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URINARY STEROID PROFILES IN DOPING TESTING

- In relation to natural variation and drug administration

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Oliver and Lucas

ABSTRACT

To detect doping with endogenous steroids, such as testosterone, biomarkers in urine are measured. These biomarkers include testosterone and some metabolically related steroids. The measured steroids are combined into ratios and together they make up the steroid profile. This steroid profile is followed over time in the Steroid Module of the Athlete Biological Passport. The software used for the passport, calculates individual reference ranges based on the previous results and gives atypical findings if one or more biomarker goes outside of the reference ranges.

All passports are evaluated by experts and all atypical findings are assessed. Evaluating steroidal passports is however difficult since factors, other than doping, can affect the biomarkers of the steroid profile. In this thesis, we evaluated natural variations of the steroid profile, including variations during the menstrual cycle and pregnancy, as well as how certain drugs, such as hormonal contraceptives and testosterone, affect the steroid profile.

In a study of over 11 000 steroid profiles we have seen that intra-individual variation in the steroid profile is large (16-27% in men and 23-40% in women), but that inter-individual variation is larger (49-76% in men and 55-84% in women). Some of this variation could be explained by annual and diurnal variation, with time of day having a larger impact on makers of the steroid profile. Another confounder to consider when evaluating passports is if the urine was collected in competition or not, a factor that could explain over 6% of the total inter-individual variation of some ratios. We have also seen that the menstrual cycle affect biomarkers of the steroid profile and that hormonal contraceptives can give patterns on the steroid passports similar to micro-doping with T. Pregnant women also show great differences in their steroid profiles as compared to non-pregnant women.

We have seen that doping with as low as 125 mg T enanthate and 100 mg T gel can be detected with the ABP. However, it is possible that the large natural variation as well as confounding factors, such as permitted drugs, will conceal the effect of doping.

The goal of studying confounding factors in steroid profiling is to provide the scientists evaluating the passport with sharper tools, not only to select the profiles suspicious of doping, but also to be able to reject and not spend unnecessary time and resources on profiles showing atypical results due to natural causes. The ultimate goal is to be able to proceed with a passport case, where the steroidal passport is the only evidence of doping.

LIST OF SCIENTIFIC PAPERS

I. **Dose-dependent testosterone sensitivity of the steroidal passport and GC-C-IRMS analysis in relation to the UGT2B17 deletion polymorphism.**
Strahm E*, Mullen JE*, Gårevik N, Ericsson M, Schulze JJ, Rane A, Ekström L, *Drug Test Anal.* 2015 Nov-Dec;7(11-12):1063-70. doi: 10.1002/dta.1841.

II. **Sensitivity of doping biomarkers after administration of a single dose testosterone gel**
Mullen J, Börjesson A, Hopcraft O, Schulze JJ, Ericsson M, Rane A, Lehtihet M, Ekström L, *Drug Test Anal.* 2017 Nov 18. doi: 10.1002/dta.2341

III. **The impact of genetics and hormonal contraceptives on the steroid profile in female athletes.**
Schulze JJ*, Mullen JE*, Berglund Lindgren E, Ericsson M, Ekström L, Hirschberg AL, *Front Endocrinol* (Lausanne). 2014 Apr 10;5:50. doi: 10.3389/fendo.2014.00050.

IV. **Urinary steroid profiles in females – the impact of menstrual cycle and emergency contraceptives.**
Mullen JE, Thörngren JO, Schulze JJ, Ericsson M, Gårevik N, Lehtihet M, Ekström L., *Drug Test Anal.* 2016 Oct 18. doi: 10.1002/dta.2121.

V. **Pregnancy greatly affect the steroidal module of the athlete biological passport**
Mullen J*, Gadot Y*, Eklund E, Andersson A, Schulze JJ, Ericsson M, Lindén Hirschberg A, Rane A, Ekström L. *Drug Test Anal.* 2018 Jan 18. doi: 10.1002/dta.2361.

