PERFORMANCE ENHANCING HORMONE DOPING IN SPORT

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Published 25 April 2015

ABSTRACT

Sport is the organized playing of competitive games according to rules. Hence doping represent drug cheating, a fraud on competitors, the sport and the public. The charter of the World Anti-doping Agency (WADA) forms a harmonized Code that authorizes an annually updated list of prohibited doping substances and methods as well as accrediting national anti-doping labs around the world. Sports performance has 4 major components: skill, strength, endurance and recovery, with each sport employing a distinct combination of these elements. These performance characteristics also correspond to the most potent and effective forms of doping. Sports requiring explosive power are most susceptible to androgen doping through their effect on increasing muscle mass and strength whereas sports that require endurance are most enhanced by hemoglobin (blood) doping which increases oxygen delivering capacity to exercising tissues. Performance in contact sports and those involving intense physical activity or training may also be enhanced by growth hormone and its secretagogues through speeding of tissue recovery from injury. Hormones remain the most potent and widely detected doping agents being responsible for about 2/3 of anti-doping rule violations detected by increasingly sophisticated detection methods. At present, the vast majority of positives are still due to a wide variety of androgens, including marketed and illicit (nutraceutical, designer) synthetic androgens as well as exogenous natural androgens, while the peptide hormones (erythropoiesis stimulating agents, growth hormone and its secretagogues) and autologous blood transfusion remain difficult to detect.

INTRODUCTION

Across the world, sport is a ubiquitous human social activity that forms an unique intersection of health, recreation, entertainment and industry[1]. It is both a major economic activity as well as a profound influence on social behaviour of individuals at home, work and play. One practical and concise definition of sport is *the organised playing of competitive games according to rules*. In that context, rule breaking is cheating to achieve an unfair competitive advantage whether it involves using illegal equipment, match fixing, banned drugs or other means.

The illicit use of banned drugs (doping) to influence the outcome of a sporting contest, constitutes a fraud against competitors, spectators, sport, sponsors and the public no different from other personal, professional or commercial frauds. While performance enhancement is almost invariably the intent of cheating, impairing performance is also well known in horse racing and even, rarely, in human elite sports (e.g. drink-spiking of banned drugs, physical assaults). Rules of sporting contest may change by agreement, but once set, represents the boundaries of fair competition. Nevertheless, fairness is an elastic, socially constructed concept which may change gradually over time. For example, a century ago deliberate training itself was

considered an ungentlemanly breach of fairness as competition was then envisaged as a contest based solely on natural endowments and, similarly, some sports once maintained a distinction between amateurs and professionals. The philosophical foundations of the concept of fairness is a deep and complex issue [2, 3] where the focus has been mainly on distributive justice. Less attention has been given to the philosophical basis of fair competition in sport where the prior distribution of talent and training and the outcome of contest are intended to provide equality of opportunity, but not of outcome, between contestants.

Naïve arguments have been made that deny doping is cheating, or unsafe or violates the spirit of sport and asserting that drugs should be freely available or under medical supervision[4-6]. However removing prohibition on doping would immediately render drug taking as pervasive as training in elite sport extending to promising underage and subelite athletes. Ensuing demands for excessive, often massive, drug doses without medical indications could not be ethically or safely prescribed by doctors. This could convert sporting participation into a potentially dangerous rather than a healthful activity. In practice, creating enforceable boundaries for drugs in sport is unavoidable whether it is prohibition or, even under the most idealistic libertarian scenarios, by age or dosage. These philosophical issues are not considered further here and, recognising that sport requires concrete, practical decisions, the establishment and enforcement of agreed rules is the basis of fair competition.

It is well understood that individual human genetic endowments are unequal and, among these, sporting prowess is at least partly genetically determined[7]. However, little is still known of the genotype-phenotype correlations that underlie beneficial genetic endowments for sports performance. Natural genetic advantages are recognized in height (tallness for basketball, shortness for jockeys and motor-cycle riders) and hereditary erythrocytosis where a high circulating hemoglobin (due to a high affinity EPO receptor[8]) for endurance sports, or conversely genetic disadvantage such as the common α-actinin-3 deletion genetic polymorphism which limits anaerobic, explosive power[9]. More examples of genetic (dis)advantages for sports performance are likely to be identified as genomics continues to expend our understanding of the biological basis of health, including natural human sporting prowess. In the context of sports doping, however, a person's natural genetic endowment is a given creating a natural boundary whereby the use of exogenous drugs or chemicals (including DNA) may constitute drug cheating or doping.

WORLD ANTI-DOPING AGENCY (WADA) AND THE GLOBAL ANTI-DOPING REGULATORY ENVIRONMENT

Cheating is as old as sport itself, yet the present endemic of doping using pharmaceutical drugs to boost sports performance is largely a Cold War legacy. Eastern European national doping programs were established by governments aiming to achieve a short-cut propaganda victory over their Western rivals, a challenge quickly reciprocated and then taken up by individual coaches and athletes, starting with power sports[10], from which the epidemic became entrenched as an endemic in sufficiently affluent circles. In 1967, following the introduction of anti-doping rules by some other federations, the International Olympic Committee (IOC) established its Medical Commission, which published their first list of prohibited substances. During the 1970's

the IOC Medical Commission took an increasingly active role by banning androgens which required developing standardized, valid methods to detect and deter androgen doping. After discarding alternatives such as immunoassays and blood sampling, mass spectrometry (MS)-based tests became the standard for detecting synthetic androgens in urine.

In 1999, the IOC established the WADA based in Montreal to be equally supported by governments and sporting organisations with its charter, the WADA Code, representing a harmonised set of global anti-doping rules introduced in 2004, revised in 2009 and 2015 [11]. WADA also publishes an annually updated Prohibited List of Substances and Methods, accredits anti-doping labs together with their operational anti-doping testing framework and established the Court for Arbitration in Sport (CAS) to settle anti-doping legal disputes as sport's "Supreme Court". By 2013, the Code was adopted by 204 Olympic Committees, 89 Olympic and 239 non-Olympic national federations and is implemented by 129 National Anti-Doping Organisations. The WADA Code prohibits substances or methods which meet 2 of 3 criteria comprising:
(i) enhance performance (cheating),
(ii) harmful to health (safety) or
(iii) violate the spirit of sport (unsporting).

Although the primacy of penalising cheating is widely understood, these criteria recognize ethical and practical difficulties in proving ergogenic effects of increasing numbers of illicit and/or non-approved substances for which safety is unknown so that human testing is not feasible and athlete safety is an additional important consideration. Crucially, the Code imposes strict liability on individual athletes so that a positive anti-doping test (including refusal or avoidance of testing or possession, attempts, trading and tampering with banned drugs) constitutes an anti-doping rule violation (ADRV), regardless of intent or negligence. Sanctions involve suspension from any elite competitive sport and extend to support personnel and teams. Suspensions were typically 2 years but were increased to 4 years from 2015, which is believed to be longer than the ergogenic benefits of doping, although recent evidence suggests that episodic androgen effects on muscle may have durable or even permanent effects[12].

