occurrences), LGD-4033 (22 occurrences), RAD-140 (3 occurrences), and S-23 (2 occurrences). Despite the lack of marketing approval, growing

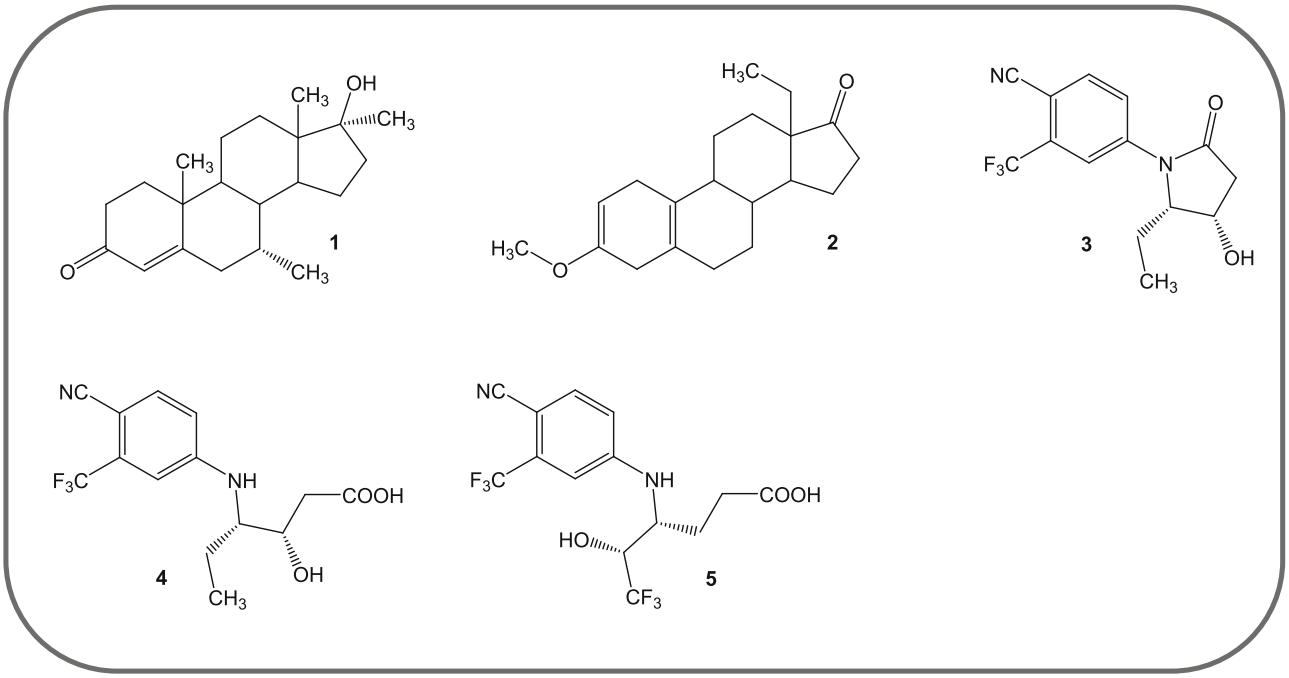

FIGURE 1 Structure formulae of bolasterone (**1**, mol wt = 316.24 u), methoxydienone (**2**, mol wt = 300.21 u), selective androgen receptor modulator (SARM) 2f (**3**, mol wt = 298.09 u), SARM 2f M3 (**4**, mol wt = 316.10 u), and main LGD-4033 metabolite with revised structure (**5**, mol wt = 370.08 u).

concerns especially regarding misuse of SARMs have been communicated,[127] taking into account the increasing body of evidence linking SARM misuse to a variety of conditions and injuries such as drug-induced liver injuries, tendon rupture, or rhabdomyolysis events and, at the same time, the soaring interest in SARMs as deduced from social media posts.[128,129] Hence, investigations into new emerging SARMS, their availability, metabolism, and, where possible, urinary elimination are critical to sports drug testing programs.

Möller et al. presented first insights into the biotransformation of a novel SARM referred to as 2f (Figure 1, **3**), which was subjected to in vitro metabolism experiments using human liver microsomal preparations.[58] Seven metabolites were identified using LC-HRAM measurements, including products resulting from oxidation, hydroxylation, hydration, and/or conjugation reactions yielding both glucuronides and sulfates. Three tentatively characterized metabolic products were subsequently synthesized, with the hydration product (Figure 1, **4**) representing a promising urinary target analyte in accordance to the metabolic fate and main metabolite reported for the structurally related SARM LGD-4033 (Figure 1, **5**). For that, the synthesis and full structure characterization of the predominant urinary metabolite was recently accomplished by Pitsinos et al.,[59] providing the means for formerly inaccessible reference material. The importance of that specific metabolite for the detection and confirmation of LGD-4033 administrations was underlined by Kwiatkowska et al., who summarized metabolic profiles of various LGD-4033 AAFs and hypothesized about potential effects of co-administered drugs on the urinary elimination pattern of the SARM.[60] In all cases reported in their study, the metabolite depicted in Figure 1 (**5**) was the most abundant species, regardless of the concomitantly detected drugs including aromatase inhibitors, growth hormone secretagogues, or other SARMs.

Furnishing the development of advanced in vitro metabolism studies, Wagener et al. compared the biotransformation capability and comparability of different approaches with human urinary metabolic patterns, using the SARM RAD-140 as model compound.[61] The anabolic agent was subjected to conventional subcellular human liver fractions, electrochemical conversion (EC), and human liver spheroids in an organ-on-a-chip system, yielding a total of 13, 7, and 14 metabolites, where especially the organ-on-a-chip approach proved highly comparable with human urinary metabolic patterns found in authentic AAFs reported for a doping control sample. All detected metabolites were tentatively identified by LC-HRAM MS/MS, and while incubations with subcellular human liver fractions and EC experiments are particularly quick and practical in providing first data on metabolic conversions of a drug or drug candidate, the advantage of the more laborious organ-on-a-chip approach is its capability of producing a more comprehensive picture of metabolites, including those appearing only after several days of exposure to human metabolic activity. This information is particularly important when selecting descriptive and sensitive target compounds (TCs) for ITPs performed on unknown samples, which originate from an unknown timepoint from drug administration.

Metabolite profiles and patterns could play a major role also in differentiating doping scenarios from interindividual (cross-)contamination scenarios as argued in various cases of AAFs and the context of the athlete's exposome.[6] The occurrence of anabolic agents such as stanozolol and LGD-4033 (and corresponding metabolites) in

ejaculate after oral application of 0.33 and 0.11 mg/kg, respectively, was recently demonstrated by means of a boar administration study.[62] Blood plasma and ejaculate were collected before and 24, 48, and 72 h post-administration, and metabolic patterns as well as drug concentrations were determined in both matrices. Following protein precipitation, target analytes were identified by LC-HRAM MS/MS experiments, using a C-18 analytical column (3.0 × 50 mm, 2.7 µm) and 5-mM ammonium acetate (containing 0.1% acetic acid, solvent A) as well as acetonitrile (solvent B), coupled via ESI to a Q/Orbitrap instrument. With limits of detections (LODs) for stanozolol and LGD-4033 at 10–40 pg/mL in both matrices, the intact drugs were detected in blood and ejaculate at all post-administration time points (up to 72 h) with maximum concentrations in ejaculate at ca. 0.25 and 0.68 ng/mL, respectively. In case of stanozolol, 9 metabolites were observed in both plasma and ejaculate, and 5 metabolites exclusively in plasma, while no discriminative ejaculate metabolite was identified. All 10 metabolites detected for LGD-4033 were present in plasma and ejaculate, indicating that further investigations appear warranted that conclusively identify ejaculate-derived metabolites to corroborate contamination of doping control urine samples, for example, via unprotected sexual intercourse. Nevertheless, the reported drug concentrations provide an estimate as to which urinary levels might necessitate consideration of a contamination scenario.

The meat contamination issue concerning clenbuterol appears to persist as deduced from a total of 102 atypical findings (ATFs) regarding clenbuterol in 2021.[126] Supporting the analytical differentiation of "animal-processed" clenbuterol intake from doping scenarios has been desirable for many years, and considering the enantiomeric ratio of R-(−)- and S-(+)-clenbuterol in urine was discussed as a potential approach. The utility of that methodology was revisited and applied to edible tissue samples collected from mini-pigs that were administered with a single oral dose of 20-mg clenbuterol per kg bodyweight.[63] After 96 h after drug intake, pigs were slaughtered, and the enantiomeric ratios of clenbuterol were determined by LC-QqQ MS. The enantiomers were separated on a chiral column (2.1 × 150 mm, 5 µm) employing an isocratic flow of 10-mM ammonium formate in methanol, and ionization as well as detection was conducted using positive ESI and tandem MS. While all clenbuterol R/S ratios remained below 1, a considerable variability depending on the tested tissue was observed spanning from ca. 0.55 to 0.95, and the authors correctly outlined the limitation that other animal species might metabolize and/or retain clenbuterol enantiomers differently and that this approach is not (yet) fit for purpose.

