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Annual Banned-Substance Review 17th Edition—Analytical Approaches in Human Sports Drug Testing 2023/2024

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ABSTRACT

The 17th edition of the annual banned-substance review on analytical approaches in human sports drug testing is dedicated to literature published between October 2023 and September 2024. As in previous years, focus is put particularly on new or enhanced analytical options in human doping controls as well as investigations into the metabolism and elimination of compounds of interest, which represent central (while not exclusive) cornerstones of the global anti-doping mission. New information published within the past 12 months on established doping agents as well as new potentially relevant substances are reviewed and discussed in the context of the World Anti-Doping Agency's 2024 Prohibited List. Thereby, analytical challenges, especially with regard to the continuously growing number of target compounds and potentially relevant drug classes as well as the exigency (and consequences) of utmost analytical retrospectivity, are thematized and contextualized. Investigations especially into anabolic agents, peptide hormones, and strategies for the detection of gene doping were identified as core areas of anti-doping research in the reviewed period.

1 | Introduction

The efforts steadily invested into improving the anti-doping system are manifold, multifaceted, and immense, featuring most diverse and yet complementary approaches towards the common goal of protecting clean athletes [1]. This endeavor has, however, proven to be a complex and presumably infinite task, necessitating (among other aspects) continuously optimized and adapted strategies as well as synergized expertise from various different scientific areas [2] and, obviously, support and commitment from sport federations, anti-doping organizations [3], governments, sport physicians, and the athletes.

Addressing the question as to *why* (and possibly under which individual circumstances of vulnerability [4]) athletes might turn towards doping warrants as much consideration as providing the means to detect doping offences, as both have been identified as essential factors in developing effective measures to minimize (or prevent) doping [5]. The environment (including the coach–athlete relationship, social norms, etc.) [6] in which athletes operate plays a critical role concerning their attitude towards doping [7], and likewise does the athletes' risk-vs.-reward assessment. The latter certainly depends on the quality of anti-doping testing programs and the available or employed analytical options [8] as deterrence via anti-doping tests necessitates the prospect of their efficiency in uncovering the use of prohibited substances and/or methods of doping [9]. Here, besides improving analytical methods in anti-doping laboratories, which were considered as material to the development of performances presented e.g. in weightlifting [10], there have been suggestions to

optimize testing strategies (i.e. selecting athletes, timepoints of sample collection, sample matrix, long-term sample storage and re-analysis programs, etc.), potentially also with support of artificial intelligence-powered analyses of the Athlete Performance Passport (APP) [11].

Mapping the prevalence of doping among elite [12–14] as well as sub-elite [15] athletes is naturally of particular interest to the anti-doping community [5]. It has hence been subject of various studies and, occasionally, also controversies, and approaching the topic with complementary strategies appears sensible as presented for instance in a recent study, where urine analysis was combined with a randomized-response questionnaire on the occasion of an ultramarathon event, aiming at opening new avenues to evaluate the effectiveness of doping prevention campaigns [12].

Advancing analytical methods for doping controls is vital for contemporary sports drug testing [16] as well as re-testing programs, as demonstrated for instance by the report on the outcome of the further analyses of samples collected on the occasion of the Games of the XXX Olympiad (London 2012) [17]. In consideration of the, at times, low *per capita* doping control sample collection frequency, an optimized analytical sensitivity can be decisive in ensuring an acceptable doping control analytical "retrospectivity". Likewise, planning and conducting testing programs with further analyses of long-term stored samples is advisable especially when improved detection methods have become available; improved with regard to sensitivity, comprehensiveness, and/or conclusiveness. Obviously, the challenge associated with the increasing capability of detecting lowest amounts of prohibited substances (or their metabolites), i.e. differentiating between inadvertent exposure and doping scenarios in case of adverse analytical findings (AAFs), must be taken into consideration [18] and has been prioritized in several anti-doping research programs and projects (*vide infra*).

In general, anti-doping research activities are commonly aligned with the World Anti-Doping Agency's (WADA's) Prohibited List [19] and its 11 classes of banned substances (S0–S9 plus P1) and three categories of prohibited methods (M1–M3) (Table 1). In comparison to the 2023 version of the Prohibited List [20, 21], the 2024 edition explicitly named additional compounds such as 2,4-dinitrophenol (DNP) and troponin activators (e.g. reldesemtiv and tirasemtiv) under S0 (non-approved substances), trestolone (7α-methyl-19-nortestosterone), dimethandrolone (7α,11β-dimethyl-19-nortestosterone) and 11β-methyl-19-nortestosterone under S1 (anabolic agents), histrelin and kisspeptin under S2.2.1 (testosterone-stimulating peptides in males), tetracosactide under S2.2.2 (corticotrophins and their releasing factors), and capromorelin and ibutamoren (MK-677) under S2.2.4 (growth hormone releasing factors). Further, under S4.4.1 (activators of the AMP-activated protein kinase) SR9011 was added as an additional example and, likewise, were conivaptan and mozavaptan under S5 (diuretics and masking agents) and 2-phenylpropan-1-amine under S6.B (specified stimulants). As announced and communicated as early as 2022 [22], the prohibition of tramadol use in-competition was implemented under S7 (narcotics) and came into effect on January 1st 2024. Tramazoline was added as an exception from S6, and also plasma and plasma components donation (M1, manipulation of blood and blood components) was clarified to be permitted if performed in a registered collection center.

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 continued to be conducted also in 2024. Being a naturally produced ingredient of various edible plants, the interpretation of prevalence data and (estimated) concentrations of athletes' doping control urine samples needs to take into account potential dietary sources and the respective distribution of ecdysterone levels, the extent of ecdysterone's oral bioavailability, potential factors influencing the metabolism and elimination, etc. as demonstrated in recent studies e.g. by Isenmann et al. [23] Urinary unconjugated ecdysterone concentrations of up to 5.5 μg/mL were found after consumption of 150 g of quinoa, and observations suggesting an influence of the participants' sex on urinary metabolite patterns were reported, which is in agreement with complementary studies on the human metabolism of ecdysterone presented by Piper and Thevis [24].

The in- and out-of-competition monitoring of gonadotrophin-releasing hormone (GnRH) analogs in females under 18 years and hypoxen also continued in 2024. The monitoring program was complemented by semaglutide, a glucagon-like peptide-1 (GLP-1) analog, to examine the prevalence and pattern of use in sport. While test methods for GnRH analogs have been established and employed in routine doping controls for years, data on the urinary elimination of hypoxen and semaglutide have been scarce. Here, Görgens et al. provided first insights into target analytes' characteristic for the use of hypoxen, both in samples collected in the context of controlled administration studies as well as routine doping control specimens. In conclusion, a recommendation was made to include a set of three tentatively identified metabolites into commonly applied liquid chromatography-mass spectrometry-based initial testing procedures (ITPs) [25]. In order to assess the use of semaglutide among athletes, probing for the presence of urinary metabolites consisting of fragments of the drug's lysine 26-bound side chain (composed of condensed 8-amino-3,6-dioxaoctanoic acid, glutamic acid, and octadecanedioic acid) was considered, albeit the metabolites (a product ion mass spectrum of U7 is illustrated in Figure 1) were not unique to semaglutide but were found to be produced from tirzepatide, another GLP-1 analog, as well [26].

Monitoring the β_2-agonists salmeterol and vilanterol below the minimum reporting level (MRL) [27] was discontinued and tramadol was, in accordance with its classification as prohibited narcotic (S7), removed from the monitoring program. The in-competition use of the stimulants bupropion, caffeine, nicotine, phenylephrine, phenylpropanolamine, pipradrol, and synephrine, and the narcotics codeine, dermorphin and its analogs, and hydrocodone remained as part of the 2024 Monitoring Program, complemented by tapentadol and dihydrocodeine.

Beyond substances and methods either classified as prohibited or subject to monitoring, research into the potential relevance and/or traceability of other candidates was conducted as presented

by the example of thyroid hormones. Albeit the status of thyroid hormones (i.e. not prohibited) did not change in 2024, a new testing approach and data on post-administration samples were presented. Martinez-Brito et al. established a liquid chromatography–tandem mass spectrometry (LC–MS/MS)-based assay, enabling the determination of urinary concentrations of a total of nine thyroid hormone-related analytes, and the method was applied to pre- and post-administration urine samples collected in the course of elimination studies with diiodothyronine [28], triiodothyronine, or levothyroxine [29]. Whether or not the measured analytes will allow to detect non-therapeutic use of thyroid hormones remains to be shown; but the option of monitoring a large array of relevant markers related to thyroid hormone metabolic pathways was demonstrated to be feasible. In addition, if and how -omics approaches will further contribute to both anti-doping efforts as well as assisting in optimizing athletes' performance and recovery has received considerable attention. Similarly, with the prevailing concept of the Athlete Biological Passport (ABP), the growing availability of options to profile especially the individual variability of markers in a targeted or untargeted fashion was shown to warrant consideration [30, 31].

In this continuation of the 16th edition of the *annual banned-substance review*, literature published between October 2023 and September 2024 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. The overall analytical assignment sports drug testing programs needs to (and does) continuously evolve [105] in order to ensure the required comprehensiveness of critical data, both for instantaneous result assessment as well as retrospective evaluation and, when indicated, interpretation. Exploring and exploiting alternative sample preparation [106] as well as analysis protocols and instrument technologies [107] are desirable to allow for the generation of such comprehensive datasets, and research into the utility of complementary sample matrices such as hair has received considerable attention [108] as exemplified also in the context of case reports.

2 | Non-Approved Substances

The category S0 of the WADA Prohibited List includes many different substances that comply with the criteria of being prohibited in sports, but that are not covered by any other section of the document. For these substances, no current approval is granted for human therapeutic use by any governmental regulatory health authority. Examples of compounds classified under S0 are, among others, BPC-157 and the troponin activators reldesemtiv and tirasemtiv.

With regard to BPC-157, Tian et al. reported on a novel approach to probe for the formation of metabolites of the substance using in vitro incubation experiments (human liver microsomes and human skin S9 fraction) [109]. In addition to metabolizing BPC-157, its stable isotope-labeled analog (comprising a $^{13}C_6$-$^{15}N_2$-labeled lysine) was also incubated with metabolizing matrices, and the combined mixture was analyzed on a LC-quadrupole/orbitrap system. The high resolution/accurate mass (HRAM) capability allowed for identifying signals in full scan mass spectral data by targeting ion pairs differing by 4.0071, 8.0142, or 2.6714 *m/z*, facilitating the

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

	Class	Sub-group	Examples	Prohibited	
				At all times	In-competition only
S0	Non-approved substances		BPC-157, dinitrophenol, reldesemtiv, tirasemtiv	x	
S1	Anabolic agents	1 Anabolic androgenic steroids	Androstenediol, 1-androstenediol, clostebol, danazol, dehydroepiandrosterone, metandienone, methyltestosterone, methyltrienolone, nandrolone, stanozolol, testosterone, tetrahydrogestrinone, trestolone	x	
		2 Other anabolic agents	Clenbuterol, osilodrostat, ractopamine, selective androgen receptor modulators (SARMs), zeranol, zilpaterol		

(Continues)

TABLE 1 | (Continued)

	Class	Sub-group		Examples	Prohibited	
					At all times	In-competition only
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, daprodustat, IOX2, 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	Testosterone-stimulating peptides (in males)	Chorionic gonadotrophin (CG), luteinizing hormone (LH), gonadotrophin-releasing hormone and its agonist analogs (e.g. buserelin, deslorelin, gonadorelin, leuprorelin), kisspeptin		
		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, somatrogon, 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), 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)		

(Continues)

TABLE 1 | (Continued)

	Class	Sub-group		Examples	Prohibited	
					At all times	In-competition only
S3	Beta-2-agonists			Fenoterol, higenamine, reproterol, salbutamol, 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, cyclofenil, 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, chlortalidone, 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, tramadol		x
S8	Cannabinoids			Hashish, marijuana, JWH-018, HU-210		x

(Continues)

TABLE 1 | (Continued)

	Class	Sub-group		Examples	Prohibited	
					At all times	In-competition only
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-hour period	x	
M3	Gene and cell doping	1	The use of nucleic acids or nucleic acid analogues 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	DNA, RNA, siRNA	x	
		2	Use of normal or genetically modified cells			
P1	Beta-blockers			Acebutolol, atenolol, bisopropol, metoprolol	x[a]	x[a]

[a]Depending on the rules of the international sport federations.

identification of BPC-157 metabolites for subsequent sequencing by MS/MS experiments and chemical synthesis. Metabolite profiles differed significantly between the two chosen in vitro systems but also yielded five common biotransformation products, which were eventually chosen for validation of a detection assay for urine sample analysis. After weak cation exchange (WCX) solid-phase extraction (SPE), the analytes were separated on a C-18 analytical column (2.1 × 100 mm, 1.7 μm particle size) using acetonitrile and water (both containing 0.1% formic acid) before identification by electrospray ionization (ESI)-MS/MS. LODs between 0.01 ng/mL and 0.11 ng/mL were accomplished, which should offer an adequate sensitivity for routine doping controls.

