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Effects of anabolic steroids and their detection by Athlete Biological Passport in sports

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ABSTRACT

Doping in sports has long been relevant and thereby also anti-doping. As users find ways to avoid detection, scientists have to find new or better ways of analysis. Athlete Biological Passport (ABP) was introduced in 2009 to detect blood doping by the use of individual biological markers. A new module was introduced in 2014 called the steroidal module. The module was made to detect anabolic steroid use, which is now the most used substance based on statistics from WADA. Anabolic steroid enhances strength and helps build muscle, but has many side effects. The steroidale module uses the markers Andosterone (A), Etiocholanolone (Etio), 5α-Androstane-3α,17β-diol (5αAdiol), 5β-Androstane-3α,17β-diol (5βAdiol), testosterone (T), and Epitestosterone (E) to measure deviations from previously measured values. Gas chromatography mass spectrometry (GC/MS) uses a combination of the free steroid fraction and the conjugated fraction released after hydrolysis of Escherichia coli (E-coli) with β-glucuronidase. Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC/C/IRMS) is used if the T/E ratio shows any deviation, or any testing authority wishes further examination.

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ABBREVIATIONS

List of all abbreviations in alphabetic order:

- **5αAdiol** 5α-Androstane-3α,17β-diol
- **5βAdiol** 5β-Androstane-3α,17β-diol
- **16-en** 5α-androst-16-en-3α-ol
- **A** Andosterone
- **AAF** Adverse Analytical Findings
- **ABP** Athlete Biological Passport
- **ADAMS** Anti-Doping Administration and Management System
- **ADVR** Anti-Doping Rule Violations
- **ASOIF** Association of Summer Olympic International Federations
- **ATF** Atypical Findings
- **ATPF** Atypical Passport Findings
- **C-17** Carbon number 17
- $^{13}C/^{12}C$ ^{13}Carbon/^{12}Carbon
- **CP** Confirmation Procedures
- **DBS** Dried Blood Spots
- **E** Epitestosterone
- **E-coli** Escherichia coli
- **EAAS** Endogenous Anabolic Androgenic Steroids

- **EPO** Erythoropoietin
- **EtG** Ethyl Glucuronide
- **Etio** Etiocholanolone
- **GC/C/IRMS** Gas Chromatography Combustion Isotope Ratio Mass Spectrometry
- **GC/MS** Gas Chromatography Mass Spectrometry
- **IC** In Competition
- **ITP** Initial Testing Procedure
- **NHS** National Health Service
- **NTNU** Norwegian University of Science and Technology
- **OOC** Out of Competition
- **PD** Pregnanediol
- **PT** Pregnanetriol
- **T** Testosterone

(PD), (PT), 5α-androst-16-en-3α-ol (16-en)

CHAPTER

ONE

INTRODUCTION

Doping has been used to enhance athletes since the beginning of competetions. The ancient Greeks, and later the Romans, were known for using performance enhancing drugs and stimulants for better results and for reducing fatigue [1]. In present time, the use of performance enhancing drugs is seen as a cheap way to get an extra edge over opponents. In 1928 the International Association of Athletics Federations were the first international sports federation to ban doping products [2]. A widely known drug used both in doping and medicine is anabolic steroids. It was first synthesized in Germany around 1935 and saw use in the 1954 Olympics by Russian weightlifters [3]. WADA 2021 anti doping test figures found that 972 of the 183,324 tests done in sports associated with the summer Olympics were positive, which equals 0.52% [4]. Anabolic steroids has become the most used performance enhancing drug making up 32 % of all hits from sports associated with the summer Olympic and 40 % of total sports in this report. This thesis will not focus on the medical side of steroids. The result part of this thesis is meant to represent why it is important to be able to detect the use of illegal substance use in competitions. The results section formed the basis for which performance enhancing drug was chosen to be discussed in the thesis. As mentioned above, anabolic steroids is the most used substance and was therefore chosen.

Athlete Biological Passport was introduced to be a tool in the fight against doping with its two modules, haematological and steroidal. The latter module will be the main focus of this thesis as it is used for detection of drugs in class S1, anabolic agents. The identification of endogenous steroids that has been administered exogenously will be the focus of the thesis. That means that the identification of selective androgen receptor modulators will not be included. The statistics used in the result part of the thesis is based on sports associated with the summer Olympics. These sports are diverse enough to give a representative overview of the situation regarding doping usage, while also narrowing down the amount of information that had to be collected for the thesis. The

thesis will introduce anabolic steroids and their effect as well as explain the different analytical methods used within the steroidal module for detection. These methods include Gas Chromatography Mass Spectrometry and Gas Chromatography Combustion Isotope Ratio Mass Spectrometry.

CHAPTER

TWO

THEORY

2.1 Anabolic steroids

Androgen, or androgenic hormone, binds to androgen receptors to stimulate or control the devolpment and maintainance of male characteristics [5]. This is usually a steroid hormone, and in men the primary secreted hormone is testosterone (T) which is shown in figure 2.3.1 [6]. The male characteristics include the activity of the male sex organs and the male secondary characteristics, which includes increased facial-, chest- and body hair, in addition to the ability to generate muscle mass at a faster rate than females [7]. This is called the androgenic effect. Androgens also have another effect called anabolic effect. The anabolic effect includes an increase in skeletal muscle mass and strength [8].

The anabolic effect is what people who use anabolic steroids want, as it helps building muscle and strength. Anabolic steroids are made from T in a laboratory, and it is used by both athletes and noncompeting people [9]. Noncompeting users might want bigger muscles to try to increase self esteem, a study found that men using anabolic steroids were less confident about their body appearance [10].

Anabolic steroids is widespread in competitive sports as well, as seen in tables 3.1.1 and 3.1.2. Athletes use it to gain an extra competitive edge over the other competitors. Anabolic steroids is split into two types, exogenous and endogenous steroids. Exogenous steroids are synthetically created versions of T and is not naturally produced in the body. Endogenous steroids are naturally occurring and are involved in the metabolic pathways of T [11].