VI. **Inter-individual variations of the urinary steroid profile in Swedish and Norwegian athletes**
Mullen J, Vestli Bækken L, Törmäkangas T, Ekström L, Ericsson M, Hullstein I, Schulze JJ, *Manuscript*

*The authors contributed equally to this work

Related work:

Mullen JE, Gårevik N, Schulze JJ, Rane A, Björkhem Bergman L, and Ekström L, *Perturbation of the Hematopoietic Profile by Anabolic Androgenic Steroids*, Journal of Hormones, vol. 2014, Article ID 510257, 2014. doi:10.1155/2014/510257

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LIST OF ABBREVIATIONS

19-NA	19-norandrosterone
11-OH-A	11β-hydroxyandrosterone
11-oxo-Etio	11-keto-etiocholanolone
16-en	5α-androst-16-en-3α-ol
5αAdiol (or 5α-diol)	5α-androstane-3α,17β-diol
5αAdiol/ 5βAdiol	5α-androstane-3α,17β-diol to 5β-androstane-3α,17β-diol ratio
5αAdiol/E	5α-androstane-3α,17β-diol to epitestosterone ratio
5αAND	5α-androstanedione
5α-diol/5β-diol	Same as 5αAdiol/ 5βAdiol
5βAdiol (or 5β-diol)	5β-androstane-3α,17β-diol
5βAND	5β-androstanedione
A	Androsterone
A/Etio	Androsterone to etiocholanolone ratio
A/T	Androsterone to testosterone ratio
AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete biological passport
ACTH	adrenocorticotrophic hormone
ADAMS	Anti-doping administration & management system
APMU	Athlete passport managemnet unit
AR	Androgen receptor
ATPF	Atypical passport finding
CV	Coefficient of variation
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
E	Epitestosterone
EAAS	Endogenous anabolic steroids
EC	Emergency contraceptive
ERC	Endogenous reference compound
Etio	Etiocholanolone
FSH	Follicle-stimulating hormone
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GnRH	Gonadotropin-releasing hormone
Hb	Hemoglobin
HC	Hormonal contraceptives
HPG-axis	Hypothalamic-Pituitary-Gonadal axis
IC	In competition
IRMS	Gas chromatography-combustion-isotope-ratio mass spectrometry
LH	Luteinizing hormone
LOQ	Limit of quantification
OOC	Out of competition
PD	Pregnanediol
rtPCR	Real time polymerase chain reaction
SG	Specific gravity

SHBG	Sex hormone binding globulin
SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
T	Testosterone
T/E	Testosterone to epitestosterone ratio
TC	Target compound
TD	Technical Document
TMS	Trimethylsilicyl
TUE	Therapeutic use exemption
UGT	Uridine diphospho-glucuronosyl transferase
WADA	World Anti-Doping Agency
WADC	World Anti-Doping Code

1 PREFACE

Steroid profiling is the current method in doping analysis for detecting the use of steroids identical to endogenous steroids, e.g. Testosterone (T). In steroid profiling, several steroids that are metabolically related to T are measured and followed over time in the Athlete Biological Passport (ABP). Testing for substances identical to those of endogenous origin is challenging since they by definition already exist in the body. To longitudinally study the steroid profile and look for atypical patterns has proven to be a sensitive way to detect these substances. However, steroid levels fluctuate naturally and are affected by internal as well as external factors. Consequently, the steroid profiles are not easy to interpret and it is of interest for those evaluating the profiles to know the extent of these variations, what they depend on and how they change when someone is doping with T.

This thesis investigates the stability of the steroid profiles during different conditions. In the first part of this thesis (Study I and II), low doses of testosterone were administered to healthy men and the steroid profile was longitudinally monitored. In **Study I**, three doses of T enanthate were injected to 25 healthy subjects and in **Study II**, a one-time dose of T gel was given to 8 subjects. In the second part of this thesis (Study III-V), the steroid profile was studied in women in relation to hormonal contraceptive use (**Study III**), menstrual cycle and emergency contraceptive use (**Study IV**) as well as during pregnancy (**Study V**). Lastly, the general stability of the steroid profile was investigated in a large population of athletes (**Study VI**).

For purposes of this thesis, "exogenous" refers to a substance which is not naturally produced by the body, whereas "endogenous" refers to a substance which is naturally produced by the body, even when administered externally (i.e. T).

2 INTRODUCTION

2.1 TESTOSTERONE DOPING

T and its synthetic analogues, which together make up the group of anabolic androgenic steroids (AAS), are included in the World-Anti Doping Agency (WADA) list of prohibited substances. For a substance to be included on the list of prohibited substances it has to meet at least two of the following three criteria (i) it has the potential to enhance sport performance, (ii) it represents an actual or potential health risk to the Athlete and (iii) the substance violates the spirit of sport [4]. T meets all three of these criteria.

(i) T enhances sports performance primarily by inducing muscle mass and strength [5-7]. Supraphysiological doses of T are known to increase fat-free mass, muscle size and strength especially when combined with strength training [8, 9]. Whereas lower doses of T and AAS are used in endurance sports for improved recovery. Low doses of T work to replace the T lost after overtraining-induced stress and play an important role in the regeneration of muscles after physical exercise [10]. In addition, T increases the activity of glycogen synthase, helping to refill the glycogen storage in the muscles [11].