The Prohibited List bans, at any time either in or out of competition, the use of performance enhancing hormones, including androgens, EPO and growth hormone and related substances or drugs which stimulate endogenous production of these hormones (Table 1). Among the 15 categories of prohibited substances (12) and methods (3), hormones feature prominently in S1 (anabolic agents, mainly androgens), S2 (peptide hormones, growth factors and related substances), S4 (hormone and metabolic modulators) and S9 (glucocorticoids) with S1 and S2 having important "catch-all" provision for unnamed but related substances "with similar chemical structure or biological effects". In addition, the S0 category bans non-approved substances, those without current regulatory approval for human therapeutic use. The prominence of hormones is reinforced by the WADA laboratory statistics for anti-doping tests where hormones remain the most frequently detected banned drugs (Table 2). In 2011, of ~250,000 anti-doping tests ~2% were positive with about 2/3 due to hormones, the vast majority (~99%) due to androgens. These findings confirm that the detection of androgen doping is effective whereas the low rate of detection of erythropoiesis-stimulating agents (ESA) and growth hormone may reflect the limitations of available tests for peptide hormones which require blood rather than conventional urine sampling and feature relatively low sensitivity and brief windows of detection, rather than their lack of abuse. Further use of out-of-competition testing and blood samples

together with more sensitive detection tests having longer windows of detection are required particularly for peptide hormones.

Table 1 – 2015 WADA Prohibited List of Substances and Methods

Substances:

- non-approved substances (S0)
- anabolic agents (S1)
  - exogenous & endogenous androgens
  - "others with similar chemical structure or biological effects"
- peptide hormones, growth factors and related substances (S2)
  - erythropoiesis-stimulating agents
  - hCG, LH (in men only)
  - GH, IGF-I, FGF, HGF, MGF, PDGF, VEGF
  - "others with similar chemical structure or biological effects"
- beta2-agonists (S3), beta-blockers(P2)
- hormone and metabolic modulators (S4)
  - aromatase inhibitors & anti-estrogens
  - myostatin inhibitors
  - metabolic modulators (insulin, PPARδ, AMPK agonists)
- diuretics and other masking agents (S5)
- stimulants (S6)
- narcotics(S7), cannabinoids(S8), alcohol(P1)
- glucocorticoids (S9)

Methods:

- manipulation of blood & blood components (M1)
- chemical and physical manipulation (M2)
- gene doping (M3)

Substances and Methods as described in the WADA Prohibited List with the category label in brackets.

Table 2 – Performance Enhancing Hormone Tests in WADA Labs

	2003	2005	2007	2009	2011	2013
ACCREDITED LABS	31	33	34	35	33	33

TOTAL TESTS	151,210	183,337	223,898	277,928	243,193	269,878
POSITIVES	2,447 (1.6%)	3,909 (2.1%)	4,402 (2.0%)	5,610 (2.0%)	4,856 (2.0%)	5962 (2.2%)
HORMONES	46%	55%	55%	73%	68%	57%
ANDROGENS	2389	3893	4375	5541	4800	3352
BLOOD/EPO	58	16	27	68	50	63
GH/PEPTIDES	0	0	0	1	6	0

Source: WADA website report on laboratory testing figures. See http://www.wada-ama.org/en/Science-Medicine/Anti-Doping-Laboratories/Laboratory-Testing-Figures/

Therapeutic Use Exemption (TUE).

In rare cases, an elite athlete with a genuine medical need for therapeutic use of a prohibited drug may be granted a TUE [13]. This exempts the athlete from the Code's strict liability provision and permits them to compete during ongoing treatment. WADA provides medical guidelines that standardize the evaluation and management of TUE applications for a range of medical illnesses. A TUE is granted by a national anti-doping organisation based on an independent, expert review of valid, documented diagnosis, appropriate clinical indications and dose for hormonal treatment with a view to facilitating essential medical treatment but avoiding unjustified use or over-dosage. After stringent review TUE's may be granted for treatment with testosterone, glucocorticoids and insulin but there are unlikely to be valid medical indications for EPO or, in adults, for growth hormone or IGF-1 in elite athletes. For example, TUE's are usually justified for young male athletes with genuine androgen deficiency, occurring in ~1:200 men[14], due to organic pituitary-testicular disorders with an established pathological basis (eg bilateral orchidectomy, severe mumps orchitis, Klinefelter's syndrome) who require life-long testosterone replacement therapy [15]. The TUE will approve, subject to regular review, a standard testosterone replacement regimen, including dosage and monitoring, with changes to regimen requiring approval. TUEs are not granted for men with functional decreases in blood T due to non-reproductive disorders including stress ("over-training") or ageing ("andropause", "LowT","late-onset hypogonadism") or for women.

In principle, detection of prohibited substances is ideally aimed at identifying a xenobiotic substance or its distinctive chemical signature(s) which do not occur naturally in the body, thereby distinguishing it categorically from normal body constituents. Such identification of a non-natural substance that can't be of endogenous origin is congruent with the strict liability onus in proving an ADRV. Proving an ADRV is more difficult to achieve with administration of natural hormones or their analogs which must be distinguished from their endogenous counterparts. In this situation, the alternative requires developing valid biomarkers to prove the use of banned substances through their distinctive effects on the body and tissues. It is a formidable challenge to validate an indirect biomarker as proof of an ADRV capable of withstanding vigorous medico-legal challenge when a proven ADRV would prevent an athlete

from pursuing their profession. That requires rigorous standardization and harmonization of every stage of the anti-doping tests from sample collection, chain-of-custody, storage and analysis including accounting for any fixed (genetic, gender, age, ethnicity) or variable (exercise, hydration, masking vulnerabilities) factors which may impact on proposed test metrics.

Components of Sports Performance and Doping

Sports performance has 4 major dimensions – skill, strength, endurance and recovery (Figure 1). High performance in any sport requires a characteristic blend of these dimensions although individual sports differ widely in that balance. Similarly, the major ergogenic drug classes have distinctive effects aligned predominantly along one of these dimensions so that the most effective ergogenic drug classes used in doping are dictated by these dimensions of sports performance (Figure 2). While every sport requires an acquired skill, some are largely or solely based on skill and concentration (e.g. board games, target shooting, car driving, and motor-cycle riding) and may benefit from drugs that reduce anxiety, tremor, inattention or fatigue. Sports that are highly dependent on explosive, short-term anaerobic power (sprinting, throwing, boxing, wrestling), typically ones which favour a stocky, muscular build, are most susceptible to androgen-induced increases in muscle mass and strength. Other sports with an emphasis on aerobic effort and endurance (e.g. long distance or duration events), characteristically favoured by a lean build, may be boosted by hemoglobin doping (blood transfusion, erythropoietin (EPO) and its analogs or mimetics). Finally, sports that depend on recovery from major injury or recurrent minor injury during intensive training, notably contact sports, may benefit from tissue proliferative and remodelling effects of growth hormone and various growth factors.