When making anabolic steroids it is desired to have only the anabolic effect or at least close to only anabolic. Nonetheless, most anabolic steroids contain both androgenic and anabolic effects, and therefore has side effects. This is well seen in the HAARLEM study, where 100 men used anabolic steroids over a period of one year. All 100 men involved started a cycle and had a total of four visits to the lab to record results. A test

was taken at the end of the cycle and here 100% and 95% of the participants reported increase in strength and muscle mass respectively. Although all the participants grew stronger, they all experienced at least one negative effect during the cycle[12].

2.2 Side effects

There are many side effects associated with the use of anabolic steroids. The most frequent side effects according to Mottram and George[13] are listed below.

2.2.1 Cardiovascular

According to the National Health Service (NHS) cardiovascular disease is a general term for conditions affecting the heart or blood vessels [14]. As seen in the case of the 32 year old man who used steroids over a 4 year period, his heart experienced a slight enlargement that led to heart failure [15].

2.2.2 Liver problems

The liver is a vital organ for humans and is actively involved in detoxifying and metabolic functions while training. In a study by Arazi H. investigated, among other things, the effects of anabolic steroid use on the liver. The results show that the users had an elevated enzyme activity in the liver [16].

Elevated enzyme activity relates to inflammation or damages to cells in the liver. The reason for the increased activity is that damaged cells leak higher amounts of certain chemicals, including liver enzymes. This leaks into the blood and makes it possible to see the increased activity on blood tests [17].

2.2.3 Sexual side effects

One of the sexual side effects that relates to anabolic steroid use, is erectile dysfunction. There are many factors that might cause erectile dysfunction, like age and vascular problems, and anabolic steroids is known to cause side effects in the male reproductive system. Long term use of anabolic steroids may lead to decreased sperm count, acne, shrinking testicles, enlarged breasts, testicular cancer and male-pattern baldness [18].

2.2.4 Effects on libido

Libido is considered the sexual drive of a person or desires to engage in sexual activities.The HAARLEM study performed a one year long study on 100 men who used

anabolic steroids. At the end of the study, 58 % of the participants reported decreased libido [12].

2.2.5 Gynecomastia

Gynecomastia is increased breast size for men and is one of the sexual side effects of anabolic steroid use. It triggers when the T hormone decreases compared with estrogen in the body. This imbalance leads to an increase in the amount of breast gland tissue [19]. In the HAARLEM study, 26% of participants reported that they experienced an increase in breast size.

2.2.6 Tendon damage

There have been reports of increased tendon damage for anabolic steroid users. This is mostly in the case of bodybuilders. Anabolic steroid users increase their strength and muscle mass rapidly, but the steroids do not have any effects on the tendons. The combination of increased strength and using heavier weights may lead to damages in the tendons. In addition, anabolic steroids may inhibit the formation of collagen, which is important for the tendons. The steroids may also alter the plasticity of the tendons by inducing changes in the arrangement and contractility of the collagen fibrils in tendons [13].

2.2.7 Blood glucose regulation

The use of anabolic steroids may lead to increased resistance to insulin. This leads to a reduced tolerance to glucose and long term use can result in secondary diabetes with type II symptoms [13].

2.2.8 Psychiatric and behavioural effects

Anabolic steroids are not only effecting the physical aspects of a person. According to the National Institute on Drug Abuse, long term drug abuse may lead to the psychiatric effects; aggression, mania and depression [20]. This is also backed by Kanayama G. et al. although they mention that not all studies they found had documented these mood changes [21]. Usage of anabolic steroids may also lead to depression, but it is less certain and is backed by less evidence. Users often struggle with their self-image which may contribute to a possible depression.

2.2.9 Miscellaneous effects of anabolic steroids

It is not only the drug that has negative effects. If a sample is fake or contaminated with impurities this could lead to unintended effects. Injection with a needle contaminated by another persons blood may lead to spreading of illness.

2.3 Modification

T is produced in the body in both men and women, but plays a big part as the major male sex hormone. If T is non modified, then it can not be taken orally. The methods used to administer non modified T are intramuscularly, sublingually or by transcutaneous patch. Intramuscularly is done by injecting a needle 5 to 10 mm into the muscle tissue. Sublingually is done by placing a tablet under the tongue, where it is dissolved and absorbed by the tissues under the tongue. Transcutaneous patch is a patch placed on the skin. The patch contains medication and is absorbed over time. At the carbon number 17 of T, as seen in figure 2.3.1, there are two available modifications. One method is to modify T through C-17β esterification as seen in figure 2.3.3. The T still contains the virilizing effect, where women develops male hormones (androgens), but the potency has been increased and the effects lasts longer [22]. Another modification is C-17α alkylation. This makes it so it can be taken orally, because it inhibits the metabolic deactivation in the liver. The C-17α modified T, shown in figure 2.3.4, is often well tolerated and has a limited virilizing effect[23]. Both of these modified Ts are still linked with implications. C-17α is linked with liver injury including prolonged cholestasis and hepatocellular carcinoma. C-17β is also linked with liver injury, but at a lower rate than the C-17α modification

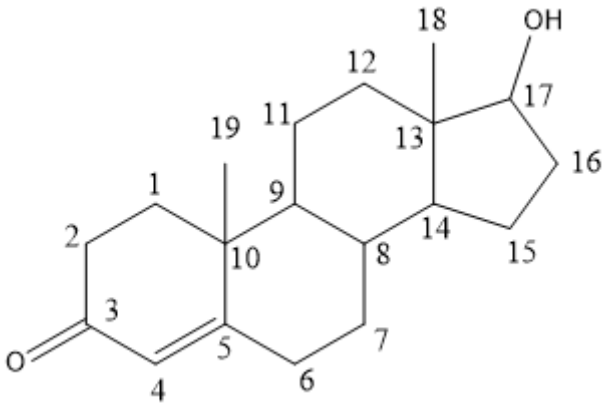

Figure 2.3.1: Testosterone hormone including the numbering of the carbons. Numbering based on figure by Mottram and George [13].