Reldesemtiv and tirasemtiv were subject of metabolite identification studies conducted by Euler et al. [110], who utilized both in vitro and (orally microdosed) in vivo experiments to produce first insights into the human metabolism of these compounds. While tirasemtiv primarily yielded a glucuronic acid conjugate as well as an oxygenated and glucuronidated species, reldesemtiv produced a series of biotransformation products from both phase-I and phase-II metabolism, with one metabolite (hydroxylated 3-fluoro-2-(3-fluoro-1-methylcyclobutyl) pyridine) representing a suitable target analyte for routine initial testing procedures in urine samples. Using the identified metabolites, the administration of 50 μg of either drug was traceable up to 72 h, employing an assay based on SPE and subsequent LC–MS/MS analysis. The LC was equipped with a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size) and operated with 0.1% formic acid (solvent A) and methanol (containing 0.1% formic acid, solvent B). The MS used ESI in positive ionization mode and a Q/orbitrap analyzer, allowing for LODs of 0.01 ng/mL and 0.2 ng/mL for reldesemtiv and tirasemtiv, respectively.

3 | Anabolic Agents

3.1 | Anabolic-Androgenic Steroids

Anabolic-androgenic steroids (AAS) have owned a central role in history of doping, despite the still growing body of evidence regarding short- and long-term consequences of AAS abuse, spanning from a dysfunctional hypothalamus-pituitary-testicular axis to cardiovascular and/or musculoskeletal diseases as well as psychological disorders [111–113].

Based on the 2022 anti-doping testing figures, anabolic agents (and in particular AAS) were by far the most frequently detected prohibited substances in routine doping controls worldwide [114]. A total of 42% of all reported AAFs accounted for anabolic agents, and more than 86% of these were attributed to AAS, highlighting the importance of research into further strengthening the testing capabilities of anti-doping laboratories in that regard. As in previous years, studies published in 2023/2024 centered around an improved understanding of the metabolism of AAS, exploring and exploiting the added value of longitudinal marker monitoring as well as complementary test matrices (such as e.g. capillary blood), and new or advanced means that facilitate the differentiation of natural and endogenously produced steroids from synthetic analogs.

3.1.1 | Initial Testing Procedures and Studies on Metabolism

Various projects were dedicated to investigations into the metabolism of methyltestosterone, using both in vitro and in vivo approaches. For instance, Angelis et al. revisited the metabolic biotransformation processes of methyltestosterone with particular focus on those structures that feature a 17,17-dimethyl-18-nor-Δ13 C/D-ring composition combined with a fully reduced steroidal A-ring [32]. Predicted structures were chemically synthesized, and samples from HepG2 incubation experiments as well as urine specimens collected after oral administration of 25 mg of methyltestosterone were analyzed using gas chromatography (GC)-electron ionization (EI)-MS/MS. A total of six new metabolites was presented, two of which (17,17-dimethyl-18-nor-5β-androst-13-en-3α-ol and 17α-methyl-5β-androstane-3α,16α,17β-triol) were shown to offer a prolonged detection window of up to 14 days using conventional sample preparation and analysis conditions (i.e. enzymatic hydrolysis of the glucuronide-conjugated fraction, liquid–liquid extraction (LLE), and trimethylsilylation). In addition, the

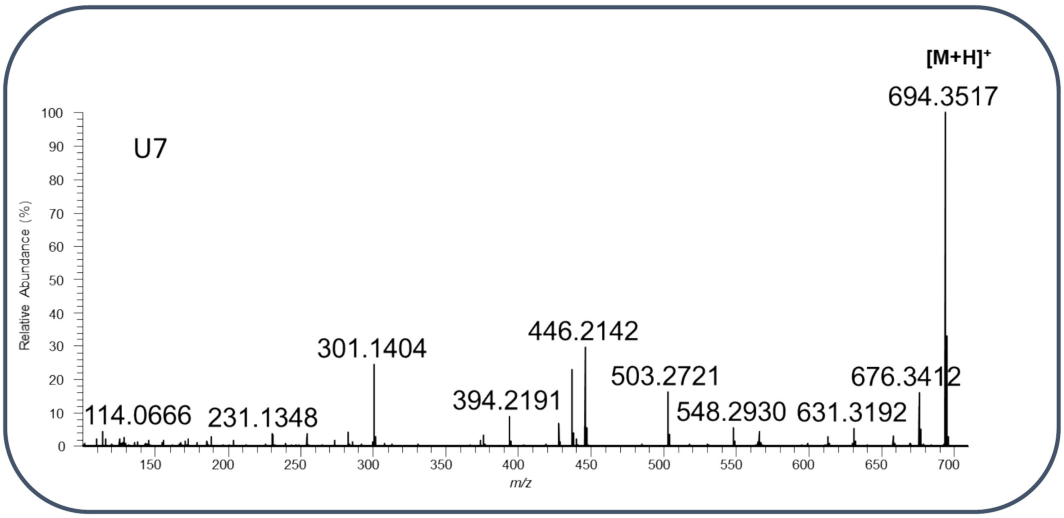

FIGURE 1 | Product ion mass spectrum of $[M+H]^+$ of a urinary metabolite of semaglutide referred to as "U7".

TABLE 2 | References to new data and/or improved screening and confirmation methods regarding human sports drug testing published in 2023/2024.

	Class	Sub-group		References			
				GC/MS (/MS)	LC/MS (/MS)	GC/C/or LC/IRMS	Complementary methods & general
S1	Anabolic agents	1	Anabolic androgenic steroids	[32, 33]	[34–44]	[45–48]	[32, 49–51]
		2	Other anabolic agents	[52]	[53]		[54–61]
S2	Peptide hormones, growth factors, related substances and mimetics	1.1	Erythropoietin-receptor agonists				[62–67]
		1.2	Hypoxia-inducible factor (HIF) activating agents		[68]		[69, 70]
		2.1	Testosterone-stimulating peptides		[71]		
		2.3	Growth hormone (GH), its analogs and fragments		[72, 73]		
		2.4	GH releasing factors		[74–76]		[77]
S3	Beta-2-agonists				[78]		[79, 80]
S4	Hormone and metabolic modulators	3	Agents preventing activin receptor IIB activation				[81]
		4	Metabolic modulators			[82]	[83–86]
S5	Diuretics and masking agents				[76]		[87, 88]
S6	Stimulants					[89]	[90–92]
S9	Glucocorticoids					[93]	
M1	Manipulation of blood and blood components	1	Administration or reintroduction of any quantity of blood or blood products				[94–98]
		2	Artificially enhancing the uptake, transport or delivery of oxygen		[99]		
M2	Chemical and physical manipulation	1	Tampering				[100]
M3	Gene and cell doping	1	The use of nucleic acids or nucleic acid analogues that may alter genome sequences and/or alter gene expression				[101–104]

co-isolation and detection of an intact sulfoconjugated metabolite tentatively assigned to 17,17-dimethyl-18-nor-5α-androst-13-en-3ξ-ol was observed, complementing study results presented by Sun et al., who corroborated the 17α-methyl-5β-androstane-3α,17β-diol sulfate conjugated at the 3-OH group as promising target analyte for doping control purposes [34]. A series of sulfoconjugated stereoisomers of methyltestosterone metabolites was prepared and analyzed as reference standards or monitored in post-administration urine using LC-quadrupole/time-of-flight(Q/TOF)-MS as well as triple quadrupole (QqQ)-MS systems. Chromatographic separation was accomplished on a C-18 analytical column (2.1 × 100 mm, 1.9 μm particle size), employing 0.01% formic acid (solvent A) and acetonitrile (containing 0.01% formic acid, solvent B) for elution into the ESI source. Assessing further the metabolism of methyltestosterone, complementary chromatographic separation strategies were employed by Bredendiek and Parr, who utilized supercritical fluid chromatography (SFC) to ensure an adequate resolution of the 2α-, 2β-, 4-, and 6β-hydroxylated methyltestosterone species [49]. A cyclodextrin-based stationary phase was employed with compressed CO_2 plus modifier (methanol) as eluent, and the efflux was directed into a QqQ analyzer to determine the quantity of the target analytes in human liver microsomal preparations. Here, the predominant formation of 2β- and 6β-hydroxylated methyltestosterone was confirmed, further complementing the overall metabolic picture of the AAS in humans.

To which extent the route of drug administration is critical regarding the urinary elimination of AAS and respective metabolites has become a main subject of the exposome and anti-doping research [115]. Characterizing the share of skin cell-borne biotransformation of a transdermally administered drug is important, especially when assessing potential drug application or exposure scenarios and resulting urinary metabolite patterns. In that context, Liu et al. investigated the contribution of human skin to the metabolism of a subset of six structurally related AAS using an in vitro model [50]. The substances testosterone (T), metandienone, methyltestosterone, clostebol, dehydrochloromethyltestosterone (DHCMT), and methylclostebol were subjected to cultivated human fibroblasts and keratinocytes for 24 h, and the media were liquid–liquid extracted followed by trimethylsilylation of the target analytes and detection of steroidal compounds by GC-Q/TOFMS analysis. With the exemption of DHCMT and metandienone, all AAS underwent reduction reactions, predominantly attributed to 5α-reductase activity, representing early steps in the overall metabolic conversion sequence of AAS when administered transdermally.

3.2 | Steroid Profiling in Urine and Blood

The athlete biological passport (ABP) is a central tool of routine doping controls and includes, among others, the steroidal modules for urine and since August 2023, also for serum analysis. Urine samples of sports drug testing programs are routinely analyzed for a panel of endogenous steroids after (enzymatic) hydrolysis of the glucuronic acid conjugates of the target compounds, followed by trimethylsilylation and GC–MS/MS measurements. An alternative approach employing acetylation of the urinary steroid profile target analytes combined with SFC and ESI-MS/MS determination was presented by Langer et al. [35] Upon cleavage of glucuronides, steroidal compounds were enriched by supported liquid extraction (SLE), acetylated, and separated on a 1-aminoanthracene-modified stationary phase (3.0 × 100 mm, 1.8 μm particle size) using CO_2 as mobile phase A and 20 mM ammonium formate in methanol/water (98:2, v:v) as mobile phase B. Ionization was accomplished in positive ESI mode and analytes detected on a QqQ-based mass spectrometer in multiple reaction monitoring (MRM) mode. The assay allowed for LOQs of 1 ng/mL for testosterone (T) and epitestosterone (EpiT), 2.5 and 5 ng/mL for 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol), respectively, and 50 ng/mL for androsterone (A) and etiocholanolone (E). In comparison to GC–MS/MS data, quantitative results of the SFC-ESI-MS/MS showed a consistent overestimating bias (< 10%) but analyte ratios (as utilized in the steroidal module of the ABP) were consistent with GC–MS/MS-derived values.