Insert figure 1 here
Insert figure 2 here

ANDROGENS

Although the ergogenic effects of androgens were discovered empirically soon after the identification of testosterone in 1935[16], their applications to elite sport performance were mainly developed during the Cold War by trial and error experiments undertaken on unknowing elite athletes[10, 17, 18]; however, the scientific basis of androgen doping was only objectively proven in the 1990's. Until that time, the settled consensus was that exogenous androgens had no effect in eugonadal men whose androgen receptors were already saturated by endogenous testosterone (T)[10, 19, 20] and that the alleged benefits of androgen doping were misattributed placebo responses together with training and nutritional effects. Using a placebo-controlled, randomized clinical trial design with supra-physiological testosterone doses, Bhasin et al showed that T increased muscle mass and strength in eugonadal young men to a similar extent as exercise alone and with additive effects when combined with exercise[21] (figure 3). Subsequent dose-response studies showed that administration of T increased muscle mass and strength by 10% without and 20-37% with exercise (where exercise alone increased them by 10-20%) together with additive effects from 3% increase in circulating hemoglobin. These benefits extended from below to well above physiological T doses or blood levels without evidence of plateau[22, 23] and regardless of age[24].

Insert figure 3 here

Androgen doping may be either direct or indirect (Table 3, figure 4). Direct androgen doping involves administration of synthetic androgens whereas indirect androgen doping includes a variety of non-androgenic drugs which increase endogenous T. Direct androgen doping originally involved all pharmaceutically marketed synthetic androgens but has extended to non-marketed designer and nutraceutical androgens as well as exogenous administration of natural androgens (T, DHT) and pro-androgens (androstenedione, DHEA). Indirect androgen doping involves use of hCG, LH, anti-estrogens (estrogen receptor blockers, aromatase inhibitors), opiate antagonists and neurotransmitters involved in neuroendocrine regulation of endogenous LH and T secretion[25-27].

Table 3 – Direct and Indirect Androgen Doping and Detection Methods

Substance	**Detection method**
Direct	
Synthetic androgens	L/GC-MS
Natural androgens	L/GC-MS, T/E, CIRMS
Designer & nutraceutical androgens	L/GC-MS (bioassay)
Indirect	
hCG (urinary or recombinant)	hCG immunoassay (LC-MS)
hLH (recombinant)	hLH immunoassay (LC-MS)
Anti-estrogens	L/GC-MS
GnRH analogs	L/GC-MS
Opioid antagonists & neurotransmitters	L/GC-MS

Insert figure 4 here

Detection of direct androgen doping using steroids of known chemical structure is highly effective using gas or liquid chromatography MS[28, 29]. Traces of synthetic androgens or their metabolites may remain detectable for periods up to months after last administration. Recent developments including the identification of long-term metabolites has further widened the detection windows for synthetic androgens[30-32]. Challenges to detection of synthetic androgens have included the development of non-marketed designer and nutraceutical androgens, the use of natural androgens and pro-androgens, masking methods, restricting use to out-of-competition training or micro-dosing. Designer and nutraceutical androgens are typically non-marketed

synthetic androgens based on structures and synthesis methods recovered from largely forgotten patent literature of the 1960-70's. These are now synthesized by unregulated non-GMP chemical manufacturers to be sold over the internet or over-the-counter as nutritional supplements, which may contain undeclared steroids[33]. However, once the chemical structures of any synthetic androgens are known, they are easily detectable although the sheer profusion of such chemicals represents an ongoing challenge. Nevertheless, despite their novelty, there is little evidence designer androgens have been much used after they are discovered from when there is a high likelihood of detection so that virtually all ongoing androgen ADRVs are still due to conventional marketed synthetic androgens.

Distinguishing between the exogenous and endogenous steroids:

Administration of natural androgens (T or DHT) or pro-androgens (androstenedione, DHEA), raises the problem of distinguishing between the exogenous and endogenous steroids. Exogenous T administration can be detected by the urine T/E ratio, the ratio in urine of T to its 17α-epimer epitestosterone (E). Both T and E are co-secreted by Leydig cells and excreted in urine consistently so that the urine T/E is usually stable for any individual over time, being typically around 1. Administration of exogenous T, which is not converted to E, increases the urine T/E ratio and, when it exceeds a specified threshold, is evidence for administration of exogenous T. The urine T/E ratio thresholds were originally population-based, set initially at 6 and then subsequently lowered to 4. However, the possibility of false negatives and false positives of population-based thresholds are limitations which may require further analysis to confirm or refute T doping in individual cases. These considerations have led to establishment of the steroid module of the Athletes Biological Passport (ABP), a compendium of serial observation of any individual's tests which creates adaptive individual-specific T/E ratio threshold. This substitution of an individual's own person-specific, in place of the population-based, thresholds allows for more sensitive and accurate detection of individual deviations in urine T/E ratio as evidence of T doping.

One limitation of the urine T/E ratio is a genetic polymorphism of the uridine 5'-diphospho-glucuronosyltransferase (UGT) 2B17 gene which encodes a phase II hepatic enzyme that glucuronidates T rendering it more hydrophilic to facilitate urinary excretion. This polymorphism comprises a genetic deletion which, in homozygotes, produces a non-functional enzyme that reduces urinary T (but not E) excretion to near zero producing an extremely low T/E ratio (<0.1). While this genetic polymorphism has no apparent biological effect on T action, it is unevenly distributed geographically being much more frequent in South East Asian populations[34]. This biological false negative means that administration of exogenous T will not exceed the usual population-based T/E ratio thresholds[35]. On the other hand, it will exceed any individual's own specific urine T/E ratio ABP threshold so that genotyping and/or Bayesian profiling of serial T/E ratio provide complementary evidence[36, 37].

Administration of exogenous T may also be identified by carbon isotope ratio MS (CIRMS) which can distinguish endogenous from exogenous T according to the C^{13}/C^{12} ratio of urinary T[38, 39]. Commercially, steroids are manufactured from starting material of plant sterols produced by photosynthesis which exhibit distinctly lower C^{13}/C^{12} ratio (typically, -26‰ to -36‰ relative to the global standard) compared with mammalian T biosynthesis (between -16‰ to -26‰)[40, 41]. Hence, a significantly depleted C^{13}/C^{12} ratio of urinary T, exceeding 3‰ relative to endogenous reference steroids, indicates that urinary T originates at least partly from exogenous chemical

manufacture from plant sterols. CIRMS can also be applied to detect administration of other natural androgens or pro-androgens including DHT and DHEA[42], androstenedione, or even attempted masking by administering E (to lower urine T/E ratio)[40]. A few T products have recently emerged with a less depleted, more mammalian-like C^{13}/C^{12} ratio for urine T[43] creating a challenge for CIRMS detection; nevertheless, extended isotope profiling of other steroid precursors and metabolites provides additional reference biomarkers[44]. Furthermore, development of hydrogen ion ratio mass spectrometry has further enhanced the ability to distinguish between endogenous and exogenous steroids even when the carbon isotope ratio is non-informative[45-47]. Suppression of urine LH excretion may also provide corroborative evidence for the use of exogenous T or other synthetic androgens[48, 49].