2.3 | Steroid profiling in urine and blood

The ABP was recently complemented by the inclusion of two serum steroid markers, that is, T and androst-4-ene-3,17-dione (Adione; A4), to further strengthen the ABPs capability of detecting the administration of endogenous AAS, especially in females.[130] The intraindividual stability of urinary steroid profiles was found to be less robust in female than in male athletes,[34] and despite the use of concentrations and concentration ratios of a variety of steroidal analytes such as T, epitestosterone (EpiT), androsterone (A), etiocholanolone (E), 5α-androstane-3α,17β-diol (5αAdiol), and 5β-androstane-3α,17β-diol (5βAdiol), several confounding factors as for instance time of day, in- versus out-of-competition sample collection, and hormonal contraception have been shown to affect urinary steroid profile data,[35] and consensus exists in the added value of serum steroid analyses in the context of sports drug testing.[36] Accounting for confounding factors in ABP result interpretations is vital and likewise is the optimization of analytical tests. Martinez-Brito et al. revisited the options of determining T/EpiT ratios and compared strategies of data correction of peak integrals (i.e., elimination of isotopic contributions) and use of a verified concentration ratio of deuterated T and EpiT.[37] In addition, potential effects of employed analyzers, that is, GC–MS and -MS/MS as well as LC–MS/MS, were taken into consideration, and the obtained results suggest superior T/EpiT ratio accuracy when using the deuterated internal standard approach, especially in analytical settings employing LC–MS/MS. Visconti et al. concluded similarly in their study on calibration approaches for the LC–MS/MS-based determination of endogenous steroids (in serum), where utilizing a one-point stable isotope-labeled internal standard as calibrant proved to provide results comparable with those obtained by external calibration approaches but with much lower analytical effort.[43] It was stated that the methodology might have a limited range of optimal performance in the light of, for example, ion suppression effects, but in consideration of the commonly observed endogenous steroid concentrations, the arguably limited range might well be sufficient for the intended purpose.

In consideration of the above aspects, various studies were conducted and published, providing optimized analytical approaches and furnishing the anti-doping community (as well as the public health sector[44]) with material data to advance the serum steroid ABP and extract additional information from the available sample matrix. For instance, Langer et al. reported on a strategy on how to combine the quantitation of the endogenous steroids T, Adione, and dihydrotestosterone (DHT) with 18 xenobiotic steroids plus 11 intact testosterone esters as well as 5 further AAS esters.[45] A volume of 200 µL of serum was prepared by supported liquid extraction (SLE), concentrated, and analyzed by LC-ESI-QqQ MS for the aforementioned serum steroid ABP markers and the non-esterified target analytes. Here, chromatographic separation was accomplished on a C-18 analytical column (2.1 × 100 mm, 1.7 µm) with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), and LODs were validated between 50–500 pg/mL. The remaining sample extract was subsequently evaporated to dryness and subjected to GRT derivatization (cf. Section 2.1.1) prior to a second LC-ESI-QqQ MS analysis, enabling the sensitive detection of intact steroidal esters with LODs determined at 50–500 pg/mL. The complementary analysis was conducted using a C-8 analytical column (2.1 × 100 mm, 1.7 µm), using also 0.1% formic acid as solvent A but methanol (with 0.1% formic acid) as solvent B, accounting for the considerably different physicochemical properties of this subset of analytes. The applicability of the assay was shown by measuring serum samples of 19 male volunteers who

received 80 mg of testosterone undecanoate orally at two occasions, demonstrating a detection window of 12 h for the derivatized ester. Whether or not the analytical approach can be transferred to plasma or dried blood spots (DBS) was further assessed by Salamin et al., who compared a set of 100 serum, plasma, and DBS specimens collected from elite athletes at a major athletic competition especially with regard to the serum steroid ABP markers T and Adione.[46] Of note, a bias of 10–20% towards an underestimation of plasma steroid concentrations (compared with serum) was observed. Pairing serum and DBS results demonstrated a similar underestimation of T in DBS while Adione was found to yield similar concentrations in these matrices, and the potential influence of the hematocrit on such measurements was found to be sufficiently addressed when applying an average value of 45%.

Expanding further on the utility of serum steroid profiling, Ponzetto et al. reported on an advanced LC-ESI-QqQ MS-based methodology that allowed for the simultaneous determination of 27 endogenous steroidal compounds, including 19 substances of immediate relevance for anti-doping purposes with 7 glucurono-, 7 sulfo-, and 5 un-conjugated analytes.[47] Employing SPE, 200 µl of serum was prepared for analysis, where particular emphasis was put on the chromatographic separation of isomeric and isobaric steroids. A C-18 analytical column (2.1 × 150 mm, 2.6 µm), 10-mM ammonium acetate (solvent A), and methanol (containing 10-mM ammonium acetate, solvent B) were found to enable the required separation power. All analytes of interest were monitored in a single analytical run, via ESI with polarity switching at excellent limits of quantifications (LOQs), which allowed for assaying a reference cohort of serum samples collected from 40 male and 40 female blood donors, and the consideration of isomeric phase-II metabolites of 5αAdiol and 5βAdiol is expected to improve result interpretation, for example, with regard to routes of drug administration, genetic predisposition, or confounding factors. Among those, combined oral contraceptives might necessitate consideration as investigated by Knutsson et al., where a double-blind and placebo-controlled study concerning the effect of levornorgestrel and ethinylestradiol on serum steroid biomarkers was conducted.[48] T, Adione, DHT, and DHEA plus the 3- and 17-O-glucuronide of 5αAdiol were monitored, demonstrating that under oral contraception medication, all serum steroid levels were reduced. Because the ratio of T/Adione remained unaffected in either study group (placebo and contraceptive), the marker was considered robust for anti-doping purposes. Creating an even more robust approach was the goal of a project reported by de Figueiredo et al., who combined serum and urine steroid profiles into a multivariate score.[49] In a model cohort of 14 female volunteers, serum (T, Adione, DHT, DHEA, and T/Adione) and urine (T/EpiT, A/T, A/E, and 5αAdiol/5βAdiol) steroid profiles were monitored before and after transdermal testosterone administrations, and serum steroid biomarker monitoring outperformed the urinary steroid profile in a unimodal approach. Further, when combined to compute a multimodal score, a true positive rate of 83% was accomplished, and contributions of individual parameters resulting in the flagging of a sample as atypical are available as residuals, that is, the element-wise squared differences between the current dataset and the individual's reference range. Of note, the administration of human chorionic gonadotrophin (hCG) did not affect urine T/EpiT or serum T/Adione to an extent that would trigger follow-up investigations, as demonstrated by Goodrum et al. who analyzed urine and serum samples of 10 recreational male athletes receiving 250 µg of hCG every 3 days for 3 weeks.[57] Instead, serum T/luteinizing hormone (LH) was suggested to represent an interesting addition.

2.4 | Confirmatory testing procedures—Isotope ratio mass spectrometry (IRMS)

Eventually, in case of suspicious steroid profile data, confirmatory analyses by means of GC/combustion (C)/IRMS are sought to determine whether significant differences exist between the carbon isotope signatures of TCs and endogenous reference compounds (ERCs). Such TCs and ERCs are well established for doping control urine samples, but the applicability of GC/C/IRMS to serum steroids has received little attention, particularly concerning samples collected from females. Hence, Andersson et al. assessed the utility of GC/C/IRMS to determine the transdermal administration of testosterone in the context of a placebo-controlled study.[38] A total of 24 female participants applied 10 mg of testosterone gel daily for 10 weeks, and both urine and serum were sampled, with serum being subjected to GC/C/IRMS analyses. A and epiandrosterone (EpiA) sulfates as well as cholesterol and DHEA sulfates were selected as TCs and ERCs, respectively, and evaluation criteria for $\Delta\delta^{13}C$ values were established for each analyte pair, ranging from 2.3 to 4.5. Applying the specified IRMS positivity criteria, 29% of all analyzed post-administration serum sample returned AAFs, with A being the metabolite of choice whereas EpiA did not contribute to any of the determined AAFs.

The availability of 11-ketotestosterone (11-KT) and some of its prohormones via internet-based providers has also necessitated investigations into the detection and confirmation of a potential misuse of these in elite sport. By means of an exploratory elimination study including one male and one female participant as well as a reference cohort composed of doping control urine samples from 104 female and 116 male athletes, the metabolic fate of 11-KT was revisited, and due to the natural/endogenous abundance of 11-KT and its metabolites, recommendations as to when a urinary finding warrants follow-up investigations were made.[50] Pursuing an analytical strategy similar to those commonly employed for the determination of T administrations, urine samples were fractionated and prepared for GC/C/IRMS analyses, focusing on 11-KT and 11-ketodihydrotestosterone as well as 11-OH-androsterone, 11-OH-etiocholanolone, and 11-ketoetiocholanolone as TCs. Consequently, independent ERCs such as, for example, pregnanediol (PD), pregnanetriol (PT), or 3α-hydroxy-5α-androst-16-ene (16-ene) were required, and fitness-for-purpose of the established approach was shown by fulfilling positivity criteria up to 20 h post-administration, particularly for 11-KT as TC. In order to effectively preselect urine samples for GC/C/IRMS confirmation, an ITP screening urinary 11-KT levels was proposed, triggering follow-up investigations above a

suggested concentration of 40 ng/mL. Alternatively (or complementarily), 3 ng/mL of 11-ketodihydrotestosterone was also proposed.