Aiming at determining the intact phase-II metabolites by LC–MS/MS, Pfeffer et al. assessed the utility of methylating intact glucuronides and sulfates of T and EpiT in order to improve chromatographic and electrospray ionization properties of the analytes [36]. In a proof-of-concept study, urine samples were solid-phase extracted and, by means of trimethylsilyl diazomethane, converted into their respective methyl esters prior to separation on a C-18 analytical column (2.1 × 100 mm, 1.9 μm particle size) using 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B). Substantially improved peak shape and abundance were obtained for T glucuronide and sulfate as well as EpiT glucuronide, allowing for limits of quantification (LOQs) better than 0.5 ng/mL when using a Q/orbitrap mass analyzer operated in positive ESI and parallel reaction monitoring (PRM) mode; however, EpiT sulfate was found instable under the chosen conditions.

In contrast to the urinary steroid profile, the serum steroid profile is (to date) composed of only the two analytes T and androst-4-ene-3,17-dione (Adione; A4). Moreover, most analytical approaches presented in the past 12 months employed LC–MS/MS-based methods for monitoring T and A4, as for instance, in the study by Okano and Shiomura, who investigated the utility of the blood steroidal passport in uncovering testosterone abuse in the Asian population [37]. The analytical approach consisted of serum dilution/protein precipitation using phosphoric acid and subsequent SPE of the supernatant, and concentrated extracts were injected onto a C-18 analytical column (2.1 × 50 mm, 1.7 μm particle size) operated with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B). The effluent was introduced into a QqQ-MS by positive ESI, and target analytes were detected in MRM mode. Studying a reference population of 510 individuals (256 females, 254 males), no significant difference in serum steroid markers between different *UGT2B17* genotypes were observed, demonstrating the robustness of the serum markers against this variable, which is a major confounding factor for urinary excretion of T. Further, serum samples collected in the context of intramuscular administrations of 100 mg of T enanthate to 10 healthy female individuals (including six *del/del* subjects, three *ins/del* subjects, and one *ins/ins* subject) were analyzed, presenting a significant increase of the individual T/A4 ratio in all volunteers up to 15 days.

A variety of questions concerning the blood steroid profile analyses were to address, especially regarding the exploitation and further utilization of samples and matrices available to the anti-doping laboratories and anti-doping organizations. Here, König et al. assessed the possibility of using "ABP blood samples" (collected in EDTA tube) complement the serum analysis-based blood steroid profile of athletes [38]. Samples were prepared by simple protein precipitation (using zinc-trifluoroacetate in methanol) and injected onto a C-8 analytical column for gradient elution with mobile phase A (consisting of 95% water, 5% acetonitrile, 2 mM ammonium acetate) and mobile phase B (95% acetonitrile, 5% water, 2 mM ammonium acetate). By means of a QqQ-MS operated with positive ESI and in MRM mode, LOQs of 0.1 ng/mL and 0.2 ng/mL for T and A4 were accomplished. Using that approach, a total of 36 paired serum and "ABP blood samples" were analyzed and demonstrated a high agreement in hormone levels, irrespective of the degree of hemolysis and sample storage for up to 3 months at +4 °C, corroborating the value of "ABP blood samples" for further use in steroid profiling.

Whether or not steroid profile data from serum collected via venipuncture, capillary blood collection devices, and DBS are comparable was assessed by Mazzarino et al. [39]. The employed testing approach used protein precipitation with phosphoric acid followed by SLE, concentration, and reconstitution for liquid samples, as well as ultrasonication-supported extraction of DBS into methanol for the subsequent LC-ESI-MS/MS analysis. The instrumental setup was composed of a C-18 analytical column (2.1 × 100 mm, 1.8 μm particle size) operated with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), interfaced via positive ESI to a QqQ-based MS operated in MRM mode, and an LOQ of 0.1 ng/mL for T and A4 was accomplished for all matrices. Applied to a study monitoring seven individuals (five female, two male volunteers) for three weeks with two sampling sessions per week, no significant differences were observed in T and A4 levels measured from dried (fixed volume) blood spots and liquid matrices. An underestimation of steroid concentrations in capillary blood plasma (less than 15%) was found when compared to venous serum, and the T/A4 ratio was comparable in all matrices with a bias < 5%, demonstrating further the complementary utility of different sample types for doping controls, as also reported by Goodrum et al. [40] Here, 20 study volunteers (10 female, 10 male) were sampled weekly over a period of six weeks, and venous and capillary serum specimens were collected for T and A4 profile analyses. Samples were diluted and subjected to SLE prior to LC–MS/MS analysis. Analyte separation was accomplished on a C-18 analytical column (2.1 × 50 mm, 1.7 μm particle size) with 0.1% formic acid and acetonitrile as mobile phases A and B, respectively, directed via ESI into a QqQ-based mass spectrometer that operated in MRM mode. The working range of the test method was 0.1–10 ng/mL, and comparable results were obtained for all paired specimens, demonstrating the possibility to use venous and capillary serum interchangeably for T and A4 in the context of blood steroid profile analyses.

The option to determine an extended steroid profile with a total of 18 target analytes from DBS was assessed by Ponzetto et al., aiming at including (among others) T, A4, dihydrotestosterone (DHT), the sulfates of dehydroepiandrosterone (DHEA), T, EpiT, A, and E, as well as the glucuronides of T, A, and E [41]. Fixed volume (30 μL) DBS devices were extracted into methanol with ultrasonication, concentrated/reconstituted, and analyzed by LC–MS/MS. The LC column contained a C-18 stationary phase (2.1 × 100 mm, 1.6 μm particle size), operated with 2 mM ammonium fluoride (solvent A) and methanol (solvent B), and analytes were ionized by ESI with polarity switching for subsequent detection in a QqQ mass spectrometer using MRM. LOQs ranged from 50 pg/mL to 20 ng/mL (depending on the analyte), and the utility and applicability of the developed assay was demonstrated by the analysis of samples obtained from two cohorts of 10 male and 10 female study volunteers. For the stability study, the samples were stored for up to 100 days at room temperature and frozen conditions, and no significant changes were observed in steroid concentrations, also when applying three freeze–thaw cycles.

Overall, the blood steroid profile appears to offer considerable added value to anti-doping programs; and yet the extent of potential confounding factors necessitates consideration. Ethanol consumption has been a well-established confounding factor of the testosterone metabolism (and thus the urinary steroid profile) and, hence, Thieme et al. assessed the effect of 0.5 mg/L blood alcohol (determined via breath analysis) on T and A4 in a study with 10 male and 10 female participants [51]. The volunteers received ethanol intravenously, and a constant blood alcohol level was maintained for 120 min, within which blood samples were collected every 10 min and analyzed for T and A4 using a previously published LC–MS/MS-based method. While (in males in particular) the determined serum T concentrations were largely unaffected by the intervention, an ethanol-dependent and significant increase of the T/A4 ratio (mostly attributable to a decrease in A4) was observed in 14 out of 18 study participants, with up to +303% in the male and +385% in the female cohort, indicating that, in line with observations in urine, also serum steroid profile measurements are susceptible to alteration by alcohol (and potentially food intake in general).

Further to analyzing serum and DBS for T, A4, and potentially other steroidal markers, the determination of testosterone, nandrolone, and boldenone esters has received considerable attention. Martinez-Brito et al. presented an approach for the detection of 13 T esters plus quantification of (among others) T and A4 from 100 μL of serum using GC-Q/TOF MS. [33] A volume of 100 μL of serum was subjected to protein precipitation with acetonitrile, the supernatant was concentrated and liquid–liquid extracted, and the obtained target analytes were finally trimethlysilylated for injection into the GC-Q/TOF instrument. A dimethylpolysiloxane capillary column (17 m, 0.2 mm inner diameter, 0.11 μm film thickness) was used to separate the target analytes, which were ionized with low-energy EI at 15 eV and detected in full scan mode at a resolving power of 30,000, enabling LODs for the T esters between 0.1 and 0.5 ng/mL and LOQs for T and A4 of 0.3 ng/mL and 0.2 ng/mL, respectively. In a proof-of-concept study, Langer et al. highlighted the complementary utility of such serum analyses for T esters, in this case T undecanoate, as determined by LC–MS/MS. [42] Employing a two-step sample preparation, 200 μL of serum were first pre-purified by SLE for serum profile analysis, and remaining volumes were subsequently concentrated and derivatized with the Girard T

reagent for T undecanoate analysis. Chromatographic conditions differed in both scenarios using either a C-18 or a C-8 analytical column (in both cases with 2.1 × 100 mm, 1.7 μm particle size) for unconjugated T, A4, and DHT, and derivatized T undecanoate, respectively. Solvent A was 0.1% formic acid for both analyses, while solvent B was either acetonitrile or methanol, both containing 0.1% formic acid. Samples collected from 19 study volunteers who received two oral doses of 80 mg of T undecanoate were analyzed, and the intact ester was detected up to 24 h post-administration, offering an alternative to prove the use of a prohibited T formulation without the need of IRMS measurements. However, it was noted that IRMS analyses appeared to allow for slightly longer detection windows regarding the use of the T ester in urine, underlining the importance of combining different matrices for most effective sports drug testing strategies.

Using DBS, Yan et al. established a test method covering a total of 22 steroidal esters (nine testosterone, six nandrolone, and five boldenone esters), measured on a LC-Q/orbitrap system [43]. Spots of 20 μL of dried blood were incubated in sodium carbonate buffer and methanol, liquid–liquid extracted into ether, and the obtained organic layer was concentrated to dryness before derivatization of the analytes with methoxylamine. The resulting solution was injected onto an analytical column of 2.1 × 100 mm (1.9 μm particle size), and gradient elution with 10 mM ammonium formate (containing 0.1% formic acid, solvent A) and methanol (containing 0.1% formic acid, solvent B) was conducted. For all analytes, targeted as steroid oxime derivatives, E/Z isomers were characterized with UV spectroscopy and computational analysis using density functional theory calculations, were detected by PRM at LODs of 0.3 ng/mL for T esters, 0.3–0.6 ng/mL for nandrolone esters, and 0.6–1 ng/mL for boldenone esters. Similarly, Miyamoto et al. presented a comprehensive DBS testing procedure for a total of 28 steroid esters including 13 testosterone, nine nandrolone, and six boldenone esters, employing an LC-ESI-QqQ-based analytical strategy [44]. DBS were extracted twice into a mixture of *tert*-butyl methyl ether/methanol/2-propanol supported by ultrasonication, the extracts were combined and concentrated and eventually derivatized with 100 mM methoxylamine. Using a C-8 analytical column (2.1 × 100 mm, 1.7 μm particle size), target analytes were separated with 0.1% formic acid (solvent A) and acetonitrile (solvent B), and detected using positive ESI and MRM. Testosterone esters were detected at LODs at 0.1–0.4 ng/mL, and nandrolone and boldenone esters at 0.4–0.9 ng/mL, and testosterone enanthate was detected in post-administration DBS samples (100 mg injected intramuscularly) in seven male study volunteers for up to 216 h (9 days).