(ii) AAS have been linked to multiple severe adverse effects. Cardiovascular side effects for AAS include morphological and functional changes of the heart, enhanced pro-thrombotic state as well as increased risk of coronary artery disease and life-threatening arrhythmia ([12] – and references therein). Behavioural side effects include aggression and violence [13], possibly reflecting connectivity reductions of brain networks [14]. There are also adverse endocrine effects of AAS including infertility [15]. The studies investigating side effects are often listing AAS as one group, not specifying T induced side effects. Some side effects might be specific for certain AAS but the effects of overstimulating the androgen receptors should hold true for supraphysiological doses of T. For example, just one single dose of 500 mg T enanthate increased total cholesterol [16] and seem to induce endothelial dysfunction [17] so it doesn't seem far-fetched that continuous use increases the risk of vascular disease.

(iii) Athletes who take T seek to gain an unfair advantage over their competitors, which undermines their competitors' hard work and therefore violates the spirit of sports. According to the Code issued by WADA, the sprit of sport is "the pursuit of human excellence through the dedicated perfection of each person's natural talents" [4]. The purpose of the ban of T and other doping substances is to protect the athletes' fundamental right to participate in doping-free sports and thus promote health, fairness and equality for athletes.

2.1.1 History of Testosterone as a drug

The tremendous biological effect of the testes has been known since the antiquity. Because it was known that removal of the testes caused symptoms of hypogonadism, ingestion of testes and testicular extracts were recommended as a treatment for hypogonadism in the Roman Empire, in the 700's Arabic medicine as well as in ancient China [18]. These remedies continued to be prescribed up into the twentieth century. However, these remedies certainly only had placebo effect at best. This is due to the fact that the testes do not store T and therefore contain a relatively low concentration of T [19]. In addition, the amount that was to be consumed would be inactivated during the first-pass metabolism in the liver.

It was not until 1935 that T was isolated and synthesized [20, 21]. Soon thereafter T pellets became clinically available, then injectable esters and from the mid-1950's the longer acting T enanthate appeared [18]. At this point the pharmaceutical industry started altering the chemical structure of the T molecule to increase its anabolic effect and decrease the androgenic effect, resulting in the making of the first AAS. In the late 1970's, more than a 1000 different AAS had been described [18].

All available preparations at this time resulted in unphysiologically high or low serum levels of T, leading to the development of new administration forms [18]. In the mid-1990's T patches became available [22] and in the year of 2000, T gels entered the market [23]. Finally, in 2004, T undecanoate became available as intramuscular injections [24]. In parallel to this development of T for clinical use, was the introduction of these substances to healthy individuals to increase physical performance.

2.1.2 Prevalence of Testosterone doping

There is no reliable information on how common doping is, let alone doping with endogenous anabolic androgenic steroids (EAAS). In 2016, 1.6% of all tests issued under WADA showed an adverse analytical finding [25]. However, this includes all forms of doping and not all of these will end up being anti-doping rule violations since some of these adverse analytical findings are covered by therapeutic use exemption (TUEs). On the other hand, doping can only be detected for a limited time and sometimes not at all, so this is most likely an underestimation of the prevalence of doping among elite athletes. A retrospective study, investigating steroid profiles of 879 European professional football players, used a statistical approach to the question and they concluded that the prevalence of steroid doping within this group was undoubtedly lower than 7.7% [26]. When athletes of the 2011 World Championship in Athletics were asked in a survey if they had "knowingly violated anti-doping regulations by using a prohibited substance or method within the last year" more than 30% said yes, however, the kind of doping was not specified [27].

Consequently, the prevalence of doping with AAS, or any other substance, is unknown and likely different in different countries and sports. However, as evident by the McLaren report about state-sponsored doping in Russia, there is no question that doping still remains a problem in sports and that most of it fails to be detected by today's Anti-Doping regimes [28].