Differentiating the misuse of synthetic nandrolone (doping scenario) from an intake of nandrolone via the consumption of edible tissue from non-castrated pigs (exposure scenario) has been an ongoing challenge, and follow-up investigations with a consumption study and subsequent GC/C/IRMS analyses were presented by Nair et al.[51] A total of 13 study participants consumed sausages prepared from heart, kidney, lung, liver, shoulder, stomach, and testes, and urine samples were collected pre- and post-ingestion up to 72 h. In accordance with the relevant WADA technical document,[131] samples exceeding a urinary 19-norandrosterone concentration of 2.5 ng/mL were subjected to GC/C/IRMS analyses. From all collected specimens, only 2 surpassed that concentration, yielding $\delta^{13}C$ values of −16.6 and −16.7‰ for 19-norandrosterone, while ERCs (PD and 16-ene) were found between −19.8 and −20.3‰. Given the fact that the $\Delta\delta^{13}C$ values are absolute values, positivity criteria were formally fulfilled in both cases, and findings would have entailed the reporting of an AAF if those samples were routine doping control specimens. The outcome of this study suggests that AAFs with 19-norandrosterone from meat consumption cannot be excluded; however, the number of occurrences was low considering the arranged scenario.

Boldenone and its main metabolite, 17β-hydroxy-5β-androst-1-en-3-one, necessitate GC/C/IRMS confirmation of their isotopic signature whenever the urinary concentrations are found between 2.5 and 30 ng/mL.[52] In order to simplify the sample preparation while meeting the required analytical sensitivity, Wen et al. proposed a protocol that employs SPE, enzymatic hydrolysis, LLE, and then a combination of two LC columns to fractionate the TCs systems from an original volume of 12–24 mL of urine.[132] The LC stationary phases were composed of phenyl (4.5 × 250 mm, 5 µm) and C-18 (4.6 × 250 mm, 5 µm) resins, operated with water (solvent A) and acetonitrile (solvent B), and allowing to obtain relevant fractions within a 64-min LC run. The assay was fully characterized and represents a valid alternative to routine sports drug testing.

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

3.1 | EPO-receptor agonists (ERAs) and hypoxia-inducible factor (HIF) activating agents

Within the class S.2 of WADA's Prohibited List, ERAs represented 70% of all reported AAFs in 2021,[126] suggesting that ERAs still play an important role as illicitly administered drugs in sport, despite the risks purportedly and evidently associated with their non-therapeutic use.[133] Test methods regarding ERAs have been continuously optimized and complemented concerning enhanced anti-doping applications and additional strategies to determine the presence of a natural variant of EPO (c.577del) and in the light of medicinal research[134] and pharmaceuticals potentially influencing the EPO biosynthesis in humans[135–137] To verify the presence of a single nucleotide polymorphism (SNP) in the c.577del *EPO* gene, which results in a frameshift and, consequently, in an increased molecular mass of the corresponding EPO protein, Yi et al. presented a testing protocol a employing clustered regularly interspaced short palindromic repeats (CRISPR)/dCas9-based testing approach.[70] With designated primers, the relevant SNP-modified *EPO* sequence is polymerase chain reaction (PCR)-amplified from 3 µL of blood and the amplicons and incubated with a complex formed from dCas9 and a variant-specific single-guide DNA. Only the variant amplicons bind to the ribonucleoprotein, which is subsequently visualized by native polyacrylamide gel electrophoresis and enables the identification of c.577del *EPO* gene variant carriers to support result interpretations of routine analyses concerning recombinant human EPO (rhEPO). How the presence of the genetic variant would affect the capability of routine test methods to identify microdosed rhEPO was investigated by He et al., who recruited 5 c.577del *EPO* gene variant carriers for an administration study with a single subcutaneous dose of rhEPO at 50 IU/kg.[71] Both urine and blood samples were collected, and while both matrices were tested for intact rhEPO, blood was additionally analyzed after *N*-deglycosylation of rhEPO. The latter approach in particular, when applying a density ratio of the two hEPO bands separated and visualized by routine sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blotting, proved useful in detecting the microdosed rhEPO administration for at least 3 days.

Aiming at identifying metabolomics signatures indicative for rhEPO administrations, Lima et al. conducted a controlled administration study with 18 male endurance-trained participants receiving rhEPO at 50 IU/kg bodyweight for 4 weeks on every second day.[64] Urine, plasma, and serum were sampled at baseline, during, and after the administration phase; all matrices were analyzed by hydrophilic interaction liquid chromatography (HILIC)-HRAM MS; and diagnostic features associated with erythrocyte membrane functions as well as erythropoiesis in general were identified by PLS-DA. The most significantly affected metabolites included orotate and dihydroorotate as well as palmitoyl-carnitine and elaidic carnitine, indicative for (drug-induced) alterations in pyrimidine and acyl carnitine metabolic pathways, and follow-up investigations as to the robustness of these markers towards confounding factors and the option to combine such markers with established markers of the ABP appear warranted.

Considerable advances concerning the analysis of peptidic and protein-based drugs and drug candidates by mass spectrometric approaches have been reported in the past[138]; however, the detection and differentiation of rhEPO from natural/endogenous human urinary EPO (hEPO) at relevant concentrations in doping control matrices has not been accomplished yet. Nevertheless, a step towards an LC–MS/MS-based test method was achieved by Seo et al., who characterized a glycopeptide common to various rhEPOs whose glycosylation does not naturally occur in hEPO.[65] The amino acid sequence HSCLNE-**N**ITVPDTK enclosing the glycosylated Asn38 was found to feature a *tetra*-sialylated glycan structure unique to recombinant EPOs and, hence, representing a viable target for routine doping controls. The

glycosylated peptide sequence was produced by sequential enzymatic digestion of rhEPO and hEPO after ultrafiltration-assisted concentration and immunoaffinity-purification of 15 mL of (spiked) human urine, followed by nanoflow LC-HRAM MS/MS analysis. Here, chromatography was conducted with 0.1% formic acid in 98% water/2% acetonitrile (solvent A) and 0.1% formic acid in 80% acetonitrile (solvent B) employing C-18 nanoLC columns of 75-µm inner diameter. The Q/Orbitrap MS was operated in targeted selected ion monitoring (t-SIM) and MS/MS mode, allowing for a validated LOD of 500 pg of rhEPO per mL of urine. While this result is commendable, the authors concluded that the applicable minimum required performance level (MRPL)[139] of 1 IU/L (equaling 6.6 pg/mL) was not met and that, to date, the assay is not fit-for-purpose for sports drug testing programs.

Cobalt, as a representative of the HIF activating agents, is commonly analyzed by inductively coupled plasma (ICP) MS approaches. With ICP-MS being rarely available in routine doping control laboratories, dos Santos et al. assessed the possibility of applying a complexation reaction of cobalt with diethyldithiocarbamate (DDC) and subsequent LC-HRAM MS/MS analysis for quantifying urinary cobalt concentrations in the context of sports drug testing.[66] Homogenized urine was treated with 65% aqueous HNO_3 in a microwave, and after neutralization of the solution with sodium hydroxide, complexation of the liberated inorganic cobalt was conducted and the complex extracted into *tert*.-butylmethyl ether. After concentration, the analyte was quantified on a C-18 analytical column (2.1 × 50 mm, 1.7 µm) using an isocratic flow of 0.012% formic acid (with 5-mM ammonium formate) and acetonitrile (30/70, *v*/*v*), directed via ESI into a Q/Orbitrap MS. Parallel reaction monitoring (PRM) allowed for an LOQ of 2.5 ng/mL, thus providing a tool accessible to doping control laboratories to monitor urinary cobalt levels.

The orally available HIF activating drug roxadustat has been included in comprehensive routine doping control analytical assays applied to urine[67] and DBS,[68] and its traceability in hair was also demonstrated in a recent study by Alvarez et al.[69] Hair samples collected from a patient undergoing continued roxadustat therapy (100–120 mg on 3 days per week) were shown to result in findings at 41–57 pg/mg as determined by LC–MS/MS. Following removal of potential external contaminants, the matrix was extracted with dichloromethane, the organic phase concentrated and measured on a C-18 analytical column (2.1 × 100 mm, 1.9 µm) interfaced via ESI to a QqQ MS system. Solvents used were 2-mM ammonium formate/0.1% formic acid (A) and acetonitrile (B), and the assay was shown to offer an LOD and LOQ of 0.1 and 0.5 pg/mg, respectively, complementing datasets and anti-doping testing options.