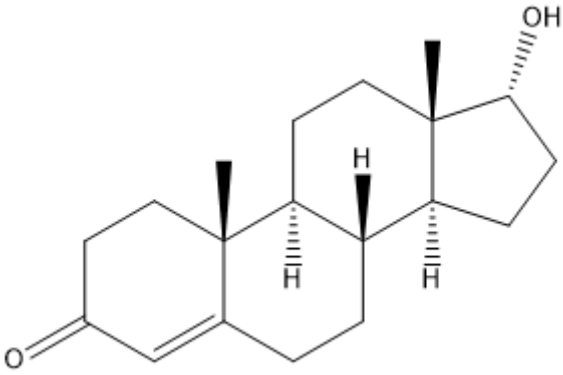

Figure 2.3.2: Epitestosterone, an 17α-epimer of testosterone. It is used in conjunction with testosterone to detect doping.

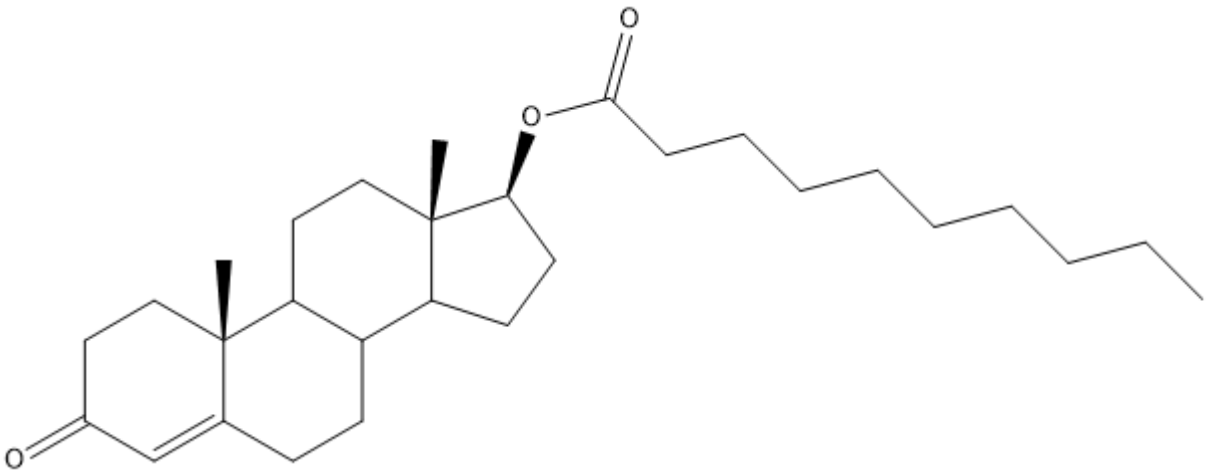

Figure 2.3.3: Nandrolone

Nandrolone, a C-17β esterification of testosterone. Figure adapted from National Institute of Diabetes and Digestive and Kidney Diseases [23]

2.4 Method of usage

Three known methods of usage include ”cycling”, ”stacking” and ”pyramiding”. The method ”cycling” is based on an active period and a rest period. The active period is when users take drugs. ”Stacking” is a method where users take multiple types of anabolic steroids. When combining ”cycling” and ”stacking” it creates ”pyramiding”. In ”pyramiding” users starts of with a low dose of one or more anabolic steroids and gradually increases the dose. When the dose has been increased to the maximum, the user switches to a rest period. The reason these methods are used is to reduce the side effects of the anabolic steroids, although there is no evidence for this.

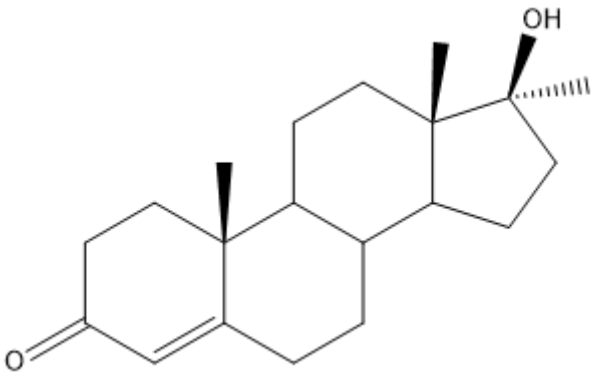

Figure 2.3.4: Methyltestosterone
Methyltestosterone, a C-17α alkylation of testosterone. Figure adapted from National Institute of Diabetes and Digestive and Kidney Diseases [23]

CHAPTER

THREE

RESULTS

3.1 Results

Doping has long been a part of sport, where athletes push themselves to the limit. As athletes push themselves to the absolute limit to become best, it might seem tempting to have a little help. Some athletes resort to performance enhancing drugs to get an extra edge over other competitors. This chapter contains test data from 2021 from sports affiliated with the summer Olympics. The collected data was used to form a basis for the thesis by representing the need for anti-doping. The data was additionally used to choose the most reoccurring performance enhancing drug, which is shown to be anabolic steroids.

Adverse Analytical Finding (AAF) and Atypical Finding (ATF) are used in table 3.1.1. AAF is defined in the World Anti-Doping Code as ”A report from a WADA-accredited Laboratory or other WADA-approved Laboratory that, consistent with the International Standard for Laboratories and related Technical Documents, identifies in a Sample the presence of a Prohibited Substance or its Metabolites or Markers (including elevated quantities of endogenous substances) or evidence of the use of a Prohibited Method” [4]. Due to some athletes using medication with approval, the AAF does not necessarily reflect the number of sanctioned cases. ATF is defined in the World Anti-Doping Code as ”A report from a WADA accredited Laboratory or other WADA-approved Laboratory which requires further investigation as provided by the International Standard for Laboratories or related Technical Documents prior to the determination of an Adverse Analytical Finding” [4]. 2 or more ATF values may correspond to an athlete as in the cases of longditudal studies on testosterone.