The importance of being able to complement IRMS testing approaches to differentiate natural endogenous steroid biosynthesis from synthetic exogenous steroid administrations has been stressed repeatedly in the past, especially in consideration of the apparently growing availability of steroid preparations featuring human endogenous carbon isotope ratios. As exemplified by Polet et al. concerning nandrolone [45], the prevalence of such preparations has apparently increased, which necessitates consideration and addressing for routine doping control applications. While carbon isotope ratio analyses of steroids are, most commonly, done using gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) instruments, the advent of LC-IRMS systems could complement the analytical options in the future as presented by Honesova et al. After identifying suitable LC columns capable of satisfyingly separating steroidal target analytes under high temperature chromatographic conditions [46], an assay allowing to determine the $\delta^{13}C$ values of T, 5αAdiol, 5βAdiol, and pregnanetriol was established [47]. Using 25 mL of urine and consecutive SPE, enzymatic hydrolysis, LLE, and acetylation, the four target analytes were prepurified by off-line LC-based fraction collection. For improved chromatographic properties under exclusively aqueous high-temperature LC conditions, the acetylation of the analytes was reversed by chemical hydrolysis and the analytes were once more solid-phase extracted prior to LC-HRMS analysis. High-temperature LC was done on a titanium dioxide stationary phase (4.6 × 20 mm, 3 μm particle size) using a temperature gradient for the elution solvent (water) from 50–200 °C, and the eluent was directed into an oxidation reactor interfaced to the sector field IRMS system. LODs of 10 ng/mL for T, 5αAdiol, 5βAdiol, and 50 ng/mL for pregnanetriol were accomplished, enabling the use of this complementary approach for authentic routine doping control samples.

Focusing on less common target analytes with steroid profile and IRMS measurements, Piper et al. investigated the use of 11-oxo-testosterone (11-oxo-T) as target analyte for detecting the abuse of adrenosterone (androst-4-ene-3,11,17-trione) [48]. In the context of an elimination study with an oral administration of 100 mg of adrenosterone, urine samples were collected prior to and up to 48 h post drug ingestion and subjected to a steroid profile screening procedure including 11-oxo-T. In addition, doping control urine samples of 3647 male and 1558 female athletes were analyzed using the same analytical approach and a tentative trigger value of 130 ng/mL was established for 11-oxo-T, initiating follow-up IRMS analyses. Here, monitoring the Δδ-value of pregnanediol and 11-oxo-etiocholanolone was suggested to be of superior retrospectivity, allowing to detect the exogenous nature of the metabolite's precursor for up to 33 h.

3.3 | Other Anabolic Agents

Other anabolic agents accounted for a total of 151 AAFs in 2022 [114], including 47 occurrences of clenbuterol and 104 findings of selective androgen receptor modulators (SARMs) such as enobosarm (49 occurrences), LGD-4033 (36 occurrences), RAD-140 (9 occurrences), andarine (five occurrences) and S-23 (5 occurrences) [114], which is a considerable overall increase compared to the 2021 statistics, where 106 AAFs for this class of prohibited substances were recorded. Of note, a very similar composition of SARMs findings with LGD-4033, RAD-140, enobosarm, and S-23 (plus YK-11) was observed in a study that included 651 Swedish individuals, where urine samples of participants that are not subject to organized sports drug testing programs but enrolled in a healthcare survey testing were analyzed [116]. The particularly easy access to and availability of SARMs e.g. via internet-based suppliers has contributed to an apparently increasing prevalence of these anabolic agents (e.g. in dietary supplements), and testing strategies for biological samples as

well as confiscated materials besides routine doping controls are to be addressed. Alternatives to conventional LC–MS/MS approaches for fast and predominantly qualitative analyses of drugs have been sought and found in ambient ionization mass spectrometric methods as reported by Nemeskalova et al. [54] Hyphenating either desorption atmospheric pressure photoionization or dielectric barrier discharge ionization to Q/TOF and Q/orbitrap mass analyzers, a fast and reliable identification of drugs such as LGD-4033, RAD-140, and enobosarm was shown to be feasible when amounts exceeded 1 mg per capsule. While being inferior regarding throughput, analyzing SARM products (and doping control urine samples) for analytes such as enobosarm by improved chromatographic systems offers additional information, e.g. on their isomeric composition as presented by Krug and Thevis [53]. Products obtained through internet-based suppliers and routine doping control urine samples found to contain enobosarm were subjected to chiral chromatography using a CHIROBIOTIC T2 chiral analytical column (4.6 × 250 mm, 5 μm particle size), operated under isocratic conditions with 46% of 0.1% aqueous formic acid (solvent A) and 54% methanol (containing 10 mM ammonium formate, solvent B). With negative ESI, the target analytes (i.e. *S*- and *R*-enobosarm) were detected using Q/TOF-MS/MS, and in all cases of enobosarm findings (both, urine and obtained products), exclusively the pharmacologically more active *S*-enantiomer was identified.

There have been substantial advances in testing approaches for (the continuously growing variety of) SARMs in routine doping controls as summarized by Sliz and Mikus [117]. Studies confirming the selection of particularly long-lasting metabolites [118] and/or defining today's technological standards and possibilities regarding accomplishable analytical sensitivity [119] were published, emphasizing and underscoring both the considerable analytical retrospectivity of doping controls as well as the need to take unwitting exposure scenarios of athletes into consideration when AAFs are documented. Routine sports drug testing assays capable of detecting low (if not sub-) pg/mL levels of enobosarm in human urine were reported, and with the proven occurrence of enobosarm in oral fluid (at ng/mL concentrations) [55] and on personal items [56] or household utensils of "users" [57], the consideration of cross-contamination scenarios and supporting evidence [58] appears warranted, if the plausibility of the argued order of events and their compatibility with the reported AAF are given.

Further data on the characterization of phase-I metabolites of LGD-4033 (Ligandrol) and complementary detection strategies were presented by Angelis et al. [59] Besides the previously reported and most commonly employed long-term metabolite ((4R,5R)-4-((4-cyano-3-(trifluoromethyl)phenyl)amino)-6,6,6-trifluoro-5-hydroxyhexanoic acid, Figure 2a), the pyrrolidinone-like product 4-((R)-2-oxo-5-((R)-2,2,2-trifluoro-1-hydroxyethyl)pyrrolidin-1-yl)-2-(trifluoromethyl)benzonitrile (Figure 2b) was confirmed as a robust metabolite via chemical synthesis and analysis of post-administration urine samples, offering comparable detection windows and compatibility with GC–MS/MS procedures. Enzymatically hydrolyzed urine samples were liquid–liquid extracted, concentrated and trimethylsilylated prior to injection into a GC-QqQ-MS system. Chromatography was done on a 5% phenyl-polysilphenylene siloxane capillary column (30 m, 0.25 mm inner diameter, 0.1 μm film thickness), and diagnostic precursor/product ion pairs were monitored using the MRM mode, enabling a limit of identification (LOI) of 0.25 ng/mL.

Also, the metabolism of the yet rarely detected SARM ACP-105 (Figure 2c) was revisited by Broberg et al. [60], yielding a total of 18 in vitro-derived metabolic products. Using human urine collected in the context of a microdose administration study (100 μg, orally administered ACP-105), seven metabolites were described, with those being monohydroxylated and glucuronic acid-conjugated representing the most abundant and therefore most promising target analytes for routine doping controls. While tentatively confirmed using LC-HRMS/MS data, concise structures of these analytes are still to be verified and metabolite patterns monitored over a prolonged period of time are warranted.

Sobolevsky et al. reported on the first AAF concerning the steroidal SARM YK-11 (Figure 2d), employing previously identified and corroborated urinary metabolites [61]. Using a dimethylpolysiloxane capillary column (17 m, 0.2 mm inner diameter, 0.11 μm film thickness) and a GC-EI-QqQ mass spectrometer, 19-nor-5β-pregnane-3α,17β,20-triol and 19-nor-5β-pregnane-3α,17β-diol-20-one were detected. Therefore, urine samples were first solid-phase extracted (cation-exchange SPE), the retained material was eluted and subjected to enzymatic hydrolysis, followed by anion-exchange SPE and finally trimethylsilylation, confirming the capability of routine ITPs to flag suspicious doping control samples and the unequivocal confirmation of diagnostic metabolites in human urine.

In order to prepare anti-doping laboratories for implementing the steroidal SARM S42 (Figure 2e), for which no information on human or animal metabolic profiles exist today, into future sports drug testing assays, Wen et al. conducted an in-depth characterization of the compound's mass spectrometric behavior under EI conditions [52]. The molecule was selectively deuterated at C-17 and C-21 as well as C-1–C-3 and C-6/C-7, and EI-induced dissociation pathways were elucidated for both the underivatized and the trimethylsilylated species of S42, in consideration of the commonly applied analytical strategies used in routine doping control laboratories. Central fragment ions attributable to the steroid core structure (such as *m/z* 211) and dissociation routes of the underivatized analyte were characterized, and also E/Z isomeric structures resulting from the trimethylsilylation of the C-20 oxo-function were assigned, facilitating future investigations of metabolite compositions.

4 | Peptide Hormones, Growth Factors, Related Substances, and Mimetics

4.1 | Erythropoietin-Receptor Agonists and Hypoxia-Inducible Factor (HIF) Activating Agents

In the context of establishing complementary detection methods for the use of erythropoietin-receptor agonists (ERAs), the trend towards utilizing and exploiting capillary-derived blood (either serum or DBS) has also been recognized, with a variety of studies highlighting the competitiveness or even superiority of

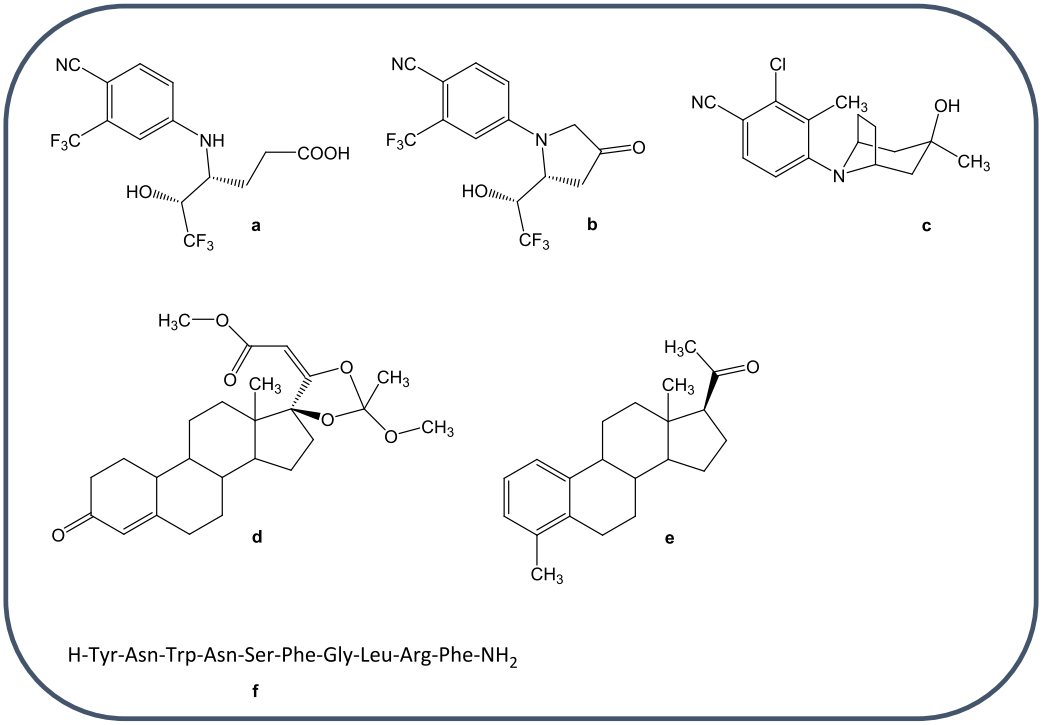

FIGURE 2 | Structure formulae of the established main LGD-4033 metabolite (4R,5R)-4-((4-cyano-3-(trifluoromethyl)phenyl)amino)-6,6,6-trifluoro-5-hydroxyhexanoic acid (a, mol wt = 370.08 u), a new LGD-4033 metabolite 4-((R)-2-oxo-5-((R)-2,2,2-trifluoro-1-hydroxyethyl)pyrrolidin-1-yl)-2-(trifluoromethyl)benzonitrile (b, mol wt = 352.06 u), the SARMs ACP-105 (c, mol wt = 290.12 u), YK-11 (d, mol wt = 430.24 u), and S42 (e, mol wt = 296.21 u), and the testosterone-stimulating peptide kisspeptin (f, mol wt = 1301.63 u).