While MS is highly effective for detecting specific androgens, it requires knowledge of the chemical structure to be detected and otherwise cannot be applied. This applies to never-marketed designer or nutraceutical androgens sold over the internet or in unregulated over-the-counter nutritional supplements with unlabelled steroid content. A potential solution is the modern in vitro androgen bioassay that incorporates the human androgen receptor together with a convenient transactivation chemical read-out signal into a host yeast or mammalian cell. This has the generic capacity to detect all bioactive androgens regardless of structure with a sensitive dose-response signal proportional to the potency of the bioactive androgen[50-53]. Yeast host cells have high specificity for detecting androgens but are less sensitive than mammalian cells, which express native steroid mechanisms including steroidogenic enzymes and/or other steroid receptors. Mammalian in vitro androgen bioassays can also detect pro-androgens, steroids lacking intrinsic androgenic bioactivity but which are converted into androgens by the mammalian cell. Hence, while mammalian host cells sacrifice specificity for higher sensitivity, they can also detect pro-androgens[54]. Hence yeast and mammalian in vitro androgen bioassays are complementary in detecting both androgens and pro-androgens.

The limitations of in vitro androgen bioassays are their susceptibility to matrix effects and difficulties in standardizing bioassay-based test so they may be best applied to characterize products and substances for androgens or pro-androgen content rather than to biological samples. Hence the yeast androgen bioassay was decisive in the first conviction for use of a designer androgen by proving that tetrahydrogestrinone (THG) was a potent androgen[55] and has also been used to screen synthetic progestins to show that, unlike the original androgen-derived progestins, the modern generation of progestins are not androgenic[56].

An attractive option to detect androgen doping is the use of hair samples. These have the advantages of easy, observable and minimally invasive sampling and simple, convenient storage featuring a potentially very long window of detection, according to hair growth rates[57]. MS-based methods have been reported to detect exogenous[58-72] and endogenous[61, 73-76] androgens in human hair following long-term, but not single dose[77], exposure. However, hair analysis tests have yet to undergo sufficient standardization and validation to become acceptable anti-doping tests. Problems that remain to be fully overcome include matrix effects, low recovery and limited sensitivity as well as the impact of age, hair colour, alopecia, and shaving or passive chemical (cosmetic) contamination of hair. Additionally nails and skin could also provide analogous information on recent past androgen exposure but suitable tests are yet to be convincingly developed[78]. In theory, androgen-induced gene expression in circulating leukocytes might provide an additional biomarker of androgen action if specific and reproducible signatures can be defined[79]; however, as direct detection of androgens is feasible and

preferable for proving an ADRV, a role for gene expression biomarkers of androgen action remains to be established for anti-doping.

Indirect androgen doping

This strategy aims to increase endogenous T production and thereby evades detection by routine screening tests for exogenous T such as urine T/E ratio or CIRMS. Urine hCG is detected by commercial hCG immunoassays using immunoassays specific for intact heterodimeric hCG (including its nicked variant) which, if positive by exceeding a detection threshold (>5 IU/L), requires confirmation by a second immunoassay for intact heterodimeric hCG which is required to prove hCG use. A key issue is to distinguish a positive hCG urine test, presumptively indicating hCG doping, from early trophoblastic tumor or immunoassay artefacts. As hCG doping is not effective in women and urine hCG screening can detect early pregnancy, an unwarranted privacy intrusion, hCG testing is restricted to male athletes[27]. Similarly, urine LH can be measured by some commercial LH immunoassays, none marketed for urine samples, validated by individual anti-doping labs. Although direct LH doping is an implausible doping threat[80], suppressed[48, 49, 80] or elevated urine LH may be useful for confirming any form of direct or indirect androgen doping[26, 27, 48, 81]. Anti-estrogens (estrogen receptor antagonists) or aromatase inhibitors, which can cause reflex increases in serum and urine LH and testosterone[26], are detected by MS-based chemical detection methods.

Overall, detection of direct androgen doping is now so effective that in WADA-compliant elite competitions it is restricted to the ill-informed, often using counterfeit or unlabelled products[82]. Yet the potency of androgen doping in power sports continues to prompt development of novel androgen doping strategies. These will include use of undocumented synthetic androgens or novel indirect androgen doping methods, especially micro-dosing during out of competition training. There remains a need to maintain deterrence by effective detection methods for evolving new androgen doping threats.

HEMOGLOBIN (BLOOD) DOPING

Hemoglobin doping involves either direct blood transfusion or indirect methods of increasing hemoglobin via stimulating erythropoiesis by administration of erythropoietin, its analogs or mimetics (see excellent reviews[83, 84]) (Table 4). Boosting hemoglobin is advantageous in aerobic, endurance sports such as road cycling, distance running and cross-country skiing. In addition to cardiac output, maximal oxygen consumption (Vo_2) is principally determined by blood oxygen transfer with a lesser contribution from tissue oxygen transfer[85]. Experiments on exercise tolerance and blood transfusion were first reported in 1945[86, 87] but the scientific basis of hemoglobin doping via enhanced tissue oxygen transfer was firmly established in 1972 by the work of Ekblom et al reporting experiments in healthy volunteers who underwent venesection and/or re-transfusion of 1, 2 or 3 units (400 mL) of blood with repeated testing of maximal exercise-induced oxygen consumption before and after each procedure[88]. This proved unequivocally that the maximal oxygen consumption was highly correlated with acute changes in hemoglobin (figure 5). Subsequently, during the 1970-80's before its banning in 1988, blood transfusion became a prevalent surreptitious practice in road cycling and cross-country skiing and the apparently low prevalence among distance runners may be an underestimate[89]. Modelling of historical performance in European road cycling from 1993 onwards shows a unique progression averaging an improvement of 6.4% corresponding closely with the

performance enhancement (6-7%) due to rhEPO administration, which is sustained for at least 4 weeks afterwards[90-92].