Table 3.1.1: Total samples analyzed in ASOIF sports in 2021. Samples was taken out of competition (OOC) and in competition (IC). The samples were from urine, blood or dried blood spots (DBS). The tests indicated adverse analytical findings (AAF) and atypical findings (ATF). Table modified from WADA [4]

	Urine						Blood						DBS						
	IC			OOC			IC			OOC			IC		OOC		Total	Total	%
Sport	Sample	ATF	AAF	Sample	ATF	AAF	Sample	ATF	AAF	Sample	ATF	AAF	Sample	AAF	Sample	AAF	Samples	AAFs	AAF
Aquatics	5302	1	71	9434	3	17	248	1	-	1139	-	-	-	-	120	-	16,263	88	0.5%
Archery	514	5	11	562	-	-	-	-	-	7	-	-	-	-	7	-	1,090	11	1.0%
Athletics	11633	25	103	16467	21	70	532	-	1	2419	-	10	3	-	123	-	31,178	184	0.6%
Badminton	532	-	1	930	1	1	14	-	-	141	-	-	-	-	14	-	1,631	2	0.1%
Basketball	2563	1	19	2545	5	5	56	-	-	186	-	-	-	-	9	-	5,371	24	0.4%
Boxing	1124	1	28	2663	4	8	41	-	-	265	1	-	24	-	29	-	4,146	36	0.9%
Canoe	1673	3	14	2957	6	5	78	-	-	430	-	-	-	-	33	-	5,171	19	0.4%
Cycling	8800	10	112	9471	12	28	714	2	2	1599	1	4	-	-	33	-	20,617	146	0.7%
Equestrian	345	-	5	561	3	1	-	-	-	30	-	-	-	-	-	-	936	6	0.6%
Fencing	623	2	3	1084	6	-	2	-	-	98	-	-	-	-	18	-	1,825	3	0.2%
Field Hockey	494	-	4	1099	-	-	26	-	-	64	-	-	-	-	19	-	1,706	4	0.2%
Football	18134	15	58	11406	17	10	669	-	-	1431	-	-	-	-	31	-	31,671	68	0.2%
Golf	300	-	2	263	-	5	1	-	-	13	-	-	-	-	4	-	571	7	1.2%
Gymnastics	1152	5	12	2243	4	3	55	-	-	168	-	-	-	-	23	-	3,641	15	0.4%
Handball	1475	-	11	2023	3	3	37	-	-	213	-	-	-	-	-	-	3.747	14	0.4%
Judo	1266	3	17	2725	3	4	31	-	-	241	-	-	-	-	6	-	4,269	21	0.5%
Modern Pentathlon	249	-	-	495	-	1	1	-	-	68	-	-	-	-	4	-	817	1	0.1%
Rowing	1134	1	6	3560	1	4	85	-	-	615	-	-	-	-	70	-	5.465	10	0.2%
Rugby Union	2139	2	42	3920	3	17	40	-	-	191	-	-	-	-	13	-	6,622	59	0.9%
Sailing	387	-	-	777	-	1	2	-	-	46	-	-	-	-	14	-	1,226	2	0%
Shooting	921	-	7	1204	-	3	-	-	-	26	-	-	-	-	25	-	2,176	10	0.5%
Table Tennis	313	-	3	670	2	-	13	-	-	53	-	-	-	-	8	-	1,057	3	0%
Taekwondo	610	1	4	1125	6	4	33	-	-	87	-	-	-	-	14	-	1,870	8	0.4%
Tennis	3989	2	27	1358	-	3	5	-	-	899	4	-	-	-	5	-	6,256	30	0.5%
Triathlon	1335	1	18	1373	1	8	122	-	-	222	-	3	5	-	9	-	3,166	29	0.8%
Volleyball	1595	1	11	1941	3	5	70	-	-	204	-	-	-	-	21	-	3,831	16	0.4%
Weightlifting	3541	7	72	5359	10	23	714	-	-	905	2	3	4	1	39	1	10,602	100	0.9%
Wrestling	2324	7	43	3537	9	11	105	-	-	418	2	2	-	-	23	-	6.407	56	0.8%
Total	74,567	93	705	91,761	123	240	3,694	3	3	12,500	10	22	76	1	726	1	183,324	972	0.52%

Table 3.1.2: Samples analyzed from drug classes in ASOIF sports in 2021. Table modified from WADA [4].

Sports	Drug Classes												Findings
	S1.	S2.	S3.	S4.	S5.	S6.	S7.	S8.	S9.	P1.	M1.	M2.	
Aquatics	11	2	9	33	6	26	-	1	2	-	-	-	90
Archery	4	-	-	-	4	1	-	-	1	3	-	-	13
Athletics	97	29	5	23	13	22	2	5	16	-	4	-	216
Badminton	-	-	-	-	2	-	-	-	-	-	-	-	2
Basketball	3	1	-	2	2	5	-	10	3	-	-	-	26
Boxing	8	1	2	7	15	3	-	4	2	-	-	-	42
Canoe	7	-	2	10	3	4	-	-	2	-	-	-	28
Cycling	51	29	11	11	13	48	1	4	25	-	-	-	193
Equestrian	-	-	1	-	-	3	-	1	2	-	-	-	7
Fencing	-	-	1	-	-	1	-	1	-	-	-	-	3
Field Hockey	-	-	-	-	-	3	-	-	1	-	-	-	4
Football	15	3	3	4	9	23	1	5	11	-	-	-	74
Golf	-	-	-	1	4	3	-	-	-	-	-	-	8
Gymnastics	4	-	1	1	3	4	-	-	5	-	-	-	18
Handball	4	-	-	2	1	11	-	1	2	-	-	-	21
Judo	6	-	-	4	9	4	-	1	2	-	-	-	26
Modern Pentathlon	1	-	-	-	-	-	-	-	-	-	-	-	1
Rowing	1	-	-	4	1	6	-	-	-	-	-	-	12
Rugby Union	19	1	1	8	7	15	-	11	9	-	-	-	71
Sailing	-	-	-	1	-	1	-	-	-	-	-	-	2
Shooting	-	-	-	-	6	1	-	-	-	4	-	-	11
Table Tennis	-	-	-	-	2	2	-	-	-	-	-	-	4
Taekwondo	2	-	-	-	4	1	-	1	-	-	-	-	8
Tennis	8	-	-	3	4	13	-	1	2	-	-	-	31
Triathlon	9	12	4	1	-	6	-	-	1	-	-	-	33
Volleyball	7	-	-	4	3	2	-	4	2	-	-	-	22
Weightlifting	77	5	2	16	13	14	1	3	5	-	-	-	136
Wrestling	42	4	2	10	8	9	1	2	2	-	-	-	80
Total	376	87	44	145	132	231	6	55	95	7	4	0	1182
Total %	32%	7%	4%	12%	11%	20%	1%	5%	8%	0.6%	0.3%	0%	

Table 3.1.3: Drug classes tested for in ASOIF sports in 2021. The drug classes are described in table 3.1.3.