alternative specimens, especially with the perspective of testing at remote locations. For instance, Miller et al. reported on an administration study with epoetin alpha (single dose of 40 IU/kg, 12 volunteers), where volunteers provided venous and capillary serum as well as urine samples once daily for up to 7 days [62]. Despite major differences in available volumes, the detectability of the recombinant erythropoietin (rEPO) was identical across the tested matrices with at least 168 h post-dosing. In order to mimic different sample delivery/pre-analytical conditions, urine samples were stressed under different storage conditions (one and two weeks at 4 °C, one and two weeks at room temperature, three days at 37 °C) where, in total, eight out of 12 samples showed degradation of EPO when stored at 37 °C (three days) and six out of 12 specimens when stored at room temperature up to 2 weeks, while serum samples were not subjected to stability studies.

Heiland et al. assessed the traceability of an epoetin theta microdose administration (15 IU/kg, five volunteers) in urine and DBS, which were collected up to 72 h after subcutaneous injection of the rEPO [63]. Of note, DBS were found to be of superior sensitivity compared to urine at 1 h and 72 h post-administration, where 40% (vs. 0%) and 60% (vs. 40%) returned suspicious ITP results, using (collection device-specific) either two DBS of 20 μL each or one DBS of 50 μL. In a similar fashion, an administration study of a single epoetin alpha microdose (10 IU/kg, two volunteers) was conducted and venous blood that was used to prepare DBS afterwards was collected up to 72 h post-s.c. injection. In addition, capillary blood DBS were collected in an in-competition setting from 111 athletes, 34 of which also provided matching urine samples, and five DBS from c.577del EPO gene variant carrying individuals were acquired [64]. Using two DBS (i.e. 40 μL of blood), rEPO was detectable up to 72 h and endogenous human EPO was found in all 111 athletes' DBS samples. Conversely, only 62% of the concurrently collected urine samples were found to contain measurable EPO, which is in part attributable to urinary instability of EPO as discussed above. DBS further facilitated the identification of c.577del EPO gene variant carriers by the detection of two separated blood EPO species, indicating the heterozygous genotype of the tested individual.

An alternative approach towards corroborating the presence of a c.577del EPO gene was presented by Leuenberger et al., who quantified the recovered DNA content of 191 routine doping control urine samples as well as 20 urine (4 mL aliquots) and DBS (20 μL) collected from study volunteers, and further subjected the DNA extracts to Sanger sequencing [65]. With a DNA extract concentration of 0.024 ng/μL or higher, the c.577del EPO genotype was conclusively confirmed in 80% of all samples, and these concentrations were accomplished for 85% of all tested routine doping control urine samples when preparing 4 mL aliquots. In addition, all DBS, which allowed for median DNA extract concentrations of 6000–10,800 ng/μL, were successfully sequenced, confirming DBS as a useful source of material to probe for an athlete's EPO genotype for sports drug testing purposes.

Besides methods that aim at the direct detection of prohibited ERAs in routine doping control samples, indirect ABP-based approaches such as the hematological module of the ABP have demonstrated their utility for several years. To which extent the use of biomarkers selected from the plethora of RNA species can support the anti-doping efforts, especially with regard to the

abuse of ERAs (or blood doping in general) in sport, was recently reviewed by Loria et al. [66] Also, and more specifically, in a comprehensive intervention study the utility of a passport-like monitoring of the two mRNA biomarkers 5′-aminolevulinate synthase 2 (*ALAS2*) and carbonic anhydrase 1 (*CA1*) in differentiating ERA abuse and altitude exposure was assessed [67]. In two cohorts, DBS were collected weekly prior to, during, and after a 4-week period at sea level or high altitude (2320 m). During the 4-week period, participants received either epoetin alpha at 20 IU/kg i.v. every other day for three weeks (11 injections) or a placebo injection accordingly. DBS were then extracted into lysis buffer, and purified and extracted into RNAse-free water for subsequent reverse transcription quantitative polymerase chain reaction (RT-qPCR). The obtained results suggest that the selected mRNA biomarkers are particularly sensitive to epoetin administrations as indicated by the analyses of samples collected in the course of the rEPO microdose intervention, and altitude (as a confounding factor) had little effect on the longitudinal data evaluation and interpretation. However, peak readouts of the biomarkers' curves were found predominantly when the athletes were receiving the drug and such high frequency of DBS sample collection is still rarely available in authentic doping control settings. Therefore, to assess the full potential of these complementary parameters, more data and studies are required.

In the processes underlying the physiological response of enhanced erythropoiesis to environmental conditions such as reduced partial pressure of oxygen (e.g. at high altitude), hypoxia-inducible (transcription) factors (HIFs) play a central role and have thus been identified, exploited, and debated as drug targets for anti-anemia therapeutics in the past [120, 121]. A series of drug candidates and approved HIF stabilizing (or activating) therapeutics have been developed, and also AAFs were investigated, documented, and arbitrated in the context of routine doping controls [122, 123], highlighting the relevance of test methods that allow for comprehensively and sensitively detecting HIF activating agents in sports drug testing programs. Employing a cell-based in vitro bioassay, Janssens et al. presented proof-of-principle data for an improved untargeted urine analytical approach, designed for the detection of substances facilitating the formation (and stabilization) of a heterodimeric HIF complex [69]. Subunits of the split-nanoluciferase were coupled to HIF1α/2α and HIF1β, respectively, and in the presence of HIF-activating agents (e.g. roxadustat), the subunits are positioned and maintained in close proximity, enabling the nanoluciferase to restore measurable activity. Applied to spiked and liquid–liquid-extracted urine, the presence of roxadustat was robustly determined, albeit at urinary concentrations that do not yet allow considering the assay as fit-for-purpose with regard to required minimum required performance levels (MRPLs) [124]. Nevertheless, further refinement of the technology could add fast and comprehensive screening options in the future as doping control specimens containing HIFs (or their metabolites) that are not explicitly included in routine ITPs could be flagged for follow-up investigations.

In an opposite manner, i.e. targeting a specific HIF activator with utmost sensitivity, Sobolevsky et al. provided information on an unexpectedly long elimination and traceability of roxadustat in human urine [68]. Patients who received roxadustat in cumulative dosages between 840 and 11,900 mg over a period of 28–185 days, and whose washout periods ranged from 184 to 540 days, provided spot urine samples for research purposes. In six of seven patients, roxadustat was detected between 2 and 50 pg/mL when preparing their latest urine sample (i.e. collected 184–540 days after drug cessation) by enzymatic hydrolysis followed by weak cation-exchange SPE, and subsequently measuring the extract by LC–MS/MS analysis. A C-18 analytical column (2.1 × 100 mm, 2.7 μm particle size) operated with 0.1% formic acid (solvent A) and methanol (solvent B) was used, and detection of the target analyte was accomplished in positive ESI MRM on a QqQ-based instrument. Although compounds in group S2. are always prohibited, the knowledge of such detection windows can be decisive in case management and result interpretation, underscoring once more the importance of continued research into drug elimination kinetics and constantly optimizing testing strategies in anti-doping. Such optimizations also include revisiting metabolic pathways as presented for the HIF activator IOX2 by Taoussi et al., who corroborated the suitability of established target analytes (i.e. the glucuronic acid conjugate of IOX2 and its quinolinone-hydroxylated phase-I metabolite) for routine doping controls by in silico and in vitro metabolism studies [70].

4.2 | Peptide Hormones and Their Releasing Factors

Detecting the abuse of growth hormone (GH) in sports relies on a dedicated isoform differential immunoassay, which employs both permissive and highly specific monoclonal antibodies to allow for calculating ratios between the predominant GH isoform of 22 kDa and other variants. The long-acting GH referred to as somatrogon, which is a fusion protein of the 22 kDa human GH and the C-terminal peptide of human chorionic gonadotropin (hCG) β-subunit at the N- and C-terminus of the GH molecule, became commercially available in 2022. The structural modifications of GH in somatrogron were found to prolong the serum half-life of the drug and, at the same time, hamper the utility of the isoform differential immunoassay in confirming the presence of the GH analog, which is why a mass spectrometric testing approach was developed by Walpurgis et al. [72] In order to enrich the target analyte from serum, samples were subjected to the recombinantly produced GH receptor coupled to magnetic beads, allowing to concentrate somatrogon for subsequent tryptic digestion and mass spectrometric analysis. Diagnostic glycosylated peptides attributable exclusively to the fusion protein composed of hCG and 22 kDa GH were characterized and specifically determined on a LC-ESI-Q/orbitrap MS system. The LC consisted of a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size), operated with 0.1% formic acid (containing 1% dimethylsulfoxide (DMSO), solvent A) and acetonitrile (containing 0.1% formic acid and 1% DMSO, solvent B). The mass spectrometric detection was accomplished in positive ionization mode and targeted single ion monitoring (tSIM) as well as MS/MS experiments, allowing for an LOD of 10 ng/mL for three proteotypical peptides. The assay was applied to a series of post-administration samples collected from healthy individuals who received a single dose of s.c. injected somatrogon 0.66 mg/kg, and the drug was traceable for 96 h. With one kit

of the isoform differential immunoassay capable of indicating the presence of somatrogon in human serum, the MS-based method provides a confirmatory procedure for the unequivocal identification of the prohibited substance in doping control samples.

The option of employing MS-based testing methods also for the detection of recombinant GH administrations was revisited by Krombholz et al., who combined a top-down (intact) analysis of the 22 kDa GH with a bottom-up (digest) analysis of a combined fraction of GH isoforms [73]. Serum GH was enriched by means of immunopurification using a polyclonal GH antibody, and one aliquot of the isolated isoforms was subjected immediately to LC-HRMS(/MS) analysis while a second aliquot was trypsinized and then measured by LC-HRMS(/MS) with fully ^{15}N-labeled 22 kDa GH as internal standard. The analytical setup was identical to the aforementioned somatrogon test method (with the exception of solvent B containing 2% DMSO), and LODs of 0.5 ng/mL and 0.1 ng/mL were obtained for the intact GH and the "total GH" as determined via the common T1 peptide, respectively. In addition, 20 kDa GH was individually determined by a proteotypical peptide with an LOD of 0.2 ng/mL. Proof-of-concept samples of patients with acromegaly, healthy individuals, and serum samples collected 4 h after the last dose of a 17-day intervention study (1.7 mg/day, s.c. injection every 2nd day) were analyzed, demonstrating the capability of the assay to determine ratios of GH species in most of the specimens but also an inferior performance in differentiating natural GH isoform distributions from doping scenarios when compared to the established isoform differential immunoassay.