3.2 | Peptide hormones and their releasing factors, growth factors, and growth factor modulators

Recent advances concerning analytes of the category of peptide hormones and corresponding releasing factors focused predominantly on gonadotrophin-releasing hormone (GnRH) and its synthetic analogs,[140] human growth hormone (hGH), and growth hormone releasing hormones (GHRHs) and secretagogues (GHS). Investigations into the detection and the human in vivo metabolism of the GnRH analogs triptorelin and leuprorelin were conducted by Saardpun et al., who confirmed the formation and extended analytical retrospectivity of a previously in vitro-derived metabolite (referred to as triptorelin [5–10]) in humans.[73] Urine samples from patients starting GnRH treatment with 11.25 mg of either leuprolide acetate or triptorelin pamoate were collected before and 3 and 6 h post-injection as well as after 30 days. Aliquots of those specimens were prepared by SPE and analyzed using LC-ion trap/TOF MS/MS. Chromatography was conducted on a C-18 analytical column (2.1 × 150 mm, 2.7 µm) using 0.1% formic acid (containing 1% DMSO, solvent A) and acetonitrile (solvent B), enabling LODs for the targeted compounds and metabolites between 20 and 80 pg/mL. In all samples collected 3 and 6 h post-administration, the intact drugs and diagnostic metabolites were detected; in addition, triptorelin (5–10) was found in 3 out of 5 patient urine samples also after 1 month of injection at concentrations of approximately 0.3–1.2 ng/mL.

Complementing testing strategies for uncovering the illicit administrations of hGH was aimed in a study by Jung et al., who developed and characterized a method employing conjugates of camelid antibodies and fluorescence tags (to form so-called quenchbodies), targeting preferentially the 22- or the 20-kDa hGH isoform.[74] Due to the instantly occurring fluorescence upon antigen binding and simple signal detection, ratios of fluorescence signals can be recorded and evaluated, indicating natural or atypical abundance ratios. The obtained LODs ranged from 2.2 to 7.0 ng/mL for the 22- and 20-kDa isoforms, respectively, and therefore are not yet in accordance with the applicable WADA technical document.[141] However, in a clinical setting as, for example, in case of overdosing/intoxication,[142] the presented approach might be useful.

Increasing plasma concentrations of hGH (and/or IGF-I) can also be accomplished by the administration of hGH releasing factors, and the detection of GHRH administrations was shown to be particularly challenging considering the extremely limited knowledge on their metabolism and renal elimination. Targeting intact analytes, Otin et al. assessed the utility of capillary electrophoresis in analyzing four GHRHs in human urine.[75] Sermorelin, CJC-1293, CJC-1295, and tesamorelin were successfully separated using dimethyl-β-cyclodextrin as chiral selector (supporting the resolution of sermorelin and CJC-1293), and employing a sample preconcentration by SPE and polarity-switching large volume sample stacking combined with UV detection, an estimated LOD of 10 ng/mL was accomplished. Further optimization might be required to mature the approach to meet current WADA MRPLs, but the superior separation power of capillary electrophoresis for selected analytes was confirmed.

The class of GHS includes compounds such as, for example, anamorelin and capromorelin (Figure 2, **1** and **2**), which stimulate hGH secretion upon binding to the growth hormone secretagogue receptor 1. New information concerning the metabolic fate of anamorelin was presented by Gameli et al., who studied biotransformation reactions in silico and in vitro (using human hepatocytes), and 4 (out of 14 tentatively identified) metabolites were suggested as markers for detecting

anamorelin administrations in doping controls.[76] Using LC-HRAM MS/MS, the formation of 3-benzyl-3[(trimethylhydrazino)carbonyl]-1-piperidine, *N*-demethyl-, *N*-hydroxy-, and indole-hydroxylated anamorelin was postulated, complementing the dataset for potential AAFs in sports drug testing. A first finding of capromorelin in a routine doping control sample triggered an in-depth investigation into the elimination of the drug after oral as well as transdermal application, based on the argument that the athlete was exposed to minute amounts of a veterinary drug containing capromorelin. Here, Sobolevsky et al. demonstrated that the oral intake of 30 µg of capromorelin can result in urinary findings of up to 7 ng/mL, and likewise, the transdermal exposure to 30 mg (in aqueous solution) led to urinary capromorelin findings of 0.7 ng/mL. Due to the particularly sensitive nature of anti-doping testing procedures concerning analytes such as capromorelin, allowing for LODs of 10–100 pg/mL, contamination scenarios necessitate consideration in case result management. Of note, the metabolite detected in conjunction with capromorelin was the *N*-hydroxylated analog, in accordance with the aforementioned in silico/in vitro study on anamorelin, thus presenting a common pathway for this class of drugs.[77]

The insulin-like growth factor-1 (IGF-1) has received much attention, both as a naturally occurring growth factor and therapeutic option for rare conditions (e.g., Laron syndrome) as well as an important biomarker in clinical as well as anti-doping contexts. Hence, substantial interest in simplified and/or superior alternatives facilitating the frequent monitoring of blood IGF-1 levels exists, and critical questions regarding the potential added value of capillary blood rather than venous blood or DBS for doping controls were addressed by Stacchini et al.[78] Four volunteers were blood-sampled using a capillary blood collection device producing up to 300 µL of heparinized material. Over a period of up to 11 weeks, samples were collected three times per day during the first 5 days and afterwards one weekly. IGF-1 concentrations were determined by a bottom-up approach detailed in respective WADA guidelines,[143] and analyses were conducted using a C-18 analytical LC column (2.1 × 5 cm, 5 µm), water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid, as eluents, and a QqQ-based MS system The LOD was determined with 25 ng/mL. Of note, stability of IGF-1 was observed at 4°C up to 72 h and at −20°C/−80°C for up to 3 months, and no indication for circadian fluctuation of IGF-1 concentrations was detected, supporting the microvolume sampling option as complementary tool, for example, for the endocrine module of the ABP.

Besides IGF-1, its analogs such as longR^3-IGF-1, R^3-IGF-1, and des(1–3)IGF-1 are also relevant target analytes for routine doping controls, and ITPs exist for both urine and blood. Mazzarino et al. assessed the utility and performance of currently available sample preparation strategies to sensitively and selectively test for the presence of these IGF-1 analogs, using either 5 mL of urine or 100 µL of serum/plasma.[79] Following preconcentration, specimens were subjected to immunoaffinity purification protocols based on either magnetic beads, pre-coated pipette tips, or spin trap columns, and extracts were then injected into an LC-ESI-Q/TOF MS/MS system. Chromatography was performed on a C-18 analytical column (2.1 × 50 mm, 3.5 µm) with 0.1% formic acid and 1% DMSO in water (solvent A) or acetonitrile (solvent B), and target analytes were detected by diagnostic precursor/product ion pairs at detection limits ranging from 0.05–0.30 ng/mL in urine and 0.5–2.0 ng/mL in serum and plasma. Under the chosen conditions, pre-coated pipette tips appeared to produce the best test results, while further multiplexing might be better accommodated with functionalized magnetic beads.

The metabolic fate of thymosin β4 was studied further by Rahaman et al. employing both in vitro and rat in vivo experiments, aiming at providing best-possible target analytes for human urine analyses.[80] The peptidic drug candidate that currently undergoes a variety of clinical trials was exposed to leucyl aminopeptidase, human kidney microsomes, and human umbilical vein endothelial cells, and was also injected to rats at 20 mg/kg body weight as a single dose. A variety of metabolites was identified, with the fragment thymosin $\beta4_{1-14}$ being a common biotransformation product in all experiments, which was further absent in tested human blank urine samples. Hence, this metabolite was considered as valuable addition to routine doping controls, and its traceability was accomplished using an LC–MS/MS approach offering an LOD of 0.2 ng/mL in spiked human urine. Chromatography was done using a C-18 analytical column (2.1 × 100 mm, 5 µm) with 0.1% formic acid (solvent A) and methanol (containing 0.1% formic acid, solvent B), and analyte detection was achieved with positive ESI and Q/Orbitrap analysis. In the animal administration study, the detection window was reported as 48 h.