Drug Class	Class Contents
S1.	Anabolic Agents
S2.	Peptide Hormones, Growth Factors and Related Substances
S3.	Beta-2 Agonists
S4.	Hormone and Metabolic Modulators
S5.	Diuretics and Other Masking Agents
S6.	Stimulants
S7.	Narcotics
S8.	Cannabinoids
S9.	Glucocorticosteroids
P1.	Beta-Blockers
M1.	Enhancement of Oxygen Transfer
M2.	Chemical and Physical Manipulation

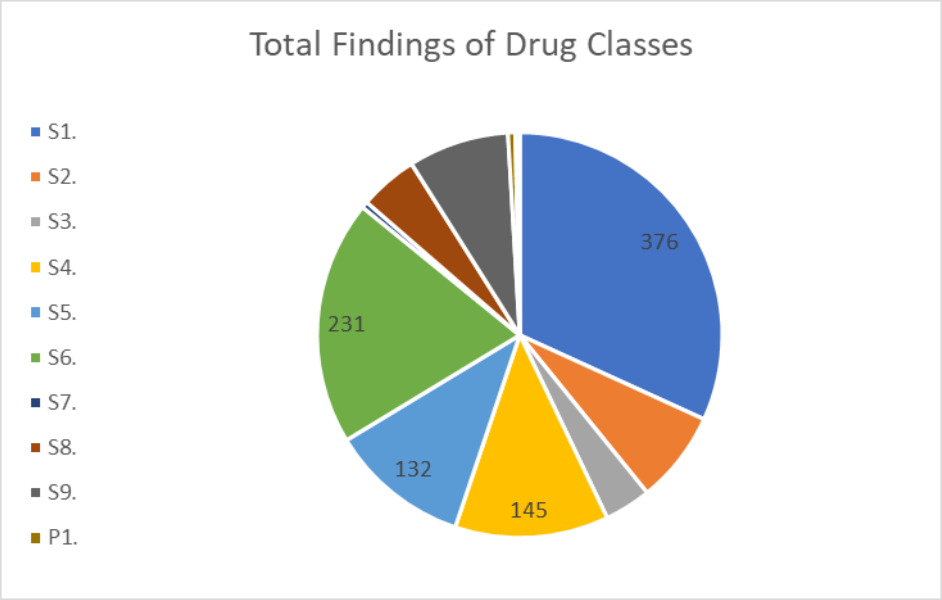

Figure 3.1.1: Findings of each drug class based on table 3.1.2. The different parameters are the drug classes and is described in table 3.1.3.

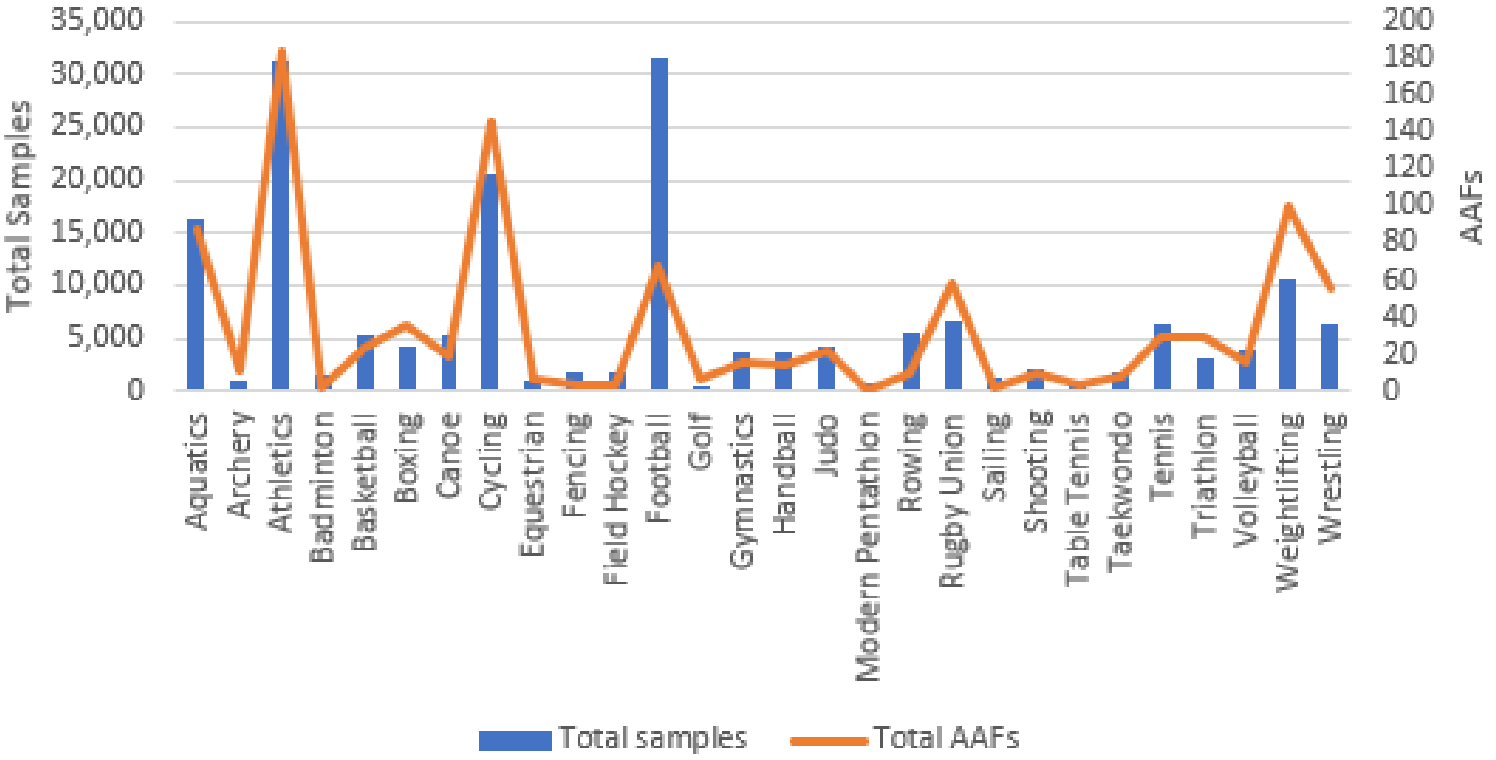

Figure 3.1.2: Total samples in conjunction with total adverse analytical findings (AAF) found in ASOIF sports in 2021.