Besides GH itself, its releasing factors and secretagogues continued to receive considerable attention in the context of (pre)clinical studies [125] as well as anti-doping efforts. Cristea et al. reported on a test method tailored to detect four synthetic GH releasing hormones (GHRHs) including tesamorelin, CJC-1295, sermorelin (1–29), and sermorelin (3–29), plus the endogenous GHRH somatorelin in human urine for doping control purposes [74]. Spiked urine samples (fortified with acetylated and D-arginine-modified sermorelin (1–29) as internal standard) were prepared by weak cation-exchange SPE, and LC–MS/MS analysis was conducted using a C-18 stationary phase (3.0 × 50 mm, 2.7 μm particle size), eluents composed of 0.1% formic acid (solvent A, containing 5 mM ammonium formate) and 90% acetonitrile (containing 0.1% formic acid and 5 mM ammonium formate), and a QqQ-based mass spectrometer operated in positive ESI and MRM mode. The straight-forward analytical setup allowed for analyte recoveries between 81 and 96%, LODs of 0.2 ng/mL for all target analytes, and sample extract stability was demonstrated for up to 24 h.

Using a similar SPE-based sample preparation but employing a mixed-mode anion-exchange resin and five different internal standards, Thomas et al. presented a comprehensive ITP method covering 23 peptidic analytes, including 20 intact compounds and three known metabolites [75]. The assay covered six GHRHs, eight insulins, three IGF-1 analogs, gonadorelin, synacthen, and two mechano growth factors, recovered from urine at rates between 11 and 86% and detected with LODs from 12.5 pg/mL to 500 pg/mL, all of which met applicable WADA requirements for routine doping control ITPs. Sample extracts were injected onto an LC system equipped with a phenyl-hexyl online trapping column (3.0 × 10 mm, 2.7 μm particle size) and a C-18 analytical column (3.0 × 50 mm, 2.7 μm particle size, using 0.1% formic acid (with 1% DMSO, solvent A) and acetonitrile (containing 0.1% formic acid and 1% DMSO, solvent B) as eluents. The effluent was electrosprayed in positive mode into a Q/orbitrap instrument, and analytes were monitored using tSIM and data-dependent MS/MS experiments. Also here, a sample extract stability for 24 h was confirmed. Further, the method was successfully applied to post-administration urine samples obtained from an insulin-dependent diabetic (using NovoLog® insulin aspart) as well as controlled elimination studies with Humalog® (insulin lispro) and luteinizing hormone releasing hormone (LHRH), confirming the principle applicability of the assay for sports drug testing purposes. However, in the absence of analytical data on elimination study urine samples with e.g. GHRHs, it remains to be clarified though if the selection of target analytes (sermorelin 1–11, 13–20, and 22–29 were shown to be stable in human serum [77]) and/or the sample matrix (i.e. urine vs. serum) might require additional consideration.

Focusing on lower molecular mass peptide drugs and their metabolites, Liu et al. reported on the development of an analytical procedure covering 63 measurands, including various growth hormone releasing peptides (GHRPs), growth hormone secretagogues (GHS), LHRH analogs, and other compounds of the WADA Prohibited List and Monitoring Program [76]. Various factors potentially affecting analyte recovery, matrix effects, ionization efficiency, etc. were assessed and optimized, yielding a sample preparation protocol based on weak cation-exchange SPE and an instrumental setup using LC-HRMS/MS with full scan and PRM data acquisition. Chromatographic separation of the target analytes was accomplished on a C-18 analytical column (2.1 × 100 mm, 1.9 μm particle size) with 10 mM ammonium formate (in 0.05% formic acid, solvent A) and methanol (solvent B) and, following positive ESI, MS data were recorded using a Q/orbitrap system. The highly multiplexed approach allowed for recoveries between 1 and 105% and LODs ranging from ca. 10 to 500 pg/mL, meeting all relevant requirements of applicable technical documents.

A recent addition to the section S2.2.1 of the WADA Prohibited List [19] are kisspeptin and its agonist analogs, a class of considerable clinical research interest (both as biomarker [126] as well as drug candidates [127–129]) which have not been considered in the above-referenced multitarget test methods but for which Colpaert et al. presented data on a test method allowing for the detection of kisspeptin-10 (Figure 2f) in human urine [71]. By means of urine dilution and injection into an LC-HRMS/MS system, the analyte was detectable at an LOD of 95 pg/mL, and in vitro-derived degradation products produced by incubation of the peptidic substrate in human serum for up to 120 h were also observed. These potential metabolites were attributed to N-terminal truncations by 1, 2, 3, and 5 amino acid residues. Chromatography was conducted on a C-8 analytical column (2.1 × 50 mm, 1.8 μm particle size) using water (A) and acetonitrile (B) (both containing 0.2% formic

acid and 1% DMSO) as eluents, and the MS system was a Q/orbitrap instrument.

5 | β_2-Agonists, Hormone and Metabolic Modulators, and Diuretics

5.1 | β_2-Agonists

With the growing body of evidence that β_2-agonists can exert performance-enhancing effects and the, albeit restricted, permission of use for selected representatives of this drug class [130], research into differentiating routes of administration or determining further details of elimination kinetics has been conducted. In this context, Harps et al. presented administration study data of interventions with single dose applications of inhaled racemic salbutamol (600 μg), inhaled levosalbutamol (630 μg), peroral racemic salbutamol (2 mg), and peroral levosalbutamol (1 mg) [78]. Using a chiral teicoplanin-based stationary phase (4.6 × 150 mm, 2.7 μm particle size) and 20 mM ammonium formate in methanol for isocratic elution, (R)- and (S)-enantiomers of salbutamol and its 4′-O-sulfate were separated and quantified using a QqQ-based MS system operated in polarity-switching MRM mode. The results demonstrated a predominant sulfoconjugation of the salbutamol (R)-enantiomer, and future studies might show whether the obtained information will aid in differentiating therapeutic use from doping scenarios.

With regard to the naturally occurring β_2-agonist higenamine, the presence and variability of concentrations in traditional Chinese medicines and herbs was corroborated by Du et al. [79] as well as Lin and Hsu [80]. Maximum levels of higenamine of up to 2.1 mg/g were observed in *Nelumbo nucifera* materials, and the authors called for awareness of risks associated with the use of such products. A "doping scenario" was mimicked in a study conducted by Stojanovic et al., where healthy volunteers received daily doses of 3 × 20 mg of higenamine over a period of three weeks [131]. Urine was collected before, during, and after the drug administration phase, demonstrating that the WADA minimum reporting level (MRL) for higenamine of 10 ng/mL was exceeded at every sampling timepoint during the intervention period and for at least 20 h after the last dose.

5.2 | Hormone and Metabolic Modulators

The category S4 of the WADA Prohibited List is composed of diverse drug classes ranging from aromatase inhibitors and antiestrogenic substance via agents preventing activin receptor IIB activation to four sub-categories of metabolic modulators.

Myostatin (or growth differential factor 8) exerts negative regulatory effects on skeletal muscle mass [132], which is why it has received growing attention both in terms of identifying genetic variants that are possibly contributive to elite athletes' potentials [133] as well as sports drug testing programs. Here, in a pilot study, Donati et al. assessed the option of detecting myostatin inhibition through monitoring a series of myokine-based biomarkers [81]. Musclin, follistatin-like 1, and oncostatin were found to cluster and correlate to myostatin, potentially offering a complementary approach to the direct detection approaches for the ever-increasing number of myostatin-inhibiting agents.

For 5-amino-4-imidazole carboxamide ribonucleoside (AICAR), an activator of the AMP-activated protein kinase (AMPK), Wang et al. established a confirmatory test method using two-dimensional liquid chromatography-based fractionation, followed by trimethylsilylation of the target analyte (while the two endogenous reference compounds (ERCs) 11-OH-androsterone and pregnanediol were not derivatized) prior to GC/C/IRMS analysis [82]. The combined use of a phenyl-based stationary phase (1st dimension) and a reverse-phase amino stationary phase (2nd dimension, both 4.6 × 250 mm, 5 μm particle size), operated with water and acetonitrile as solvents A and B, respectively, allowed for fraction collections suitable for carbon isotope signature determinations. Therefore, a 5% phenylmethylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 μm film thickness) was used, separating the target analytes from biological interferences before introduction into the combustion interface and the magnetic sector MS. From a total of eight individuals, endogenous AICAR $\delta^{13}C$ mean values of −21.1 mUr were determined, while post-administration urine samples (collected after ingestion of 3 g of AICAR) resulted in considerably enriched $\delta^{13}C$ values and, consequently, a $\Delta\delta^{13}C$ of 8 mUr within 16 h after AICAR intake.

The selective estrogen receptor modulator clomifene, and substances of the subgroup of the metabolic modulators such as GW1516 and trimetazidine were subject of case-related investigations aiming at contributing to the identification of potential, purported, or proven inadvertent exposure scenarios, complementary matrix analyses (e.g. keratinous materials) as well as discussing data interpretation approaches. For instance, clomifene was shown to be traceable in hair and nails following the intake of a single oral dose of 50 mg as presented by Gheddar et al. [83], demonstrating the incorporation and detectability of the drug in all studied specimens as well as substantially variable levels (4–486 pg/mg) depending on the anatomical origin and sampling timepoint of the collected materials.

Analytical data contributing to assessing the plausibility of argued drug exposure scenarios are essential for the management of AAFs. Whether or not intimate contact, more specifically the exposure to ejaculate of a person that uses substances classified as prohibited by WADA, can *de facto* result in an adverse finding was discussed in a case report by Breuer et al. concerning the peroxisome proliferator-activated receptor (PPAR)δ agonist GW1516 [84]. Potential scenarios of cross-contamination include, among others, the introduction of semen that was transferred through unprotected intercourse to a female and, subsequently, residues of that ejaculate were introduced into a doping control urine sample. Assays to determine semenogelin, a seminal fluid-specific protein, in human urine were presented previously and applied to urine samples of two cases of AAFs, with one confirming the presence of semenogelin. The fact that GW1516 can be found at considerable concentrations in seminal fluid of users was corroborated by the analysis of 361 clinical semen samples, one of which was shown to contain GW1516 at 48 ng/mL, and assuming a doping control urine sample volume of 90 mL, as little as 37 μL would have been sufficient to cause the reported AAF, corroborating that such a scenario

cannot be generally excluded. Also, assessing if a drug transfer between the user and the athlete via other bodily fluids (such as e.g. saliva) is the more or less likely reason for a particular test result certainly warrants further studies and consideration [134]. Further, a case of unwitting exposure to the prohibited substance trimetazidine by the administration of legitimate but contaminated prescription medicine has been reported by Kintz and Gheddar [85], where ca. 16 μg of trimetazidine was found in pills used for the management of blood vessel disorders. The combined information and plausibly matching test results obtained for the athlete's urine (i.e. the doping control sample), hair (supported by controlled administration study hair sample analytical data [86]), and the contaminated therapeutic provided robust arguments for declaring no fault or negligence for the anti-doping rule violation (ADRV).