4 | β_2-AGONISTS, HORMONE, AND METABOLIC MODULATORS

4.1 | β_2-Agonists

Asthma and exercise-induced bronchoconstriction reportedly affect 15–30% of the elite athlete population, and the use of β_2-agonists in the context of appropriate medication necessitates thorough education and awareness of permissive and prohibited therapies.[144] Concise regulations were deemed inevitable and established in the light of a growing body of evidence demonstrating the potential of β_2-agonists to positively affect athletic performance when used at dosages considered prohibited by WADA. Also, potential synergistic effects of the use of more than one β_2-agonist have required scientific and regulatory attention as demonstrated by in vitro experiments conducted by Piribauer et al.,[84] who could demonstrate increased myotube diameters and enhanced myosin heavy chain protein expression when exposing differentiated C2C12 cells to salbutamol and formoterol at the same time. Another representative of the class of β_2-agonists with a defined permissive dosing regimen (25 µg/24 h) is the ultra-long acting drug vilanterol, which is administered via inhalation.[21] To date, the MRL for vilanterol (unconjugated intact drug in urine) is established at 10 ng/mL,[145] and verification or optimization was aimed in a comprehensive study conducted by Ostergaard et al.[81] Single- and multi-dose administrations of inhaled vilanterol at therapeutic (25 µg) and supratherapeutic (100 µg) were conducted with a total of 19 study

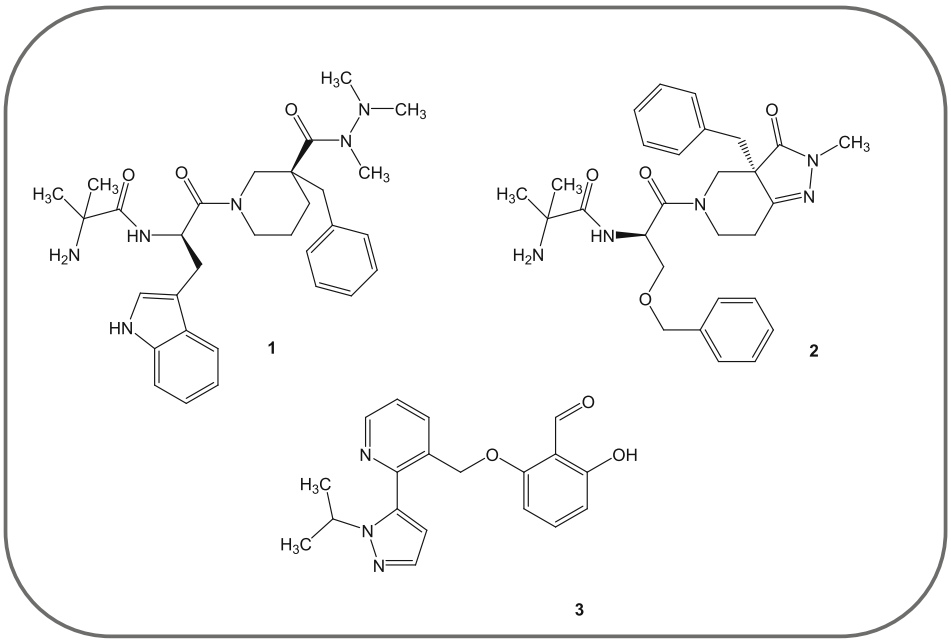

FIGURE 2 Structure formulae of anamorelin (**1**, mol wt = 546.33 u), capromorelin (**2**, mol wt = 505.26 u), and voxelotor (**5**, mol wt = 337.14 u).

participants, and mean C_{max} concentrations of 1.2 and 6.2 ng/mL were determined for single applications of 25 and 100 µg, respectively. In case of multiple applications, the corresponding mean C_{max} levels were found at 2.0 and 22.4 ng/mL. With consideration of specific gravity adjustment (1.018), the lowest possible cut-off supporting the differentiation of permissive vilanterol from a doping scenario was calculated with 3.1 ng/mL. Such a doping scenario was further simulated by Panchal et al., who enrolled 4 recreational athletes that received single or multiple doses of vilanterol (100 µg) combined with 800-µg fluticasone furoate, followed by a 1-h ergometer cycling exercise, and urine was collected up to 72 h.[82] The samples were solid-phase extracted using a mixed-mode cation-exchange resin and subjected to LC-QqQ-MS/MS analysis. Chromatographic separation was accomplished on a C-19 analytical column (3.0 × 50 mm, 2.7 µm) with 0.01% trifluoroacetic acid (solvent A) and acetonitrile/methanol (90/10 v/v, solvent B), and analytes (including vilanterol and two metabolites) were determined in multiple reaction monitoring mode at LOQs of 5–50 pg/mL. Mean C_{max} urine concentrations were at 9.5 and 18.6 ng/mL for single and multiple dosing, respectively, all observed 1–2 h post-administration. Hence, the sampling time point was seen critical with regard to establishing a meaningful cut-off, especially in consideration of the fact that β_2-agonists are prohibited at all times.

As in the previous years, the β_2-agonist higenamine continued to receiving much attention in anti-doping research, due to its natural occurrence in dietary products, its availability in therapeutic formulations, and nutritional supplements and because of its capability to induce the mRNA expression of muscle fiber hypertrophy-related genes and to increase myotube diameters in vitro.[85] The MRL established at 10 ng/mL, applicable to unconjugated higenamine in urine only,[145] is still enforced, and scenarios where athletes might unwittingly ingest higenamine at dosages that could result in AAFs were frequently discussed. Leaney et al. investigated the potential of beetroot to contribute to higenamine findings in the context of sports drug testing and demonstrated the presence of higenamine in various processed beetroot products up to ca. 300 ng/g.[86] Elimination studies with such products resulted in urinary higenamine levels below 0.1 ng/mL, that is, approximately 100-fold below the currently applied MRL of 10 ng/mL.

In another study by Rubio et al., urinary elimination profiles of higenamine and related alkaloids were investigated, both after single and multiple consumption of Annona fruit preparations as well as higenamine-containing dietary supplements.[83] Volunteers ingested fruit puree containing between 162 and 320 µg of natural higenamine, yielding C_{max} urine concentrations of 9.0 ng/mL, that is, not exceeding the relevant MRL of 10 ng/mL in this study. Conversely, single-dose dietary supplement ingestion accounting for ca. 30–44 mg of higenamine readily resulted in c_{max} urine levels of up to 434 ng/mL. Moreover, the abundant presence of reticuline and isococlarine glucuronides in urine samples collected exclusively after fruit consumption provided biomarker information that can assist in result interpretation of higenamine findings in future sports drug testing programs.

4.2 | Hormone and metabolic modulators

Aromatase inhibitors and selective estrogen receptor modulators (SERMs) continue to represent frequently observed prohibited substances, with clomifene and tamoxifen collectively accounting for 33% of all findings in the section S.4 Hormone and Metabolic Modulators in 2021.[126] In the context of investigating metabolites that support the differentiation of contamination and doping scenarios with clomifene, Euler et al. identified a new

hydroxylated clomifene species in re-tested doping control urine samples that returned clomifene-related AAFs previously.[87] The metabolite's configuration was unknown and did not match any of the confirmed hydroxylated clomifene metabolites' retention time, which is why the synthesis of (*E*/*Z*)-3′-OH clomifene and (*E*/*Z*)-4′-OH-clomifene was conducted. A LC–MS/MS-approach facilitating the separation of 8 isomers (including the above-mentioned 4 isomers plus (*E*/*Z*)-3-OH and (*E*/*Z*)-4-OH-clomifene) was then developed, using a C-18 analytical column (2.1 × 100 mm, 1.7 µm) and 5-mM ammonium acetate in 0.1% acetic acid (solvent A) and methanol (containing 0.1% formic acid, solvent B). Via positive ESI, the effluent was directed into a QqQ MS system operated in MRM mode. Target analytes were enzymatically hydrolyzed, extracted by LLE, and derivatized with dansyl chloride for optimized chromatographic separation. The metabolite observed in doping control urine samples was eventually characterized as (*Z*)-3′-OH clomifene, providing complementary information for future case managements.

As reference materials are vital for improving test methods, Kumar et al. endeavored to synthesize the common carboxyl metabolite of tamoxifen and toremifene, which represents an important addition to ITPs in multiplexed screening approaches.[88]

Inhibiting the myostatin signaling pathway has been subject of various (pre-)clinical trials aiming at identifying therapeutic means for conditions associated with, for example, cancer cachexia, and the myostatin propeptide and modified (minimum) amino acid sequences derived from the propeptide composition were suggested as promising drug candidates.[146] Internet-based suppliers have offered myostatin propeptide preparations for research purposes, and means to monitor a potential misuse in sports has been required. Here, Reichel et al. presented a comprehensive study on the composition of 12 products (only 9 of which proved to contain the labeled product) and electrophoretic approaches for their detection in human serum and urine.[90] Immunoprecipitation followed by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blotting of either 500 µL of serum or 15 mL of preconcentrated urine (ultrafiltration) allowed for LODs of 2.5 and 0.4 ng/mL, respectively, allowing for detecting potential administrations of this protein-based compound in routine doping controls. Complementarily, Walpurgis et al. focused on testing approaches concerning myostatin inhibitory peptides with molecular masses between 1.7 and 2.9 kDa.[89] Six drug candidates were subjected to in vitro metabolism studies, and the intact compounds as well as abundant biotransformation products were studied as to their traceability in spiked human serum and plasma. Due to the comparably low molecular mass, serum was merely protein-depleted with acetonitrile, and the supernatant was concentrated prior to analysis by LC-Q/Orbitrap MS/MS. Peptidic analytes were separated on a C-18 analytical column (3.0 × 50 mm, 2.7 µm) using 0.1% formic acid (containing 1% DMSO, solvent A) and acetonitrile (with 1% DMSO, solvent B), and the six model compounds were detected at LODs between 3 and 9 ng/mL. Whether any of these or related structures will advance to clinical trials remains unclear, nevertheless, both the potential misuse and the option to analytically monitor such compounds in sports drug testing programs appear warranted. Using the same analytical setup as above, Thomas et al. investigated the traceability and metabolic fate of insulin-mimetic peptides referred to as S597 and S519.[91] The analytes exhibited molecular masses of 3.8 and 4.2 kDa, respectively, and the extraction from plasma was shown to be particularly efficient when combining protein precipitation with subsequent mixed-mode cation-exchange SPE. LODs were determined with 0.5 ng/mL, and by means of isotope-labeled reporter ion screening using $^{13}C_5{}^{15}N$-labeled analogs of S519 and S597, 8 and 9 respective metabolites were identified using in vitro models employing incubations in serum, urine, or skin microsomes.