Table 4 – Direct and Indirect Hemoglobin Doping and Detection Tests

Doping Mechanism	Detection
Direct (Blood transfusion)	
Heterologous	Flow cytometry: bimodal population of blood group antigens
Autologous	No direct detection test Biomarkers: Urine phthalate excretion Total hemoglobin mass Athletes Biological Passport
Indirect (Erythropoeisis stimulation)	
Direct	
rhEpo & biosimilars (>100) Epo analogs	Urine double immunoblot, (LC-MS)
Indirect	
Hypoxia altitude training, hypoxic sleep area	Not banned
Hypoxia-mimetics hypoxia-inducible factor & stabilizers, iron chelation, cobalt, 2,3 DPG analogs	LC-MS/MS
Artificial O_2 carriers HbOC, perfluorocarbons	LC-MS/MS

Insert figure 5 here

Blood transfusion

Transfusion may involve either another person's (homologous) or the athlete's own (autologous) blood administered prior to a contest to acutely increase circulating hemoglobin. Homologous blood can be transfused at any convenient time to enhance performance in competition but

when performed by untrained personal in non-clinical environments risks transfusion reaction, blood-borne infectious disease and iron overload. By contrast, autologous transfusion reduces health risks but requires complex coordination as venesection itself is detrimental to performance, and it requires balancing recovery from blood withdrawal and loss of erythrocyte viability during long-term cryostorage with training and competition schedules. Although blood transfusion was first banned by the IOC in 1986, the first practical approach to banning blood doping was the introduction of hematocrit testing in 1997 by the international skiing and cycling federations which excluded athletes on health grounds from entering competition on the day if their hematocrit exceed a safety threshold (0.50). This encouraged hematocrit titration to just below threshold and only prevented competing until hematocrit returned under that threshold, which could be a very short period particularly if venesection was employed. The first ADRV's for blood manipulation involving hematocrit threshold and titration were in 2001.

Homologous blood transfusion creates a bimodal population of blood group antigens which is detectable by flow cytometry using a panel of 12 minor blood group antigens[93], from the wider array of blood group antigens[94], which can detect a <5% contamination of exogenous erythrocytes. Subsequent refinements simplified and improved test sensitivity so that a panel of 8 antigens can detect contamination comprising a minor admixture population of 0.3-2.0% with no false positives but high sensitivity (~80%), the latter depending on the magnitude of the minor contaminating mixture[95, 96]. Alternatives based on genotyping for the admixture population of leukocytes have also been proposed[97]. As a test proving unequivocally the presence of non-endogenous erythrocytes in the circulation, this method is definitive if performed to the required standard. A remotely hypothetical defence against a positive test, based on stable marrow chimerism from a vanished twin, was raised by a cyclist who subsequently admitted transfusion[97]. Based on risk of detection as well as to health risks, homologous transfusion has now largely disappeared in favour of autologous transfusion[98].

Insert Figure 6 here

Autologous transfusion

The biggest gap in current anti-doping tests is the lack of a specific test to detect autologous transfusion[99]. Research to identify robust physico-chemical or biological markers for direct identification of a subpopulation of ex-vivo aged erythrocytes is underway but the dilution and rapid clearance of effete erythrocytes make for challenging detection problems[100]. In the interim, other indirect methods have been developed. These include measuring urinary excretion of phthalates, plasticizers that leach out from the polyvinylchloride blood packs used to store venesected blood[101]. This test has brief window of detection (2 day) so will detect auto-transfusion during or immediately before events (characteristic in road cycling, according to convicted dopers) but may miss earlier auto-transfusion. Furthermore, the ubiquity of low level environmental phthalate exposure requires establishing detection thresholds and non-plastic blood containers can be used. An alternative is the measurement of total hemoglobin mass[102], a measure with good stability and reproducibility even during exercise and circumvents influence of variations in plasma volume such as due to dehydration or dilutional masking[102, 103]. However, as this requires inhalation of carbon monoxide, which has transient detrimental effects on performance, it is not ideal for routine anti-doping use and its sensitivity may be insufficient to detect all EPO micro-dosing[104, 105]. Nevertheless, alternative methods for serial measurement of total hemoglobin mass remain attractive. Other hypothetical methods include the detection of

microRNA[106] or immune reactions to transfusion[107] but the sensitivity and specificity of these proposed tests remains to be fully evaluated.

The best detection test for autologous hemoglobin doping at present is the hematological module of the ABP introduced in 2009[108]. Conceptually, it is a biomarker test which adopts a Bayesian approach of creating serially-adaptive, person-specific reference limits, based on using all prior testing, to supplant population-based thresholds. Combining all of an individual's previously collected hematological data creates a probabilistic test of whether any new result deviates significantly from that individual's personal reference limits[109]. These person-specific thresholds allow for ongoing refinement and reinforcement by further testing. The thresholds are calculated by a variety of algorithms incorporating routine hematological parameters, notably hematocrit and reticulocyte counts. Those were developed over the last two decades to create the ABP hematological model which is sensitive to both direct and indirect hemoglobin doping[110]. The first attempts to regulate hemoglobin doping in the late 1990's sought to prevent road cyclists or cross-country skiing athletes competing on health risk grounds when their hematocrit exceeded pre-determined, population-based safety criteria (e.g. hematocrit 0.50 or hemoglobin 170 g/L for cycling). However, while this excluded extreme hemoglobin doping only until the short period when the safety threshold was no longer exceeded, it allowed an increase in an athlete's natural haematocrit, typically averaging ~0.45, up to the permitted ceiling threshold which fostered titrated hemoglobin doping and manipulations like hemodilution by saline or plasma volume expander infusions to avoid detection[111]. More sophisticated hematological algorithms were then developed to detect hemoglobin doping initially for the Sydney 2000 Olympics[112, 113], the first generation of algorithms developing validated tests for ongoing and for recent cessation of hemoglobin doping, using a combination of biochemical variables related to erythropoiesis physiology. This approach was simplified by a second generation algorithm using only routine hematological parameters (hemoglobin, reticulocytes)[114], and was subsequently combined with the concept of a sequential development of individual-specific reference ranges[115] into a third generation algorithms[116, 117] which were refined for the ABP[108, 109]. The hematological module of the ABP currently employs an algorithm involving 8 parameters derived from routine hematological profile (hemoglobin, hematocrit, erythrocyte count, reticulocyte count and percentage, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration)[118]. This is capable of detecting any form of hemoglobin doping, whether direct or indirect, with good but imperfect sensitivity[103-105] and using only routine hematological tests. The reported increasing use of very low EPO doses ("micro-dosing") would markedly reduce the magnitude of any dose-dependent ergogenic benefits[105] while still carrying risks of detection, disqualification and disgrace.

Stimulation of Erythropoiesis

Indirect methods to increase hemoglobin include administration of recombinant human EPO or its analogs as well as hypoxia-mimetic drugs (hypoxia-inducible factor stabilisers, iron chelation, cobalt, 2,3 diphosphoglycerate analogs) or artificial oxygen carriers (perfluorocarbons, hemoglobin-based oxygen carriers). Related but non-banned methods include altitude training or its simulation by sleeping in hypoxic rooms which are less effective than hemoglobin doping[91].