CHAPTER

FOUR

DISCUSSION

When analyzing whether or not athletes are using performance enhancing drugs, samples first needs to be taken. These samples can be taken from urine, blood and dried blood spots (DBS) as seen in table 3.1.1. It is important to differentiate between the two types of anabolic steroids. As mentioned earlier, there are exogenous steroids and endogenous steroids. The exogenous steroids are made synthetically, while the endogenous steroids are naturally occurring substances involved in the metabolic pathways of T in the human body [11].

4.1 ABP

An athletes biological passport (ABP) is a electronic record that contains personalized data about selected biological values. The values are monitored over time and the use of ABP will expose any changes relating to the selected biomarker(s). This can be done because the ABP uses an Baysian statistical approach to calculate individual reference ranges based on both the population and the athletes previous results[24]. This makes it possible to pursue possible Anti-Doping Rule Violations (ADVR) in accordance with article 2.2 of the World Anti-Doping Code (Use or attempted use by an athlete of a prohibited substance or a prohibited method)[25]. ABP might be a essential tool in detecting and reducing doping in sports, as it can indirectly reveal the effects of doping without attempting to find the specific substance or method used. The passport can also be used in other anti-doping related decisions. This includes, but is not limited to, the decision to collect additional samples from an athlete to be analyzed by other analytical methods, investigate an athlete or a group of athletes, or take a specific sample to be re-analyzed at a later date[26].

4.2 ABP Modules

The guidelines and mandatory standards for the ABP was published in 2009 and was used to as a means to define a persons hematological profile. This was called the Hematological Module. In 2014, a new module was established which was called the Steroidal Module.

4.2.1 Hematological Module

The module gets it's name from hematology, which is the study of blood. This module collects information about possible blood doping by monitoring selected hematological variables. This module includes substances and methods that enhance oxygen transport or delivery. It also includes Erythropoiesis-Stimulating Agents and any form of blood transfusion or manipulation. The module primarily identifies the methods from section M1 and Erythropoiesis-Stimulating Agents, included in section S2[27] from table 3.1.1.

4.2.2 Steroidal Module

The steroidal passport of an athlete is built by using the steroid profile of urine samples over time. The steroidal module serves to gather information on markers of steroid doping, including endogenous anabolic androgenic steroids (EAAS) when administered endogenously and other anabolic agents such as selective androgen receptor modulators. The Steroidal Module has an added advantage of being capable of detecting whether the urine sample has been tampered with or exchanged with the urine of someone else. Markers considered in the steroidal module includes Andosterone (A), Etiocholanolone (Etio), 5α-Androstane-3α,17β-diol (5αAdiol), 5β-Androstane-3α,17β-diol (5βAdiol), T, and Epitestosterone (E)(figure 2.3.2). It also includes specific ratios like T/E, A/T, A/Etio, 5αAdiol/5βAdiol, and 5αAdiol/E, as detailed in the Technical Document on Measurement and Reporting of (EAAS) Markers of the Urinary Steroid Profile [27]. If any values deviate, the module triggers Atypical Passport Findings (ATPFs), which might lead to confirmation procedure (CP), target testing of an athlete or to establish a violation of article 2.2 of the World Anti Doping Code.

4.3 Measuring

The determination of the steroid profile is based on two procedures; initial testing procedure (ITP) by use of gas chromatography mass spectrometry (GC/MS) and CP by use of chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS).

4.3.1 Initial testing procedure (ITP)

The ITP is conducted to estimate the steroid profile by the use of gas chromatography mass spectrometry (GC/MS). GC is used by evaporating substances and then separating the compounds by propelling them through capillary columns coated by a stationary phase. The compounds are propelled by the use of inert gas like nitrogen, hydrogen or helium. The separation is based on the compounds polarity and boiling point, as they elude at different times. The separated compounds are then ionized and fragmented by the use of a mass spectrometry. The ions are separated based on their different mass-to-charge by acceleration through the mass analyser. The result of GC/MS is many different peaks in the GC and a unique mass spectrum for each of the peaks [28]. This makes GC/MS a sensitive method that can be highly specific when used in identification and quantification of steroids in urine [29]. The steroid profile markers A, Etio, 5αAdiol, 5βAdiol, T and E, are determined by a combination of free steroid fraction and conjugated fraction released after hydrolysis of *Escherichia coli* (E-coli) with β-glucuronidase. In addition to the markers themselves, ratios between markers is determined and analyzed. The ratios are: T/E, A/T, A/Etio, 5αAdiol/5βAdiol, and 5αAdiol/E. The T/E is the primary parameter and will be discussed later in the thesis. The other ratios are evaluated by Athlete Biological Passport Managment Unit and might lead to further analysis or actions, if ATPF is triggered by deviating values.

4.3.1.1 Free steroid fraction

Steroids are only biologically active when they are free, unbound. They are lipophilic and can therefore diffuse across the plasma membrane [30, 31]. The results from measuring the fraction of free steroids reflect the amount of steroid that are biologically active. By using free steroids instead of total steroids, one might get more information as a majority of steroids are inactive.

4.3.1.2 Conjugated fraction

Conjugated steroids need to be deconjugated before they can be analyzed by GC/MS. This is because conjugated steroids degrade at the temperatures needed to perform the GC/MS analysis. This can be done by hydrolysis of the conjugate by either enzymatic (biologically) or non-enzymatic (chemically) means. Most used is the enzymatic means. There are three main sources of enzymes; Mammalian, Molluscs and bacterial. Mammalian, extracted from beef liver containing Ketodase. Molluscs, Helix pomatia plus some lesser sources. The enzymes in the bacterial source comes from E-coli and that is the source used by WADA [27, 32] in ITP. Ketodase was originally used in doping detecting due to it possessing β-glucuronide activity, but was later switched to E-coli.