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is an activator of the adenosine monophosphate-activated protein kinase (AMPK) and as such prohibited when administered. Due to its natural occurrence in humans, analytical means to differentiate between endogenous and exogenous sources are required. In order to readily identify suspicious doping control urine samples for, for example, subsequent IRMS confirmatory analyses, Sobolevsky et al. suggested the use of a ratio determined from AICAR and its structurally related precursor succinyl-AICAR (SAICAR).[92] After enriching and diluting urine with isotopically labeled internal standards, target analytes were injected onto a trifunctional C-18 analytical column (2.1 × 50 mm, 1.8 µm) interfaced via ESI to a QqQ-based MS system operated in MRM mode. Solvents used were 0.1% formic acid and methanol (containing 0.1% formic acid) as eluents A and B, respectively. Determined from a population of 5517 doping control urine samples, AICAR/SAICAR median values were measured for male (3.3) and female (4.2) athletes. In comparison, urine samples collected after the oral administration of 10 g of AICAR exhibited ratios >10 for 40 h post-administration, with a ratio of 10 representing the 95th percentile of the male athletes' reference population. Hence, in addition to urinary concentrations, the newly established ratio can contribute to targeting suspicious doping control urine samples for follow-up investigations.

5 | DIURETICS AND MASKING AGENTS, STIMULANTS AND NARCOTICS

The health risks and, occasionally, potentially also performance-limiting aspects[147] associated with the misuse of diuretics or masking agents, stimulants, and/or narcotics are well established, and yet both attitude towards their use and proven misuse exist among elite athletes and recreational sportspersons.[148–150] Consequently, comprehensive routine ITP procedures covering the enormous breadth of compounds subsumed under these drug classes at required performance levels are essential for modern anti-doping laboratories. Further to existing testing approaches, optimizations were desirable, addressing requirements of, for example, major sporting events where fast turnaround times are critical. A particularly comprehensive approach was presented by Liu et al., reporting on the test method employed at the 2022 Beijing Winter Olympic Games and Paralympic Games.[67] Here, a total of 350 target analytes were covered by

combining two analytical runs on a QqQ- as well as a Q/Orbitrap-based MS platform. Therefore, urine was prepared by producing three aliquots, one for dilute-and-inject onto the LC-ESI-QqQ-MS, one for enzymatic hydrolysis and LLE, and one to combine with the second (deconjugated) fraction for subsequent LC-ESI-Q/Orbitrap MS analysis. Two LCs were used, equipped with either a C-18 PFP (4.6 × 150 mm, solvent A: 10-mM ammonium formate with 0.05% formic acid, solvent B: methanol) or a C-18 UHPLC (2.1 × 100 mm, 1.9 µm, solvent A: 110-mM ammonium formate with 0.05% formic acid, solvent B: methanol) analytical column. Overall, 45 diuretics, 3 masking agents, 101 stimulants, and 19 narcotics plus respective diagnostic metabolites were covered, in addition to numerous additional substances from other categories of the Prohibited List and all LODs readily meeting applicable MRPL criteria.

Wüst et al. assessed the utility of supercritical fluid chromatography (SFC) tandem MS as an alternative and/or complementary tool for multi-analyte ITP applications in sports drug testing.[94] Urine samples were enzymatically hydrolyzed and diluted with tetrahydrofuran prior to injection into the SFC-ESI-QqQ-MS system, with the SFC being equipped with a 2-EP analytical column (3.0 × 100 mm, 1.7 µm) and employing supercritical CO_2 and 5-mM ammonium acetate in methanol/water (96.5/3.5, *v*/*v*) as solvent and modifier, respectively. A total of 26 diuretics, 60 stimulants, and 11 narcotics (plus numerous additional analytes from other classes of prohibited substances) were successfully included in the test method, and the comparison to established RP-LC–MS/MS approaches underlined the added value of the orthogonal testing approach especially with regards to highly polar analytes.

To further support exploiting the instrumental capabilities of multiplexing hundreds of target analytes in single analytical measurements, optimized sample preparation and extraction protocols are desirable. Here, Gonzalez-Rubio et al. reported on the use of supramolecular solvents, assisting in improving extraction yields and/or reducing matrix interferences for urine sample analyses.[95] To enzymatically hydrolyzed urine, disodium sulfate was added and dissolved, followed by the addition of 1,2-hexanediol and thorough mixing. The in situ formed supramolecular solvent was separated by centrifugation, and after dilution with water, the extract was analyzed on a LC-ESI-Q/TOF MS system. Using 80 model substances, recoveries between 70% and 120% were observed for the 84–93% of analytes demonstrating compatibility with the sample preparation approach, and matrix effects were always below 20%, thus offering an interesting alternative to routinely used sample preparation strategies.

Assessing the opposite approach, that is, omitting (largely) sample preparation and chromatography and employing desorption electrospray as well as paperspray ionization HRAM MS, was conducted by Bressan et al.[97] Urine was analyzed as raw matrix or subjected to SPE, with the urine or the extracts deposited on different supports (polytetrafluoroethylene, C-18- and cellulose-based chromatographic paper). The dried samples were then either desorbed with methanol/water (80:20, *v*/*v*) or sprayed with acetonitrile/water (85:15, *v*/*v*) into a Q/Orbitrap MS, operated in positive and negative ionization mode. Using a set of model compounds including 3 diuretics and 52 stimulants, the performance of the ambient ionization-MS strategy was tested, indicating superior datasets obtained by paperspray MS than desorption ESI, and yet, limitations in sensitivity and specificity were found. While the majority of stimulants was successfully detected at applicable MRPLs, meeting relevant MRLs proved challenging for some, suggesting an interesting potential of the testing methodology in anti-doping and the need for further optimization.

Using disposable pipette tips containing cation exchange resin for automated sample extraction, Bordin et al. presented a proof-of-concept approach for analyzing 13 stimulants with urine and sweat as matrices.[151] Following cation-exchange SPE and derivatization, the amphetamine-like target analytes were derivatized and subjected to GC–MS in selected ion monitoring mode, enabling the detection of substances at LODs between 1.5 and 2.5 ng/mL in urine and 1.5–5 ng/mL in sweat, thus demonstrating appropriate sensitivity of the assay for the selected compounds. Whether or not extending the list of analytes to the required number of additional stimulants relevant to sports drug testing programs is feasible needs to be confirmed; however, the comparison of urine and sweat testing options is certainly a valuable information for doping controls.

The mere detection of substances classified on WADA's Prohibited List in doping control urine samples does not necessarily constitute an ADRV, and aspects requiring consideration are documented predominantly in technical letters.[152] Phenethylamine is one such example, and as recently corroborated by Nara et al., its concentration in urine can vary with time and storage conditions post-sampling, which is why other analytical means are required (and applied) to routine doping control specimens to probe for the use of phenethylamine by athletes.[98] Likewise, the prohibited stimulant meclofenoxate dissociates in vivo into 4-chlorophenoxyacetic acid and 2-(dimethylamino) ethan-1-ol (deanol), with the latter further metabolizing to its *N*-oxide. Krug et al. assessed the natural abundance and utility of the deanol-*N*-oxide in human urine as additional marker for meclofenoxate administrations and provided first reference ranges for this analyte in human urine using an LC-QqQ-MS/MS approach.[96] Urine from athletes as well as post-administration samples collected after meclofenoxate or deanol ingestion were analyzed, demonstrating that deanol and meclofenoxate applications result in similar elimination profiles of deanol-*N*-oxide, suggesting that this analyte alone is not able to indicate the use of meclofenoxate. Combining findings of 4-chlorophenoxyacetic acid and deanol-*N*-oxide in human urine at concentrations indicative for recent use of meclofenoxate appears to offer a solution though, which nevertheless requires further elimination studies with the prohibited substance.

In the category of narcotics, a major change in anti-doping regulations has been the inclusion of tramadol into the Prohibited List, coming into effect in 2024.[153] Various studies aiming at identifying whether tramadol has performance-enhancing effects were conducted during the last decade, with the most recent investigation by Mauger et al. suggesting once more its potential relevance particularly in endurance sport.[99] In a double-blind, randomized, and cross-over designed study, highly trained cyclists completed time trials significantly faster at higher power output under the influence of 100 mg of

orally administered tramadol than when receiving the placebo. Despite the existence of contradictory results and scientific positions, this could be one factor that might have contributed to the comparably high prevalence of tramadol in doping control urine samples of cyclists in the past.[154]

6 | GLUCOCORTICOIDS, CANNABINOIDS, AND BETA-BLOCKERS

Whether or not glucocorticoids possess performance-enhancing properties for elite athletes has been debated extensively in the past, and a recent review by Riiser et al. concludes that currently available data suggest enhanced maximal and aerobic performance of athletes after administration of glucocorticoids.[101] Conversely, Nordsborg et al. reported on the accelerated erythropoiesis after a single intramuscular injection of 40 mg of triamcinolone acetonide to trained cyclists and triathletes, while aerobic exercise performance remained unaffected.[102] The significant increase in reticulocyte percentage (%ret) was followed by a significant increase in hemoglobin mass, and the classification of glucocorticoids as drugs being prohibited in-competition only was called into question.