The identification of the human EPO gene in 1985 led to the marketing of recombinant human EPO (rhEPO) between 1987-9. Despite the IOC's prohibition of EPO's use in sports in 1990, the

commercial availability of rhEPO created powerful new opportunities for indirect hemoglobin doping which were soon proven experimentally[119]. A drug, which circulates for hours to days, but with potent and long-lasting ergogenic effects after its disappearance due to the 4 month lifespan of erythrocytes, is both attractive for doping and a challenge to anti-doping testing. Expiry of the rhEPO patent in 2004 allowed marketing of a profusion of generic EPO ("biosimilar") products, estimated globally at over 80[120], as well as modified EPO analogs (darbepoeitin, pegylated EPO, peginesatide, EPO fusion proteins). A fatal cluster involving deaths of 18 Dutch and Belgian road cyclists, presumably due to inadvertent over-dosage during empirical attempts to maximise ergogenic effects of illicit rhEPO, was reported[121], although difficult to verify[122]. A similar excess of unexpected deaths of road cyclists was also reported again in 2003-5 when novel EPO analogs and EPO biosimilars were marketed.

Detection of EPO in urine is difficult because of the prevailing low concentrations and need to distinguish exogenous recombinant from endogenous EPO. The first effective method for rhEPO in urine was a double immunoblot[123, 124] which was capable of detecting urinary excretion of a variety of exogenous EPO products and analogs according to their differences in glycosylation side-chains, and differences in primary amino acid sequence where they exist, while distinguishing them from endogenous EPO. Although further refined[125] and extended to other EPO analogs[126], the immune-electrophoresis test is sensitive but relatively laborious and provides only a short window of detection of up to a week post-administration[127]. More sensitive methods based on proteomics (for EPO analogs with differences in primary structure) together with glycomics (for biosimilars and analogs which have host-cell specific variations in side-chain glycosylation but unchanged natural EPO primary structure[128]) are possible but not yet approved.

Other EPO mimetics such as hypoxia mimetic drugs including hypoxia-inducible factor (HIF) stabilisers and related small molecules represent growing threats as potential indirect hemoglobin doping agents[129]. These non-peptide chemicals interfere with various steps of the molecular oxygen sensing mechanism so as to mimic renal hypoxia and thereby induce EPO secretion to increase circulating hemoglobin. As a convenient orally active alternative to the lucrative pharmaceutical market for injectable erythropoiesis-stimulating peptides (~$7-8 billion[130]) to counteract anemias of chronic renal failure or marrow failure due to myeloproliferative disease or cytotoxic cancer therapy, they constitute a very active area of pre-clinical patent-based clinical drug development[129]. Experience suggests that such innovator products can enter the doping black market before marketing approval[110, 129]. Despite the profusion of pre-clinical leads, they represent families of related chemical structures disclosed in patents for which LC and/or GC-MS detection tests should, in principle, be effective. Understanding the metabolism of these drugs when they come to market may identify long-lasting metabolites that can extend the windows of detection. Coupled with evidence from the ABP, manipulation of the EPO pathway may be detected in conjunction with corroborative measurement of inappropriately suppressed or elevated endogenous EPO for the prevailing hemoglobin level.

HIF is a key generic biological mechanism for tissue sensing of hypoxia and triggering local (neovascularisation, angiogenesis) and systemic (EPO) defensive reactions. The promoter of the EPO gene contains enhancer and inhibitor regions with the hypoxia-responsive element which binds HIF and a GATA binding site which enhance and inhibit, respectively, EPO gene transcription. HIF is a heterodimer formed by constitutively expressed subunits with the β subunit in excess and availability of α subunit limiting formation of bioactive HIF. The 3 HIFα

subunit isoforms are subject to hydroxylation of specific proline residues by prolyl hydroxylase enzymes which inactivate HIFα by ubiquitination, a tag which targets it to proteosomal degradation. HIFα subunit inactivation is strongly dependent on tissue oxygenation being active during normoxia but reduced during hypoxia when persistence of HIFα stabilises the HIF heterodimer. Notably, during hypoxia the expression of HIFα in renal cortical cells stimulates EPO gene expression so that HIF stabilisation by prolyl hydroxylase inhibitors leads to increased EPO secretion and circulating hemoglobin. Hence inhibiting prolyl hydroxylase activity via blocking its required cofactors (ascorbate, ketoglutarate, iron) using cobalt, nickel, iron chelation, ketoglutarate analogs or mechanism-based chemical inhibitors can result in increased hemoglobin via stimulation of EPO secretion[129]. Similarly, small molecule GATA inhibitors potently stimulate circulating EPO, hemoglobin and performance in mice[131] although none have yet been marketed so their human efficacy and safety remain to be determined.

Another approach to increase oxygen delivery to muscle has been to exploit the ability of 2,3 diphophoglycerate (2,3 DPG), whose binding to hemoglobin reduces its affinity for oxygen with the left-shift of its oxygen dissociation curve as an oxygen unloading mechanism in tissues. 2,3 DPG analogs, developed as radiation sensitisers for hypoxic radio-resistant tumors, enhance tissue oxygen delivery in vivo[132, 133] but would feature only short-term, acute effects readily detectable by mass spectrometry[134, 135].

Adverse effects from use of rhEPO or its analogs are well known in medicine but poorly recognised in doping. They include immunogenicity (with risk of EPO autoantibody mediated pure red cell aplasia)[136, 137], cardiovascular complications (including venous thromboembolism, stroke, hypertension and myocardial infarction) and premature death[138-141]. In routine clinical use of EPO to correct renal anemia, the goal is a gradual increase to subnormal hemoglobin targets so that the excessive and/or rapid rises in hematocrit and blood viscosity[142] may explain the excess unexplained deaths among young European road cyclists in the late 1980s. In addition, use of rhEPO may deplete iron stores which limits hemoglobin synthesis so that athletes may also use oral or injectable iron supplements, which carry their own risks such as iv iron supplementation's potentially adverse effects in enhanced tissue oxidative damage and excess mortality in chronic kidney disease[143]. Although clinical safety experience with ESAs is restricted to patients with serious medical disorders, there is evidence from the general community that higher natural hematocrit is associated with worse long-term cardiovascular health outcomes[144-146].

GROWTH HORMONE

Growth hormone (GH) is a tissue growth promoter in children but after puberty it is predominantly a metabolic hormone although latent tissue growth promoting effects may be unleashed under non-physiological circumstances, such as during recovery from tissue injury. There is consistent anecdotal evidence that GH has been used in elite sports for decades[147]. Nevertheless, ergogenic effects of GH remain unproven and largely speculative as discussed in excellent recent reviews[148-150]. Claims of GH benefits in sport have included increases in muscle mass and strength, especially in conjunction with androgens, and/or improved tissue healing with more rapid recovery from either major injuries or minor repetitive injuries, such as from intense physical training allowing for more effective training. The biological basis of ergogenic effects of GH have been tested in these two different scenarios with largely inconclusive findings.