5.3 | Diuretics

Chlortalidone was the subject of elimination studies and case-related debate in the light of an increasing number of AAFs with this diuretic, where athletes argued that contamination was the reason for the respective test results. Chlortalidone was shown to exhibit a comparably slow and long-lasting elimination into urine in a study conducted by Thieme et al., who mimicked a contamination scenario by orally administering the drug at 0.2 mg/day over 5 days and collecting urine and hair samples of the study participants [87]. Urinary peak concentrations between 45 and 53 ng/mL were observed 24–48 h after the last dose, and after 10 days, still 4 ng/mL were measured. Hair specimens collected five weeks after chlortalidone ingestion were found to contain up to 4 pg/mg, which was shown to be substantially lower than what is detected under conditions of a long-term therapeutic use of chlortalidone as corroborated by the analysis of a patient's hair sample [88]. Instructions are already given by WADA on the reporting of certain diuretics, which are known to be found as contaminants of some legitimate pharmaceutical products [135], but the increasing number of incidences with chlortalidone has been of concern, especially since the source of a presumed contamination was not identified in any of the recent cases as detailed by Kintz [136]. Also, the fact that correlating the dosing regimens of diuretics (in general) with hair concentrations has been shown to be difficult has been a complication in managing ADRVs.

With regard to arginine vasopressin (AVP), Liu et al. thematized the fact that this naturally occurring peptide hormone is commonly observed in human urine, with concentrations largely ranging below 2 ng/mL as determined in more than 2000 doping control urine sample analyses [76]. Hence, a trigger level of 3 ng/mL was suggested for follow-up investigations for AVP findings.

6 | Stimulants and Glucocorticoids

6.1 | Stimulants

The class of stimulants comprises drugs with a considerable variety of pharmacophores, one of which being phenethylamine. Phenethylamine's natural occurrence in plants, animals, and humans and, thus, doping control samples necessitates doping control analytical strategies that allow for differentiating between the endogenous and exogenous nature of the analyte. In analogy to other lower molecular mass compounds such as androgens or AICAR, Piper et al. assessed the possibility of employing GC/C/IRMS to determine whether exogenous phenethylamine was administered or not [89]. Phenethylamine was isolated from urine using SPE, acetylation, and HPLC fractionation, while one of its abundant metabolites phenylacetylglutamine was directly HPLC-fractionated from urine. Interestingly, the carbon isotope ratios of endogenously produced phenethylamine (determined only from a subset of reference urine samples) were found between −28 and −31 mUr, complicating the use of this marker for doping control purposes when considering the δ [13]C values of phenethylamine standards measured between −26 and −28.5 mUr. Also, despite its significant response to phenethylamine administration, phenylacetylglutamine was not corroborated as a sensitive target compound either due to the broad distribution of its naturally occurring $\delta^{13}C$ values reaching depleted carbon isotope ratios down to −28 mUr. Hence, further research into alternative options appears necessary.

The detection of related but not naturally produced stimulants was facilitated by the synthesis of sulfo-conjuated reference materials of octopamine, norfenefrine, and etilefrine as reported by Kalita et al. [90] By selectively protecting, sulfo-conjugating, and deprotecting functional groups of the drugs, the phase-II metabolites were obtained with overall yields of 26–54% at a purity > 99%, thus qualifying them for use in sports drug testing.

In a controlled administration study conducted by Sakayori et al. with methylephedrine (150 mg) and pseudoephedrine (240 mg), the detection of the compounds in urine was only secondary [91]. Monitoring the dopamine receptor occupancy via positron emission tomography, the results suggested that pseudoephedrine reached a significantly elevated level compared to the placebo group while urinary drug concentrations remained below WADA's threshold; conversely, no significant effect of methylephedrine at the employed drug administration regimen was seen regarding the dopamine receptor occupancy, but urinary concentrations exceeded the threshold of 10 μg/mL, raising the question whether the currently enforced thresholds might warrant revision.

In addition, Lu et al. reiterated on the fact that chlorphenesin can be present as preservative in a variety of cosmetics, and a survey of products available in Taiwan was found to adhere to applicable regulations (i.e. the content was below 3 mg/g) [92]. Since it was shown to metabolize into 4-chlorphenoxyacetic acid, a marker monitored in routine doping controls to detect the misuse of meclofenoxate, concerns have been reported regarding the possibility that AAFs are the result of the use of chlorphenesin-containing products, but the concomitant screening for diagnostic chlorphenesin-specific metabolites has been shown to eliminate the issue.

6.2 | Glucocorticoids

The regulatory framework for permissive and prohibited scenarios of glucocorticoid use in sport has undergone an extensive

revision and refinement in the past, with adjusted MRLs and recommendations regarding washout periods. Prednisone and prednisolone are glucocorticoids, which have been shown to be occasionally produced by Δ1-steroid dehydrogenases from the naturally occurring cortisol, creating a situation where the differentiation of drug use from artefact formation is required. GC/C/IRMS was proven to be a useful tool for addressing this task, and Li and Liu presented an alternative sample preparation based on 2-dimensional liquid chromatography for automated and accelerated fraction collection [93]. Following SPE, enzymatic hydrolysis, and LLE, extracts were injected into an LC system operating a C-18 plus an ethyl-phenyl analytical column (both 4.6 × 250 mm, 5 μm particle size) with water and acetonitrile as mobile phases A and B, respectively, enabling the separation and fractionation of both target analytes as well as relevant ERCs (pregnanediol and 11-OH-androsterone) for subsequent GC/C/IRMS analysis. Proof-of-concept data were presented by means of urine samples collected after the oral administration of 5 mg of either prednisone or prednisolone, where $\Delta\delta^{13}C$ values were substantially greater than 4 mUr. If the artificial formation of prednisone and prednisolone would exceed the today enforced MRL of 300 and 100 ng/mL, respectively, and thus necessitating a carbon isotope ratio-based confirmation of the origin of the analytes will be shown by future routine doping control data.

7 | Manipulation of Blood and Blood Components

Developing robust and reliable means for the detection of illicit blood transfusions in sport required almost two decades since this method to enhance performance was prohibited in 1985, and comprehensive review of milestones and accomplishments particularly concerning homologous blood transfusion analyses was published by Marchand and Ericsson [137]. While a flow cytofluorimetric method that aims at identifying different populations of erythrocyte surface antigens in doping control blood samples has become the method of choice, modern analytical strategies to identify DNA from individuals other than the athlete in the respective specimen have received growing attention. In a recent exploratory study, Donati et al. assessed the sensitivity of an approach based on the genotyping of single nucleotide polymorphisms (SNP) in support of identifying the presence of DNA of different persons in an athlete's DBS sample [94]. Therefore, 36 blood samples composed of 12 single donor specimens and 24 ex vivo -mixed samples (mimicking different transfusion volumes) were prepared and deposited as DBS. These DBS were then extracted, and four selected SNPs underwent 40 cycles of PCR amplification and Taqman detection. An overall sensitivity of the approach of 79% was documented, and as little as 1% of transfused blood was shown to be traceable in 75% of the investigated specimens.

Autologous blood transfusion is primarily (if not exclusively) detected by means of the hematological module of the ABP, and the corresponding analyses are largely done from whole blood samples collected via venipuncture. The fact that this procedure could be complemented (or partially substituted) by microcapillary blood sampling devices was shown be Lewis et al., who compared the results of 58 paired blood samples collected, shipped, and analyzed under standardized test methods concerning a 16 parameter-containing complete blood count (CBC) [95]. With the exception of the platelet count, excellent agreement of laboratory test results were obtained, indicating that blood collections without requirement of venipuncture (and no phlebotomist) could be a valid alternative despite the lower volumes that are eventually available. Since the robustness of measured parameters and comparability of analysis results is essential for the applicability of the ABP approach [138], investigations into the natural physiological variability and means to correct for factors affecting selected ABP parameters have continued also in the past year. For instance, Krumm et al. determined and compared the intraindividual variability of 11 hematological biomarkers (hematocrit (HCT), hemoglobin (Hb), red blood cell count (RBC), reticulocyte percentage (Ret%), reticulocyte count, mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), mean corpuscular volume (MCV), immature reticulocyte fraction (IRF), platelet count, red cell distribution width (RDW), and white blood cell count (WBC)) plus the computed OFF score over the course of one year with 20 elite athletes and 20 moderately trained non-elite athletes as control group [96]. In addition, the plasma volume markers transferrin, creatinine, calcium, low-density lipoprotein (LDL), albumin, and total protein were measured to allow for an estimation of plasma volume variations employing a machine learning-based prediction tool trained with 769 datasets [97]. It was noted that the overall variability of biomarkers of the hematological module of the ABP was significantly higher in the elite athlete group, with the 12-months monitoring resulting in a total of 10 atypical passport findings (ATPFs) in the elite athlete population (attributable to eight individuals) and two ATPFs in the control group (two individuals). Six ATPFs (five for the elite athletes and one for the control group) were eliminated when factoring-in the plasma volume correction, supporting the notion that there is considerable added value in including plasma volume estimations into the assessment of ABP profiles.

Approaches that might potentially complement the existing ABP-based testing strategy for detecting especially autologous blood transfusions were reviewed by Hassanpour and Salybekov [139], focusing primarily on miRNA-derived biomarkers such as miRNA-26b, −30b, −30c, and −197-3p. It was noted that the window of opportunity in exploiting these markers for detecting blood doping practices exists largely within the first 24 h post-transfusion, and that increasing these markers' sensitivity for doping control purposes will necessitate a frequent and longitudinal intraindividual monitoring. In addition, additional studies characterizing factors that influence miRNAs other than blood transfusions or administrations of erythropoiesis-stimulating agents are required. With a more holistic strategy, Biasini et al. investigated the extent of erythrocyte storage lesions on the molecular level in whole blood samples [98]. Anonymized whole blood samples collected from athletes in the context of routine doping controls were enriched with buffers commonly used in blood banking practice and stored for 20 or 40 days. These as well as freshly obtained samples of the same individuals were analyzed concerning the abundances of glycophorin-A and band-3 complex (membrane proteins), peroxiredoxin-2, CD47, phosphatidylserine, and erythrocyte microparticles using immunological and flow cytofluorimetic methods. The comparison

of stored and fresh samples revealed significantly decreased levels of the membrane proteins glycophorin-A and band-3 complex as well as CD47, and concomitantly increased levels of serum phosphatidylserine and erythrocyte microparticles. Combining these markers, the identification of ex vivo -mixed blood, i.e. 90% fresh whole blood plus 10% stored whole blood (40 days), was accomplished. While a scenario where an athlete receives an erythrocyte transfusion representing 10% of the entire red blood cell population is expected to be rare, the obtained data add further options to the analytical toolbox for refining and optimizing doping control analytical approaches.

Compared to the complex challenge of detecting homologous or autologous blood transfusions, the identification of non-human hemoglobin-based oxygen carriers such as the extracellular hemoglobin of the lugworm *Arenicola marina* was shown to be readily implemented into sports drug testing programs using chromatographic-mass spectrometric methods. Walpurgis et al. reported on an approach where serum and plasma were subjected to ultrafiltration, tryptic digestion of the retentate, followed by a second ultrafiltration where the filtrate was used for subsequent LC-HRMS/MS analysis [99]. Targeting four proteotypical peptides of the lugworm globin A2 (T6) and B1 (T3 and T6) subunits as well as a linker region, limits of detection of 10 μg/mL were accomplished, which proved fit-for-purpose to detect and confirm the administration of the product to rats at 200 and 600 mg/kg for 8 h post-infusion. Chromatography was done using a C-18 analytical column (2 × 50 mm, 2.7 μm particle size) with 0.1% formic acid (solvent A) and acetonitrile (containing 0.1% formic acid, solvent B), with both eluents also containing 1% DMSO, and the detection of the peptides was accomplished after ESI in full MS and targeted MS/MS mode on a Q/orbitrap mass spectrometer.