This might be due to the high specificity of β-glucuronide when E-coli is hydrolysed. This activity allows for cleaving of glucuronide moiety which makes it possible to analyze. An example figure of testosterone glucuronide is shown in figure 4.3.1 with the glucuronide moiety on the right connected to the oxygen on C-17 on testosterone. High amounts of conjugated androgen exits the body via urine, and the reason may be because of phase II metabolism. The phase II metabolism that steroids go through is the conjugation with the glucuronide moiety. This makes the steroid more hydrophilic and promotes urinary excretion. It also works with sulfate moiety for some steroid androgens like T and A. The urinary steroids must also be derivatized before the sample can be analyzed. By using derivatisation on the sample, it will improve its volatility, thermal stability and peak shape. This method has the trade off of being time consuming and requires a lot of effort, but enhances separation and detection.

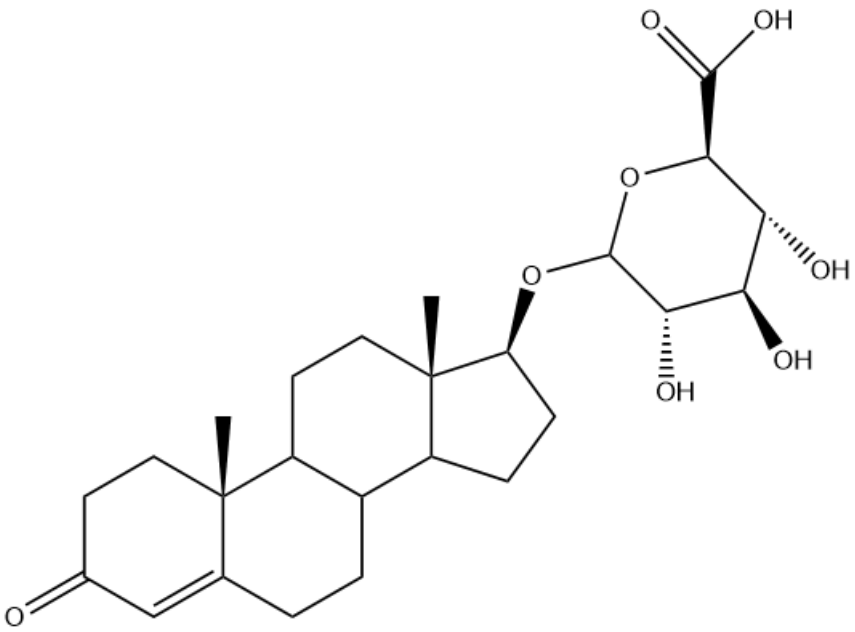

Figure 4.3.1: Testosterone glucuronide, glucuronide moiety on the right connected to the oxsygen on C-17 on testosterone. Inspired by Gomes et al.[32]

4.3.1.3 T/E ratio testing

Any APB deviation detected from the individual values of the athlete triggers an ATPF. If the ATPF is based on a high T/E ratio, then a CP will be requested. The ratio is around 1 to 1 in a male body for a non user and higher in the favor of T for users. The amount of T will increase in the case of usage of exogenous steroids, but the steroids will not increase E [32]. E is produced in the testicles in response of luteinizing hormone [33]. As the source of T is subjected to a strong negative hypothalamic feedback, the body says no more production and therefore no more production of E either. When the T/E ratio

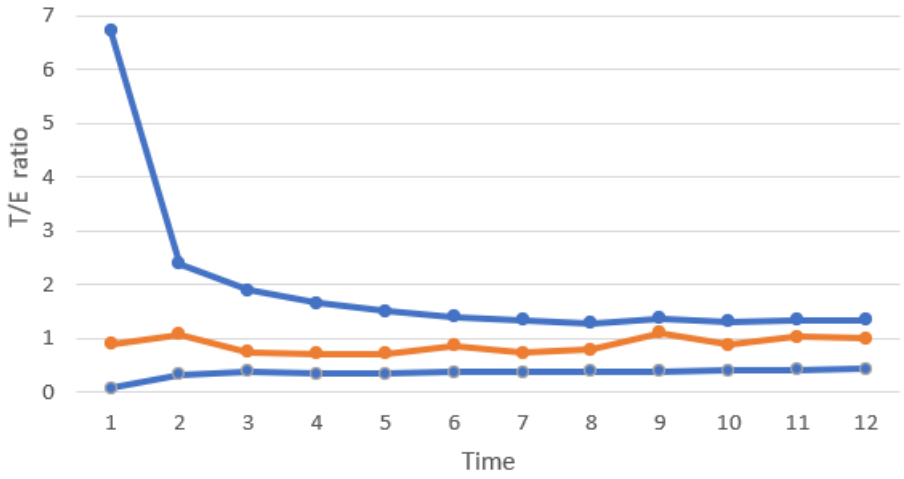

Figure 4.3.2: Example of how a steroid profile generated by the Bayesian model of the ABP for the T/E ratio parameter. The blue lines are the individual limits, and the orange line represents the measured T/E values. Time represents number of times the athlete has been tested. Inspired by Kuuranne et al.[35]

exceeds a specified threshold it can be used as evidence for doping usage. This method however can not be used for women because their T comes from three sources (instead of one), where none of the sources are subjected to strong hypothalamic feedback [34]. Another drawback is a genetic polymorphism of a specific gene that leads to a biological false negative. Because of this false negative, the threshold for the usual population-based T/E ratio will not be exceeded. However, the ratio will exceed the individuals specific ratio and the ABP detects this [34].