Evidence for direct enhancement of athletic performance by GH has been investigated in two well controlled RCTs with a primary focus on athletic performance. In one study, 96 recreational sub-elite athletes (63 male, 33 female, mean age 28 yr) were administered 8 weeks of daily sc injections of GH or placebo with the men also having weekly im injections of T enanthate or saline placebo for the last 5 weeks[151]. GH increased lean (muscle) mass (by +2.7 kg) and reduced fat mass (by -1.4 kg) while T increased lean mass (alone by +2.4 kg, by +5.8 kg with GH). The effects of GH were marginally significant for anaerobic sprint capacity (by +3.9%, p=0.05) when pooling male and female participants but this was due to significant effects in men only (by +5.5% alone and +8.3% with GH). However, there were no significant effects on maximal Vo_2 consumption, dead lift or jump height[151]. A second study involved 30 healthy non-athletes (15 male, 15 female, mean age 25 yr) who were administered daily sc injections of GH at high (4.6 mg/day) or low (2.3 mg/day) doses or placebo[152]. There was no significant effect on muscle mass or maximal Vo_2 consumption. Additional controlled studies of GH effects but with less focus on athletic performance have also shown that (a) a single dose of GH (~0.8 mg) in 9 recreational athletes did not affect maximal Vo_2 or power output in repeated 30 min bursts of bicycle ergometry[153], (b) short term (6 days), low dose GH (~1.7 mg/day) treatment of 48 male androgen abusers withdrawn from androgens for 12 weeks significantly increased maximal Vo_2 more than placebo[154], (c) daily sc injections of a GH receptor antagonist (pegvisomant) or placebo for 16 days to 20 sedentary men did not change maximal Vo_2 although time to exhaustion at 90% maximal Vo_2 was reduced[155] and (d) 4 weeks of daily sc injections of GH (~5 mg/day) increased whole body protein synthesis[156], lipolysis and glucose uptake[157] with uncertain significance for athletic performance. Overall, these studies suggest that GH has, at most, a modest ergogenic effect in men only and there through enhancing T effects.

It is also claimed that GH may enhance injury healing, thereby facilitating more intensive training and/or recovery from muscle, connective tissue or bone injury, notably in contact sports. This claim is difficult to evaluate and no well controlled studies of recovery from sports injuries or tolerance of training intensity in elite athletes are reported. The most germane surrogate evidence available arises from investigations on the use of GH in recovery from injuries due to burns, fracture or for wound healing. A recent Cochrane meta-analysis review of GH treatment effects on recovery from burns injury and healing of donor skin graft sites suggests that GH has a small benefit in skin healing with large burns and reduced hospital stay but there was no benefit in reducing mortality or scarring and adverse effects, notably hyperglycemia, were increased[158]. In practice, the increased mortality due to administration of high dose GH in critical illness[159] has led to GH treatment not being widely adopted in clinical practice of treatment of burns. Similarly, the only well controlled study of GH effects on bone healing from fracture reported that, among over 400 patients with tibial fractures treated for up to 16 weeks with GH (1, 2 or 4 mg/day) or placebo, there was no benefit of GH for overall healing[160]. Finally, while there are numerous experimental studies of GH or growth factors on wound healing in animal models a wide variety of findings are reported with detrimental, neutral or beneficial effects but no well-controlled human studies are available. In summary, the available evidence for improved tissue repair or regeneration is minimal.

Important caveats on interpreting these few well designed studies is that the effects of higher GH and T doses, as used in doping, have not been studied so that more potent higher dose and/or interactive effects cannot be excluded in the absence of well controlled studies. Anti-doping science history suggests that caution is required before rejecting evidence for claimed ergogenic effects without investigations replicating the pharmacological doses used.

Furthermore, safety analysis is not feasible based on the few, small, short-term studies of GH's potential ergogenic effects; however, there are significant safety concerns about the long-term risk of cancer following GH administration. Even standard therapeutic GH doses administered to GH deficient children are associated with increased risk of second cancers in some [161-163] but not all [164] follow-up studies although these risks appear largely confined to survivors of childhood cancers and its treatment which render them GH deficient[165-168]. Although the significant cancer risk based on uncontrolled observational cohort data using standard GH doses remains contentious[169, 170], the long-term risks of much higher GH doses used illicitly by athletes must be viewed with significant concern.

Detection of GH doping remains difficult[171]. A major challenge is the non-glycosylated primary structure of recombinant and endogenous 22 kDa GH, that lack the distinctive side-chain carbohydrate differences of exogenous glycoproteins EPO or hCG which provide a convenient basis for sensitive molecular detection tests. Nevertheless, minor infidelities in commercial manufacturing of GH may incorporate distinctive non-natural chemical features proving an exogenous origin[172-174] although these findings have not been developed into detection tests. Challenges to the detection of GH doping arise from the physiological pattern of endogenous GH secretion with its intermittent, pulsatile pattern subject to prominent influence of exercise, stress, and nutritional effects together with GH's brief circulating half-life and low urine concentrations[175, 176]. Like other major doping classes, there are both direct and indirect forms of GH doping, involving either direct administration of GH or IGF-I or their analogs and indirect GH doping involving drugs that aim to increase endogenous GH and IGF-I secretion (Table 5).

Table 5 – Growth Factors, Growth Hormone Related and Other Peptides

Growth Factors	Growth Hormone related peptides			Other Peptides
	GHRH analogs	Ghrelin analogs	Other	
FGF	GHRH	GHRP-6	IGF-1 & analogs (MGF, long R^3 IGF-1)	Thymosinß4
HGF	CJC-1295	GHRP-2 (pralmorelin)	IGF-2	
MGF		GHRP-4	Insulin & analogs	
PDGF		GHRP-5	AOD-9604	
VEGF		GHRP-1		
		Hexarelin		
		Ipamorelin		
		Alexamorelin		

The first test to detect administration of exogenous GH, the 22kD recombinant form of human GH, was based on blood sampling to measure the ratio of circulating isoforms of GH recognizing the fact that the pituitary secretes not only the major 22 kD isoform (65-80%) but also a variety of minor isoforms including a wide variety of minor isoforms and their multimeric variants [177]. Administration of exogenous GH suppresses endogenous pituitary GH secretion leading to a predominance of circulating 22 kD GH. This is the basis for the GH isoform ratio test whereby a serum sample is measured by two different GH immunoassays, one with predominant 22 kD GH specificity ("rec" assay) and the other recognizing the broad spectrum of pituitary GH isoforms ("pit" assay) and the ratio of results ("rec"/"pit" ratio) is an index to detect administration of exogenous recombinant GH[175, 178]. This ratio test then serves to detect administration of exogenous recombinant human 22kD GH analogous to detection of exogenous T by the urine T/E ratio and exogenous insulin by analysis of serum C peptide [179]. The differential GH isoform ratio test has undergone extensive validation involving standardization of the two GH immunoassays with distinctive immunoreactivities to quantify 20kD and 22kD epitopes as well as its application to various populations of elite athletes and evaluating physiological factors which might impact on the validity of test read-out. A strength of this test is that it is aimed at the exogenous doping agent itself, although it cannot definitively distinguish it from its endogenous counterpart. The major limitations of this differential isotope ratio test is its narrow window of detection (24-36 hr post administration) and its inability to detect indirect GH doping. While pituitary-derived human GH might not be detected, human pituitary GH, once obtained from national scale pituitary collection and purification programs, has not been available since 1985 when its risks of Creutzfeldt-Jakob disease were identified[180, 181] with recombinant human GH replacing pituitary-extracted GH worldwide. This differential isoform test was first introduced for the 2004 Olympics[182] and led in 2010 to the first successful detection of out of competition GH doping[183].