Also, the relevance of cannabis (and here especially the active ingredient delta-9-tetrahydrocannabinol, THC) as a doping agent has continued to be subject of controversial positions.[155,156] The 2023 and 2024 editions of the Prohibited List remained unchanged as to the status of THC and cannabinoids in general,[21,153] with cannabidiol (CBD) being explicitly excluded from the banned category in virtue of its molecular mechanisms of action.[157] Athletes might be at risk of (unknowingly) ingesting THC-containing products as reported by Wagner et al., for example, when consuming milk from hemp-fed dairy cows.[100] Levels of ca. 200 µg of THC per liter of milk (mean) were detected, and the presence of other natural minor cannabinoids must be taken into consideration in the context of anti-doping regulations, necessitating once more awareness of athletes and their entourage.

Concerning beta-receptor blocking agents (beta-blockers), Lin et al. investigated the urinary elimination profile of timolol and carteolol after acute as well as chronic ophthalmic use.[115] With acute and chronic dosages of at least 500 µg of timolol maleate/day and 2 mg of carteolol hydrochloride/day via eyedrop solution application, urinary concentrations of the target analytes exceeding the MRL of 50 ng/mL[145] were observed. Hence, when medically indicated, therapeutic use exemptions are required in particular sports, in order to comply with applicable anti-doping rules.

7 | MANIPULATION OF BLOOD AND BLOOD COMPONENTS

In consideration of the reported performance impact of "blood doping," which commonly subsumes homologous (HBT) or autologous (ABT) blood transfusion practices and illicits interventions including combinations of blood transfusions and oxygen depletion-, altitude- or drug-induced erythropoiesis, improvements of analytical strategies to detect such practices have been seen as critical for modern anti-doping efforts. A consensus appears to exist in the perception that applying direct as well as biomarker-based indirect test methods (as provided, e.g., by the ABP hematological module) enables the identification of doping practices while clearly recognizing biomarker variabilities originating from factors other than doping. However, the consensus extends also to the observation that new, complementary biomarkers would be beneficial to enhance the sensitivity of the ABP,[158–160] and intraindividual long-term biological variation is a key factor to assess for the utility and significance of data derived from the ABP. Diaz-Garzon et al. presented results from 30 triathletes that were blood-sampled over a period of 11 months (once monthly), and 9 hematological parameters were subjected to CV-ANOVA to provide estimates for the respective biological variation.[104] Neither exercise nor state of health was found to affect the biological variation, which ranged from as little as 1.3% (95% CI, 1.2–1.4) to 23.8 (95% CI, 21.6–26-3) for the mean corpuscular volume and %ret, respectively, corroborating the robustness of the existing approach.

In order to probe for the performance of the ABP under authentic drug administration protocols, Miller et al. conducted a study with 12 male recreational athletes receiving subcutaneous rhEPO "boosting" doses (40 IU/kg, eight injections over a period of 20 days) followed by 10 days of washout before administering 900 IU intravenously on 6 occasions over a period of 12 days.[105] Blood was collected before, during, and up to 28 days after the last dose (study day 70). Atypical passport findings (ATPFs) were reported for 49% of all specimens obtained during the "boosting" and first washout period, and another 24% of samples returned ATPFs during the microdose period and subsequent washout, demonstrating that, in the light of the analytical sensitivity for rhEPO with direct testing approaches (vide supra) when applied within a short period of time after drug administration, combining ABP and direct analytical methods is of utmost importance. The added value of including further markers such as the immature reticulocyte fraction (IRF, defined as the ratio immature reticulocytes/reticulocyte count) and the ratio of immature reticulocytes/erythrocyte count (IR/RBC) for detecting rhEPO microdosing by ABP approaches was investigated by Breenfeldt Andersen et al.[106] In a comprehensive study, 48 participants (24 males, 24 females) received three weekly intravenous injections of rhEPO at 9 IU/kg bodyweight for a total of 4 weeks, and established ABP analyses were conducted on samples collected up to 10 days post-treatment. ATPFs were obtained for 29% of all collected specimens, and including secondary markers such as %ret and the abnormal blood profile score (ABPS) increased the ATPF fraction to 46%. Complementing the analyses by IRF and IR/RBC, a sensitivity of 79% was reported, indicating the utility of these markers for routine ABP applications; however, their robustness concerning potentially confounding factors such as iron supplementation might require further data.[107]

To which extent other interventions such as low-volume blood withdrawal affect the ABP was studied by Krumm et al.[108] A loss (or withdrawal) of 140 mL of blood was found to significantly decrease

hemoglobin mass over a period of 7 days but not resulting in an ATPF at any point in time over the monitoring period of 21 days. Of note, ferritin concentration was reported to be significantly downregulated, thus offering means to register low volume blood withdrawals as allegedly practiced in situations of autologous blood transfusions.

HBT practices are monitored by anti-doping laboratories by means of flow cytometry, targeting 8–10 (out of 345 recognized) erythrocyte surface antigens that characterize and reflect a person's individual red blood cell population. The presence of a second population of erythrocytes with regards to two or more specified antigens results in an AAF. Mirotti et al. revisited the utility of the most commonly utilized surface antigens with a cohort of 261 athletes, originating from five different continents and representing 14 different sport disciplines, to assess the likelihood of matching phenotypes (and thus the risk of false negatives).[109] An unexpectedly high occurrence of matching patterns, higher than the published theoretical probability, was found, and complementary testing strategies such as, for example, DNA-based analyses were suggested. In an exploratory study by Marchand et al., the traceability of HBT by forensic DNA analysis, facilitated by collecting DBS, was investigated, using two male volunteers who received 150 mL of an erythrocyte concentrate.[110] The transfused volume was not leukodepleted, and yet probing for a second set of DNA in post-transfusion DBS proved incapable of detecting a 150-mL HBT event. In contrast, a flow cytometry assay comprising 9 erythrocyte surface antigens allowed for the detection of the transfusion up to 50 days after the intervention, attributable to a superior dynamic range of the approach that, if complemented by additional surface antigens, might gain the desired sensitivity for doping control purposes.

A comparably new addition to the category M1.2 of the Prohibited List is the hemoglobin S polymerization inhibitor voxelotor (Figure 2, **3**), which enhances the oxygen binding affinity of hemoglobin and was shown to increase also serum EPO levels in healthy individuals. To allow for appropriate test methods in routine doping controls, Rzeppa et al. produced metabolites in vitro by means of human hepatocells as well as human liver microsomes and characterized potential target analytes by LC-HRAM MS/MS.[103] The obtained main metabolites were attributed to hydrogenation, hydroxylation, and *O*-methylation plus glucuronoconjugation, providing a variety of biotransformation products compatible with commonly employed sports drug testing methods. Here, a C-8 analytical column was used, operated with 2-mM ammonium acetate in water/acetonitrile (95/5, *v*/*v*, containing 0.1% acetic acid, solvent A) and 2-mM ammonium acetate in water/acetonitrile (5/95, *v*/*v*, containing 0.1% acetic acid, solvent B). Following positive ESI, product ion mass spectra were generated on a Q/TOF MS system, providing characteristics of the tentatively identified voxelotor metabolites.

8 | GENE DOPING

The necessity of developing and evaluating approaches to detect the gene doping practices is evident, and a variety of options as to how such illicit activities can be addressed have been assessed over the past 12 months, targeting the most prominent methods of gene doping, that is, the introduction of transgenes into the human organism or manipulating the human genome via Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas methods.[112] Baoutina et al. reported on the successful expansion of an existing hydrolysis probe-based real time PCR assay from one (*EPO*) to a total of five targets, with the latter subsumed under "anabolic transgenes" including follistatin, growth hormone 1, GHRH, and IGF-1.[161] The applicability and robustness of the assays were demonstrated by the detection of 1500 copies of each transgene per mL of spiked blood, following routinely used extraction steps and amplification of one exon-exon junction region each, producing a fluorescence signal in the presence of the transgene. With currently one exon-exon junction being targeted per transgene, further studies were announced to complement the assays with additional junctions employing real-time PCR.

While not applied to human blood but equine specimens, the technology referred to as πCode demonstrated also multiplexing capabilities in detecting the presence of transgenes in doping controls as shown by Ohnuma et al.[162] On the surface of microdiscs (diameter 40.5 μm), DNA probes are immobilized that hybridize with defined transgene sequences. Those transgenes are extracted from blood, PCR amplified with the reverse primer being biotinylated, and subjected to the πCode surface. Only target genes are retained and subsequently capture fluorescent-labeled streptavidin for readout, and the successful detection of 1500 copies in 1.5 mL of spiked blood was reported. A method covering a total of 12 transgenes was established and proof-of-concept data provided by an *EPO* in vivo study with 20 mg of the transgene being administered intramuscularly to a horse. The transgene was detected up to 1 week using the presented approach.