4.3.2 GC/C/IRMS

Laboratories that receive the CP request confirms the previous results by GC/MS before using GC/C/IRMS. GC/C/IRMS can measure very small differences in the ^{13}Carbon/^{12}Carbon (^{13}C/^{12}C) ratio by using an endogenous reference compound. This ^{13}C/^{12}C ratio is reported as δ-value and has units per mille (‰). The reference compound needs to be a precursor of T or a member of another steroid pathway, and which is not affected by abusing T [36]. The reference compound also compensates for inter-athlete variations [37]. The calculation used for the absolute difference in $\delta^{13}C$ is

$$|\delta^{13}\mathrm{C}| = |\delta^{13}\mathrm{C}_{\mathrm{EndoRef}} - \delta^{13}\mathrm{C}_{\mathrm{TargetComp}}| \tag{4.1}$$

where the endogenous reference compounds are i.e. pregnanediol (PD), pregnanetriol (PT), 5α-androst-16-en-3α-ol (16-en), and target compounds are the markers of the steroid profile. 40 individuals (20 male and 20 female) volunteers to provide a urine sample as reference for the reference population data. All samples are analyzed using

endogenous reference compound and target component pairs. It is mandatory to use at least two reference compounds and one of them has to be PD [38]. The synthetic steroids are made commercially from plant sterols produced by photosynthesis and therefore has a slight difference in δ-value from the endogenous reference compound. Athletes might be sanctioned based on these deviations. The sample preparation also includes hydrolysis of E-coli with β-glucuronidase, but is further purified by high performance liquid chromatography prior to the analysis. The technical document for GC/C/IRMS analysis by WADA states that if an AAF is reported for sample ”A”, then GC/C/IRMS and GC/MS should be repeated on the sample ”B” if applicable [38]. This might lead to fewer false positives, as two samples gives two tries and could exclude a false AAF if the samples give different results.

4.4 Advantages and Limitations

There are certain cases where normal means of detecting illegal use of performance enhancing substances might not be the best choice. In the 1964 winter Olympics, a Finn won two gold medals in cross-country skiing, but had unusually high levels of erythoropoietin (EPO). The cause of this was a genetic mutation that caused his body to produce extra EPO which is a hormone that regulates the production red blood cells in the bone marrow and increased his oxygen carrying potential by 25-50% [39, 40, 41]. The values of his biomarkers would probably exceed the normal amount, even if he hadn’t taken any illegal substances. One solution to avoid these false positives are the ABP. This is because ABP measures values over a long time and takes into account previous values from the athlete. Athletes with mutations are not doing anything illegal just by having an innate ability to jump higher, run faster or endure running for longer periods of time. A mistake in the doping tests should be avoided as false positives might damage an athletes carrier. That is also why it is important to be able to confirm the results of the test, like ABP having an initial test and a confirmation of an eventual positive test.

In addition to a functional tool for detecting doping, APB might also have a deterring effect on athletes considering performance enhancing drugs. When considering using these substances, most athletes likely consider the chances of being caught. ABP provides a tool that even detects doping that is meant to be hard to detect by watching biological markers in the athletes body. Zorzoli and Rossi[42] indicates that the deterring effect could hold true as statistics from cycling in 2010 shows a decline in blood doping following the years after the hematological module was established.

ABP has some limitations as well as advantages. The method has only got two modules that cover only three of the twelve drug classes, that being S1, M1 and Erythropoiesis-

Stimulating Agents from class S2. That being said steroids represent around 40 % of used substances, as shown figure 3.1.1. ABP might benefit from expanding their detection to more drug classes. Krumm et al. discusses a new profile in development, the blood steroid profile, which uses ultra-high performance liquid chromatography-high-resolution mass spectrometry and will complement the steroidal module [43]. The hematological is impacted by plasma volume and erythropoiesis from for example physical exercises and altitude training respectively [43]. The steroidal module is impacted by administration of EAAS, but the profile can also be altered by other factors. Factors include, but is not limited to, exogenous factors like alcohol intake, endogenous factors like menstrual cycle, sample manipulation and administration of other anabolic androgenic steroids[27]. These factors might trigger a CP and return a negative after analysis by GC/C/IRMS. Take the example of alcohol, here the T/E ratio increases although no steroids has been used and that might lead to many unnecessary GC/C/IRMS confirmations. It has been found that the A/T ratio decreases when consuming ethanol[44] and might therefore be used as an indication. To further strengthen the probability of knowing if ethanol has been consumed, a reporting level of Ethyl Glucuronide (EtG) was implemented. EtG is a urinary metabolite and is strongly correlated with consumption of ethanol. With a high value of EtG may invalidate the related steroid profile to maintain the probative forces of the ABP. The procedure for cases with elevated T/E ratios and EtG concentrations, is to perform GC/C/IRMS the first time this happens for an athlete. It is optional to perform the test for any subsequent cases. This might be done so that athletes trying to administrate T in conjunction to ethanol to mask it.

CHAPTER

FIVE

CONCLUSIONS

Doping is used by athletes to increase their performance in competitions. Among the substances used by athletes, anabolic steroids is the most used. This is based on the WADA report for 2021[4] where results showed that usage of anabolic steroids constituted 32 % of total doping usage in sports associated with the summer Olympics. In addition to being illegal to use in competition, it is also dangerous to the health of the athletes boasting several side effects. The importance of detecting doping is vital for a fair competition and the responsibility falls on the analysts. The ABP was developed to be able to detect changes in the individual biological values of athletes with its two modules. The steroidal module makes use of GC/MS to measure the biological markers A, Etio, 5αAdiol, 5βAdiol, T and E. These are determined by a combination of free steroid fraction and conjugated released after hydrolysis of Escherichia coli (E-coli) with β-glucuronidase. Deconjugation is important for the sample so it can be analyzed by GC/MS and that is done by enzymatic hydrolysis of E-coli. A CP is requested if the initial test reveals a deviation in the ratio between T and E. The sample will then be checked by GC/C/IRMS. GC/C/IRMS is able to measure the ratio between $^{13}C/^{12}C$ by using an endogenous reference compound. The steroids are made from plant sterols and will therefore have a different ratio than the reference compound. ABP is a useful tool to detect performance enhancing drugs, as it can detect a difference in the markers without the need to know what kind of doping was used. Athletes with naturally high biomarker values could benefit greatly from ABP, as it would identify no fluctuations in their values over time and thus eliminate any doubt about their use of performance-enhancing drugs. ABP is limited in which drug classes it can detect due to only having two modules. ABP could benefit from continuing to improve the existing modules and if possible, introduce new modules.

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