2.2 ANDROGEN BIOSYNTHESIS, ACTION AND METABOLISM

2.2.1 Androgen Biosynthesis

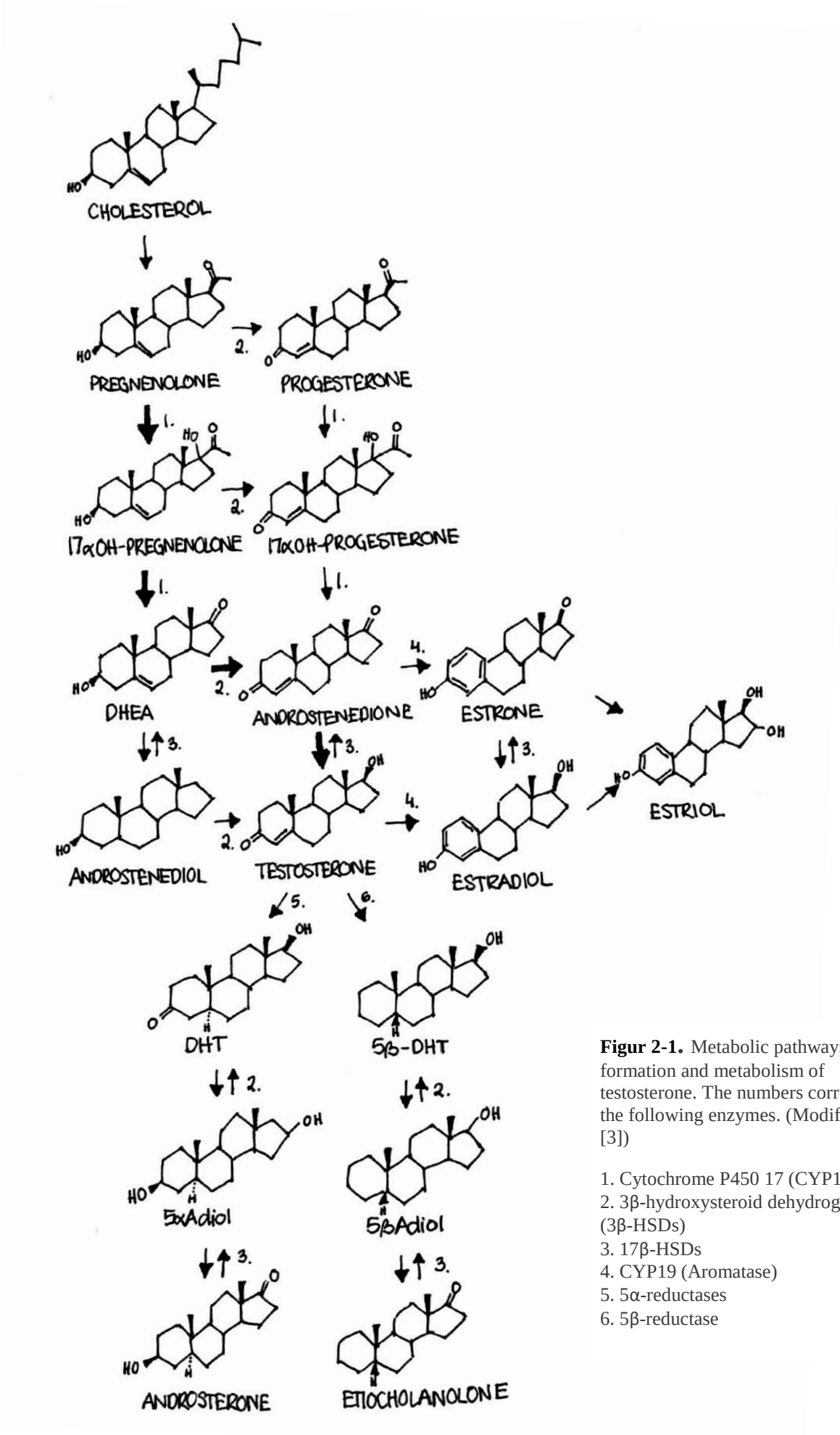
Testosterone or androst-4-en-17β-ol-3-one, is a natural androgen formed by modification of the cholesterol molecule (Figure 2-1). T can be formed through different pathways, in humans the preferred route of T formation is via pregnenolone and DHEA [29]. T is produced at a rate of about 7 mg per day in men and around 0.1-0.4 mg per day in females [30, 31]. Serum concentrations of T are typically between 10-35 nmol/L for men and <3.5 nmol/L for women [32-34].

In men, most of the natural production comes from the Leydig cells in the testes but a small amount also from the adrenal cortex [35]. In women, androgen production occurs in the ovaries, adrenal cortex and for pregnant women also in the placenta [30, 36, 37]. The adrenal cortex and ovaries however produce more androstenedione and DHEA, precursors of T, which are converted to T and the more potent dihydrotestosterone (DHT) in peripheral tissues [38-40]. In women, peripheral conversion from androstenedione contributes to about half of the circulating T, whereas approximately 25% each comes from ovarian and adrenal production [31, 41]. Virtually all T is bound to plasma protein - either strongly bound to sex hormone binding globulin (SHBG) or loosely to albumin [42].