A complementary detection test with a wider window of detection has been developed based on biomarkers of GH action. This uses two serum biomarkers of tissue GH effects, circulating IGF-1 as a short-term marker of hepatic GH action, and N-terminal peptide of procollagen type III (PIII-NP) as a long-term marker of GH-dependent collagen synthesis. In a study of 102 recreational athletes (53 male, 49 female, mean age 25 years, from 4 different European cities) randomly assigned to self-inject 2.7 mg or 5.4 mg GH or placebo once daily, measurement of serum IGF-1 and PIII-NP by specific immunoassays were able to correctly classify 86% of samples from males and 60% of samples from female using an empirical linear discriminant analysis of log-transformed serum IGF-1 and PIII-NP at the specificity of 1:10,000 required for a WADA biomarker threshold[184]. Subsequent studies have shown that additional collagen biomarkers, N-terminal propeptide and C-terminal telopeptide of type I collagen, further widen the window of detection for GH administration[185, 186]. This multiplex biomarker test, based on using standardized immunoassay antibodies, requires establishment of reliable reference range with specificity (false positive detection rate) of no more than 1:10,000 incorporating the impact of gender and age, although exercise, injury, ethnicity and sports type appear not to be confounding influences but is not yet in routine use by WADA anti-doping labs. The two GH doping test, the differential isoform and biomarker approaches, are considered ultimately complementary[187].

IGF doping

IGF-1 is a circulating marker of hepatic GH effects and mediator of GH action so the marketing in 2005 of recombinant human IGF-I alone, and later with its major binding protein recombinant human IGF binding protein 3 (IGF-BP3)[188], for treatment of diabetes, insulin or GH insensitivity or motor neuron disease, together with the availability of IGF-1 analogs for laboratory use, creates the possibility of IGF doping[189]. Time-series analysis of elite sports performance[190] is consistent with the occurrence of IGF-1 doping but its prevalence is unknown[33]. As the biological basis for ergogenic effects of IGFs is due to its GH-like effects, this remains largely speculative and accompanied by the same safety concerns. IGF-1, IGF-2 and their analogs[191] as well as insulin and its analogs[192] are all readily detectable by LC-tandem MS and preliminary evidence suggests that biomarkers for IGF-1 administration (IGF-2, IGFBP2) may widen the window of detection[193]. However, a specific test to detect IGF doping remains to be established[194].

MGF is a splice variant of IGF-I which, although not known to appear in the circulation, have any pharmacological effects or be approved for human use[195], is advertised on the black-market and internet[196] for alleged anabolic or tissue repair/regeneration benefits. Like other short peptide with known structure, it is readily detectable using LC- tandem MS[196].

Growth factors, GH releasing and other peptides

For the unscrupulous in pursuit of the unlawful, the increasingly stringent detection of the most potent ergogenic drugs used in androgen and hemoglobin doping has created a new, more speculative form of doping involving peptide growth factors and GH releasing peptides. These are within the size range of automated bulk custom peptide synthesis and are marketed cheaply by chemical manufacturers. While notionally sold solely for laboratory research, these unregulated products are available for purchase over the internet. Promoted by speculative fantasies on their mode of action coupled with testimonials to their efficacy but without objective testing or assurance of safety in humans, they are believed to be widely used by gullible and/or desperate athletes and their trainers. As unregistered drugs, this growing range of peptides appears to constitute a greater threat to athlete's health than a risk of effective cheating.

The S2.4 category of Prohibited Substances lists, in addition to GH and IGF-1, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF). The listing also contains a generic catch-all provision for unnamed growth factors and peptides which may affect connective, vascular, muscular or regenerative tissues or energy utilization and other substances with similar chemical structure or biological effects.

The major category of oligopeptides used for doping is the class of GH releasing peptides analogs of the endogenous GH releasing peptides, GHRH and ghrelin, whereby their analogs aim to increase endogenous GH secretion and are therefore banned (table 5). Most of these peptide were developed in the pharmaceutical industry from the 1990s aiming to provide cheaper, orally active, non-peptide agonists with capacity for sustained stimulation of endogenous GH secretion to "rejuvenate the GH/IGF-1 axis"[197], an unusually explicit acknowledgment of the regular nexus between hormonal rejuvenation and doping[198]. However, none of these hormonal peptides have been registered for human therapeutic use with only one

(pralmorelin) registered for single-dose, diagnostic use (for GH deficiency) in Japan. Although they may stimulate GH release initially, many failed to achieve sustained GH release due to desensitization and none achieved meaningful clinical improvements in any target diseases. If their unproven ergogenic benefits are due to sustained GH release this renders them unlikely to be beneficial; nevertheless, the caveat on not accepting negative conclusions without direct testing are also relevant to this class of peptides. Like other short peptides, once chemical structures are known, detection is readily feasible using LC-MS[199, 200]. The illicit nature of this market raises the risks of counterfeit and unsafe products with attendant risks of infection and residual toxic contaminants unlike the purity pharmaceutical product manufacturers are required to demonstrate by batch release testing.

PROGRESS, GAPS AND FUTURE PROSPECTS

Anti-doping science continues to make major progress over recent decades especially since the advent of WADA with its harmonization and focus on deterrence through standardized testing. Like any efforts to combat human malfeasance, the quest for drug-free and safe sport requires ongoing vigilance and continual renewal of intelligence-based detection testing. While great progress has been made in the two canonical forms of doping, androgen and haemoglobin doping, human ingenuity continually finds way to challenge the testing just as traditional frauds are supplanted by cyber-crime and ingenious computer hacking.

The major gaps remaining in anti-doping science are (a) the lack of a definitive test for autologous blood transfusion, (b) need for more sensitive detection tests for peptide doping with wider windows of detection and (c) more economical, affordable and robust sample handling and storage procedures. These challenges must be met by adapting novel technologies such as quantitative proteomics, genomics and metabolomics as well as implementing more out of competition and blood testing. Such progress depends on innovative applied research which is supported by WADA, Partnership for Clean Competition and certain national national anti-doping organisations together with regular peer-review research granting agencies. Finally, the development of effective forensic intelligence investigations, a slow, complex and costly process but which can have salutary effects (eg for road cycling in the Lance Armstrong case), is proving a valuable complementary approach as an adjunct to effective laboratory testing.

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