An alternative strategy allowing to detect the human *EPO* transgene was presented by Yi et al., who exploited the highly specific binding capacity of a nuclease-deficient Cas9 mutant (referred to as deadCas9) equipped with single-guide RNA (sgRNA) directed against transgene *EPO* exon-exon junctions.[163] Two deadCas9 versions were employed, one immobilized to magnetic beads as capture tool and one biotinylated for visualizing the presence of the target analyte with streptavidin-coupled horseradish peroxidase. In analogy to sandwich-based immunoassays, the strategy allowed for an LOD of 12.3 fM (equalling 740.000 copies), and installing an additional PCR amplification step prior to applying the CRISPR-based sensing approach enabled lowering the LOD to 2.5 copies in 5 μL of blood. Of note, the overall assay time was reported with 90 min, which would facilitate high throughput and fast result reporting. Yan et al. described a test method that utilizes a platform with CRISPR/Cas12a in combination with Recombinase Polymerase Amplification (RPA) to target *EPO*, *GH1*, and *IGF-1* transgenes, visualized by fluorescence detection as well as in a lateral flow test device.[164] For the first detection approach, the activation of CRISPR/Cas complex (by recognizing the design-matching exon-exon junction amplicons) triggers the cleavage of the labeled reporter TBA11 (truncated Thrombin Binding Aptamer) by way of "collateral activity" to produce a fluorescence signal for

Info Box

S1
- New metabolites, particularly bishydroxylated species, of bolasterone were identified in vitro and *in vivo*, complementing routine screening options.
- Methoxydienone was shown to metabolize in vivo to 18-methyl-19-norandrosterone, which is readily identified in routine doping control ITPs.
- Including sulfo-conjugated metabolites of AAS in ITPs using GC-MS(/MS) offer complementary and competitive detection windows as demonstrated for metenolone, mesterolone, and methyltestosterone. Alternatively, derivatizing intact glucuronides with Girard Reagent T and measuring AAS metabolites using LC-HRAM MS/MS offers appropriate LODs.
- The metabolism of a new SARM (2f) was investigated, and a similar metabolic pathway as revised and reported for LGD-4033 was established, facilitating their implementation into test methods on LC-ESI-MS/MS platforms.
- Animal models and organ-on-a-chip assays proved suitable to generate critical information on drug (and drug metabolite) formation and distribution, aiming at investigating contamination scenarios with seminal fluid.
- Complementing urine steroid profiling with serum steroidal analyses was shown to considerably increase the true positivity rate in controlled elimination studies, especially concerning female study volunteers' test samples.
- New IRMS-based test methods particularly focusing on steroids with androst-1,4-diene or 11-oxo-androst-1-ene pharmacophore are available.

S2
- Complementing the ABP hematological module data with either direct detection methods for ERAs and/or adding further markers such as the immature reticulocyte fraction (or its ratio with the reticulocyte count) increases the percentage of ATPFs in controlled hEPO administration studies.
- An assay based on CRISPR/dCas9 allows for the detection of the presence of the.577del *EPO* gene variant in 3 µL of blood.
- With deglycosylation prior to SDS-PAGE analysis, also microdosed EPO can be detected in blood samples of athletes carrying the c.577del *EPO* gene variant.
- Testing options for complexed cobalt by LC-MS/MS were presented, offering an alternative to commonly applied ICP-MS.
- Triptorelin (5-10) was identified as a potential long-lasting metabolite in human urine.
- New data on the metabolism of anamorelin are available, and the first finding of capromorelin in routine doping controls was attributable to a contamination scenario with a veterinary product.
- Metabolism studies supporting the detection of thymosin β4 administrations identified thymosin β4 (1-14) as promising target analyte candidate.

S3
- The MRL for vilanterol might be lowered to 3.1 ng/mL based on controlled elimination studies.
- Repeated and high-volume consumption of higenamine-containing fruits was shown to result in urinary higenamine concentrations below the established MRL. Moreover, probing for related alkaloids can support differentiating the source of higenamine being an enriched supplement or conventional diet.

S4
- A new metabolite of clomifene found after drug administration was identified as (Z)-3′-hydroxy-clomifene and is indicative for drug exposure rather than contamination.
- Myostatin propeptide products potentially used as doping agent were shown to be of substandard quality, and testing options for myostatin inhibitory peptides (and related metabolites) were established.
- The implementation of insulin-mimetic peptides S519 and S597 as well as in vitro derived metabolites into routine ITPs was accomplished.
- Monitoring the AICAR/SAICAR ratio in urine supports identifying samples suspicious for AICAR administrations.

S5
- Supercritical fluid chromatography was shown to be suitable for comprehensive screening methods including especially diuretics and stimulants.

S6
- Monitoring urinary deanol-*N*-oxide supports the result interpretation concerning meclofenoxate use in case of 4-chlorophenoxyacetic acid findings.

S9
- The glucocorticoid triamcinolone acetonide was shown to exhibit erythropoiesis-stimulating effects and further research appears warranted to assess whether the currently enforced prohibition of such drugs in-competition only is justified.

M1
- Ferritin monitoring might assist in identifying blood withdrawal incidents such as those expected in situations of autologous blood transfusion practices.
- Flow cytometry offered considerably longer detection windows for homologous blood transfusion events than DNA typing.

M3
- New and particularly fast methods enabling the detection of *EPO* or anabolic transgene administrations using qPCR or CRISPR/Cas methods were presented.
- Likewise, first approaches towards uncovering CRISPR/Cas-based manipulations by collateral activities of CRISPR/Cas systems were reported.

FIGURE 3 Info box on particularly relevant observations.

detection. The lateral flow test required a differently labeled reporter, and while the intact reporter is captured by streptavidin, its cleavage product containing the fluorescein (FAM) is captured on a separate site by primary and secondary antibodies. An LOD of 1 aM of amplified plasmids was determined, and an overall turnaround time of 40 min was presented, suggesting an interesting methodology for

future gene doping tests, but additional data on specificity and reproducibility might need to be provided. In a similar manner, Passreiter et al. aimed at detecting the manipulation of the human genome by CRISPR/Cas interventions rather than proving the administration of transgenic material.[113] Exploiting the conserved region of the sgRNA found in CRISPR/Cas9 systems as xenobiotic target, a protocol consisting of isolating the CRISPR RNA from human serum, reverse transcription-RPA, subsequent T7 transcription, and detection by fluorescence enabled by the amplicon-triggered activation of Cas13a and cleavage of a fluorescence-quenched ssRNA reporter. With an LOD of 100 pM, the injection of lipid-mediated CRISPR ribonucleoprotein complexes into mice was shown to be traceable for 8 h. While this detection window appears very limited, improving the LOD by advanced detectors appears possible, and thus, another detection tool for gene doping practices might become available.

9 | CONCLUSION AND PERSPECTIVES

Great advances in various areas of anti-doping testing approaches were reported and accomplished over the past 12 months, and continuing efforts in investigating (new) anabolic agents, their metabolism and elimination, and the ABP with its already well-established modules for profiling of urinary steroids and hematological parameters, as well as for the newly created blood steroid and endocrine modules, were registered. Likewise, research efforts were made in the anticipated studies exploring the options of DBS testing, their applicability, added value, and limitations in routine doping controls. For DBS in particular, their utility regarding the analysis of individual classes of analytes (e.g., ERAs[72]), comprehensive ITPs for lower molecular mass analytes in general,[68] or kinetics monitored for single drugs (e.g., trimetazidine[93]) were presented, underlining the enormous potential of this complementary test matrix for doping controls as well as biobanking of athletes' sports drug testing specimens (e.g., in the context of follow-up investigations). In addition, the robustness of DBS concerning manipulation was taken into consideration by investigating means to determine the time-since-deposition of DBS, in order to identify those samples that were not collected at the time when allegedly sampled, that is, in an attempt of sample swapping.[111]

Further, a considerable increase in studies concerning the detection of gene doping practices was registered, and even if not applied yet at the desirable frequency in routine doping controls, long-term storage programs for blood samples appear to be feasible and sensible in the light of the apparent stability of transgenes when appropriately conserved.[114] Also, additional matrices independent from individual athletes (such as, e.g., wastewater analyses were discussed in the context of monitoring patterns of drug misuse in sport,[165]) and whether or not this can contribute effectively to future doping controls need to be seen.

In summary, the ongoing efforts in optimizing and refining analytical options beyond comprehensiveness and sensitivity, particular in expanding anti-doping sciences and applications towards new biotechnological options, were registered, all of which aiming at supporting of decision-making processes in anti-doping. Key aspects of this 16th *annual banned-substance review* that has considered literature published between October 2022 and September 2023 are summarized in the Info Box in Figure 3.

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