8 | Chemical and Physical Manipulation

The most sophisticated analytical methods are of little use in uncovering doping practices if the doping control sample submitted to the anti-doping laboratory has been tampered with or was substituted. Likewise, confirming the authenticity of a doping control sample and ascertaining its origin from the intended athlete can be critical in situations where sample substitution or confusion is suspected. Here, DNA analyses have frequently been requested "after-the-fact", occasionally complicated by long storage periods or even found impossible when minimum storage periods were exceeded and samples were disposed. Here, Naumann et al. established an analytical procedure that allows for genotyping urine, blood, and DBS samples using a panel of 52 SNPs, determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry [100]. With urine being of particular interest, a method was developed requiring a volume of 12 mL to produce pelleted cells by centrifugation, which were then separated and genomic DNA was purified via spin column extraction for subsequent multiplexed PCR-based amplification of target SNPs. The reaction mixture was treated with shrimp alkaline phosphatase prior to the addition of oligomeric extension primers for single-base extension reactions (which occurs exclusively in the presence of the respective SNP-containing amplicon), and the unextended and extended primers are finally determined by MALDI-MS producing a characteristic SNP profile for the individual. With most sample preparation and analysis steps being automated, this PCR-MALDI MS-based high-throughput sample ID typing is accomplished with batch analysis times of < 11 h.

9 | Gene and Cell Doping

In the light of the considerable and rapidly evolving advances in modifying, manipulating, and influencing gene expression rates, establishing detection strategies for potential gene doping practices has, necessarily and inevitably, become a priority topic in anti-doping research. With general principles of e.g. transgene detection being in place in anti-doping laboratories, alternative and/or comprehensive complementary approaches are warranted, both in consideration of the growing list of potential target genes (presumed to positively affect athletic performance) as well as the increasing number of codon-optimized variants that might reduce or even annul the detection capacity of individual test methods [140]. At the same time, restrictions and limitations apply to non-biased genomic profiling analyses particularly for the purpose of sports drug testing [141, 142].

Sugasawa et al. established a mouse model for the assessment of detection strategies concerning administrations of transgenes via a recombinant adeno-associated virus vector (rAAV) using the example of the human *EPO* gene (rAAV9-hEPO) [101]. Following injection of the rAAV9-hEPO vector, blood samples (50 μL) were collected up to 30 days and subjected to Taqman qPCR analyses targeting two *EPO* exon-exon junctions as well as a cytomegalovirus promoter (CMVp) element, demonstrating the traceability of the transgene up to 30 days with an assay sensitivity requiring 34–104 copies of the viral genome per μL. In addition, significantly upregulated gene expression profiles were reported concerning *asparagine synthetase*, *serine hydroxymethyltransferase*, and *methylenetetrahydrofolate dehydrogenase*, and the potential of including these in future ABP monitoring programs was discussed. The necessity of robust testing approaches was underscored by a study of Naumann et al., who reported on the identification of products (advertised for performance enhancement and purchased from Internet-based suppliers) containing transgenic *EPO* DNA [102]. A test method enabling the simultaneous detection of seven transgene targets (i.e. *EPO*, *follistatin*, *GH1*, *myostatin propeptide*, *IGF1*, and the *vascular endothelial growth factors A* and *D*) was developed, employing a multiplexed PCR-based amplification of at least two exon-exon junction regions of each transgene. Amplicons were used for single-base extension reactions, and the detection of single-base extended primers as determined by MALDI-MS confirmed the presence of a transgene, as in the present case shown for *EPO*. Exploiting the specificity of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) protein-based reactions, Zheng et al. presented a fast and multiplexed testing device established for detecting six transgenes (i.e. *vascular endothelial growth factor A, HIF1, EPO, GH1, IGF1*, and *alpha-actinin3*) in whole blood [103]. In an eight-channel microfluidic chip, six channels were dedicated to one transgene each (plus two negative controls), where recombinase polymerase amplification (RPA) and CRISPR RNA (crRNA) designs specifically covering the target transgenes' exon-exon junctions were allowed to operate consecutively. In combination with Cas12a

Info Box

S0
- Specific target analytes for the newly listed troponin activators reldesemtiv and tirasemtiv were identified in vitro and in vivo, facilitating an implementation into routine initial testing options.
- Metabolites of BPC-157 determined by in vitro experiments confirmed previous structures as potential target compounds

S1
- New phase-I and -II metabolic products of methyltestosterone (e.g. 17,17-dimethyl-18-nor-5β-androst-13-en-3α-ol and sulfo-conjugated 17,17-dimethyl-18-nor-5α-androst-13-en-3z-ol) were reported as additional urinary markers for detecting its abuse
- Supercritical fluid chromatography (SFC) was shown to offer competitive and, occasionally, superior resolution for AAS, both for detecting xenobiotics as well as for determining urinary steroid profiles
- The serum steroid profile contributes critical information in support of detecting the abuse of testosterone (and its prohormones), and markers of the serum profile can reliably be measured also from EDTA plasma, capillary blood, and DBS
- Confounding factors of the serum steroid profile necessitate further investigations; e.g. ethanol was shown to increase the T/A4 ratio in females
- Implementing new SARMs and their metabolites (as e.g. reported for LGD-4033, ACP-105, S42) is essential for maintaining an optimal detection capability for these substances in sports drug testing as evidenced by the first report on a finding with the SARM YK-11

S2
- Capillary-derived blood (both serum and DBS) was shown to offer adequate detection windows for ERA administrations as demonstrated for epoetin alpha and theta (microdoses), irrespective of the presence of a c.577del EPO variant
- Further, DBS were found suitable for identifying c.577del EPO variant carriers and as doping control matrix for measuring *ALAS2* and *CA1* as mRNA biomarkers for ERA abuse
- Roxadustat was found to exhibit an unexpectedly long detection window (540 days) in urine after use in therapeutic regimens
- A new detection assay for the long-acting GH somatrogon was established by targeting a non-natural peptidic sequence using LC-HRMS/MS, enabling the detection of a single dose of the drug for up to 96 h
- First data on the detection of kisspeptin and potential metabolites were presented using LC-HRMS/MS, enabling its implementation into routine doping controls

S4
- Markers indicative for myostatin inhibition were identified with musclin, follistatin-like 1, and oncostatin, potentially adding to an endocrine passport-like monitoring
- A complementary method to differentiate endogenous from exogenous AICAR by GC/C/IRMS was developed
- Several case-related investigations concerning AAFs with metabolic modulators were presented, underscoring the growing risks of unknowing exposure to prohibited substances and resulting findings in routine doping controls

S5
- Chlorthalidone elimination kinetics and its incorporation into hair were revisited in the context of several recent AAFs, suggesting a prolonged excretion phase into urine
- Arginine vasopressin is naturally found in human urine commonly below 2 ng/mL, necessitating distinct instructions for anti-doping laboratories how and when to act on a finding

S6
- The origin (endogenous or synthetic) of phenethylamine and its main metabolite in doping control urine samples is difficult to determine via IRMS due to considerably depleted carbon isotope ratios of the naturally occurring analytes
- Pseudoephedrine occupies the dopamine receptor at significantly higher rate than methylephedrine without resulting in an AAF when the currently enforced threshold levels are applied

M1
- SNP monitoring could offer an alternative approach for the detection of homologous blood transfusions
- Capillary and venous blood samples provide hematological ABP data of excellent agreement
- Including plasma volume markers such transferrin, creatinine, calcium, low-density lipoprotein (LDL), albumin, and total protein for plasma volume shift correction in the ABP hematological module improves the robustness of the obtained data
- Lugworm hemoglobin is detectable in serum (or plasma) by characteristic peptide sequences using LC-HRMS/MS

M2
- Identifying sample swapping events is facilitated at high throughput using profiles of 52 SNPs analysed from urine samples

M3
- Using a mouse model transfected via rAAV9-hEPO, the traceability of the gene doping event was demonstrated via qPCR and gene expression profiles of *asparagine synthetase, serine hydroxymethyltransferase, and methylenetetrahydrofolate dehydrogenase*
- A test method for the simultaneous detection of seven transgene targets (i.e. *EPO*, *follistatin*, *GH1*, *myostatin propeptide*, *IGF1*, and the *vascular endothelial growth factors A* and *D*) was developed, employing multiplexed PCR-based amplification, single-base extension, and MALDI-MS analysis.
- First gene doping products (sold via the internet were confirmed
- Using CRISPR/Cas12a-based detection methods, an assay detecting six transgenes (*vascular endothelial growth factor A, HIF1, EPO, GH1, IGF1, and alpha-actinin3*) simultaneously after RPA on a chip was presented
- (RT)-RPA combined with CRISPR/Cas13a allows for the detection of gene editing events with CRISPR/Cas-based methods by targeting the common CRISPR sgRNA

FIGURE 3 | Info box on particularly relevant observations.

and a carboxyfluorescein-labeled reporter probe, test results were obtained within 40 min at a concentration of 10^{-18} mol/L. For a proof-of-concept, mice received an injection of 0.4–10 μL of a *rAAV9-hIGF1* construct (total virus titer = 5.26×10^{12} vg/mL) or a naked *hEPO* plasmid (25–100 μg), and blood samples (100 μL) were collected for 20 respectively 2 days. The analyses allowed for a clear differentiation between the negative controls and the post-administration blood samples, albeit for the *hEPO* experiment the intensity difference was discussed as less prominent compared to the rAAV-based intervention.

In addition to the option of using the CRISPR/Cas system for the detection of transgenes, it was also shown to allow for the analysis of CRISPR/Cas-based gene editing attempts as presented by Passreiter et al. [104] Employing analogous principles as described above, the target sequence of this approach was the conserved region of the CRISPR single guide RNA (sgRNA) associated with *Streptococcus pyogenes*, which was first amplified with reverse transcriptase (RT)-RPA and then exposed to CRISPR/Cas13a with a dedicated crRNA in the presence of a fluorescence-labeled reporter probe sensitive to RNAse activity. Also here, a mouse model was used to assess the applicability of the testing approach, and the intravenous as well as intramuscular injection of 600 pmols of sgRNA was detected in all blood samples collected over a period of 24 h, and the method's LOD was estimated with 1 fM when using 100 μL of serum.

10 | Conclusion and Perspectives

Awareness exists that successful global anti-doping efforts necessitate advancements at the analytical laboratory level as well as the revision of today's (and the shaping of tomorrow's) doping control strategies in the light of a "new normal". This awareness is reflected in a considerable variety and number of research and review articles, case reports, and opinions that were published between October 2023 and September 2024. On the one hand, the anti-doping system is facing a quickly increasing number of new substances and methods of doping that require undivided attention in order to provide suitable means for their detection, especially in consideration of the (still) limited number of sports drug testing samples collected per athlete per year. On the other hand, it is vital for the credibility of the doping control system to continue research efforts to maintain the critical balance between an appropriate analytical comprehensiveness plus retrospectivity and the protection of clean athletes considering the growing challenge created by an exposome, which can evidently put an innocent athlete's career at risk.

A considerable investment into complementary sampling strategies (e.g. with DBS) and new analytical approaches (or first proof-of-principle results) for established as well as emerging doping agents and methods was observed, with particular focus on anabolic agents, erythropoietin receptor agonists, athlete biological passport marker analyses, and the detection of gene doping practices. Equally, the commitment to develop, improve, and evaluate new avenues in support of an optimized anti-doping program and scientifically sound result management was evident.

In summary, significant contributions to advancing 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 for routine sports drug testing. Key aspects of this 17th *annual banned-substance review* are summarized in the Info Box in Figure 3.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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