2.2.2 Androgen Action and Regulation

T has anabolic properties, increasing muscle mass and strength [5-9], as well as androgenic properties, increasing virilization. Androgens exert their effect by binding to the androgen receptor (AR), a nuclear receptor which, when activated, works as a transcription factor for several hundred genes [43-45]. This androgen-AR complex regulates transcription of muscle-specific enzymes and structural proteins as well as noncoding RNAs, including regulatory microRNA [46]. Besides the genomic activity, there are non-genomic actions [47], such as rapid rise of intracellular calcium concentration in response androgens [48].

Testicular and ovarian androgen production is stimulated by luteinizing hormone (LH) secreted from the anterior pituitary, which in turn is stimulated by gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus upon neural signals from other parts of the brain (Figure 2-2). This so-called hypothalamus-pituitary-gonadal (HPG) axis is controlled through a negative feedback mechanism where elevated levels of endogenous steroids, including estrogens and progestogens, as well as exogenous steroids send signals to the hypothalamus and/or anterior pituitary to decrease the release of GnRH and/or LH.



Figur 2-1. Metabolic pathways of formation and metabolism of testosterone. The numbers correspond to the following enzymes. (Modified from [3])

1. Cytochrome P450 17 (CYP17)
2. 3β-hydroxysteroid dehydrogenases (3β-HSDs)
3. 17β-HSDs
4. CYP19 (Aromatase)
5. 5α-reductases
6. 5β-reductase

The release of LH by the pituitary is pulsatile in both sexes, however, the levels remain more stable in men than women due to the cyclical release for women during the menstrual cycle. GnRH, LH, follicle-stimulating hormone (FSH), estrogen and progesterone are the major hormones responsible for the menstrual cycle with great variation at different stages of the cycle. Adrenal androgen production is at least in part modulated by adrenocorticotrophic hormone (ACTH) but other factors or hormones also exist, e.g. estrogens, growth hormone and gonadotropins [30, 49].

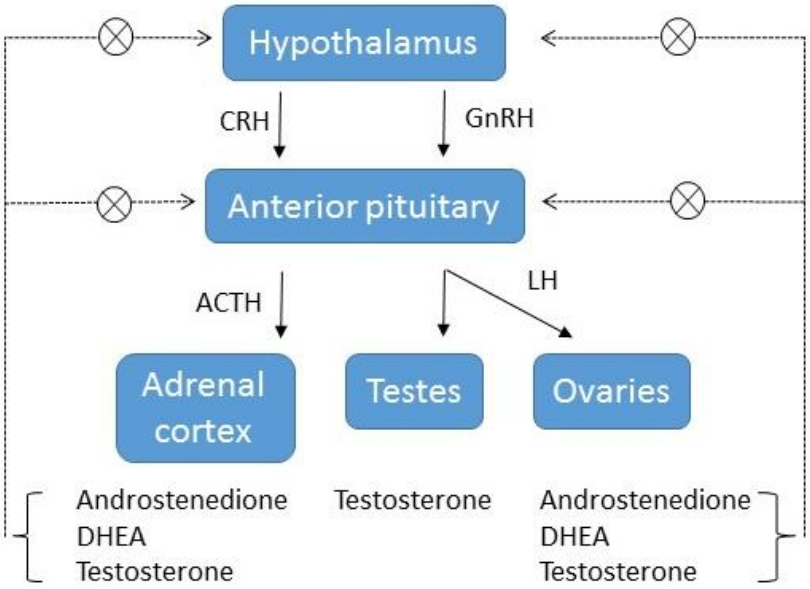


Figure 2-2. The regulatory feedback mechanism of steroids. The hypothalamus produces gonadotropin releasing hormone (GnRH) which stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). These hormones then stimulate the production of sex hormones in the gonads. The adrenal production of androgens is under control of adrenocorticotrophic hormone (ACTH) released from the anterior pituitary after stimulation of corticotropin-releasing hormone (CRH). The dotted line represents the negative feedback mechanism steroids have on the release of hormones from the hypothalamus and anterior pituitary.

2.2.3 Androgen Excretion

To be able to excrete steroids like T, the body has to convert this non-polar steroid to a more polar molecule, which can be excreted in urine. This is achieved through phase II metabolism where the steroids are conjugated mainly with glucuronic acid by Uridine 5'-diphospho-glucuronosyltransferases (UGT) [50], Figure 2-3. The steroids are also, but to a lesser extent, sulfated by members of the sulfotransferase (SULT) enzyme family [51].

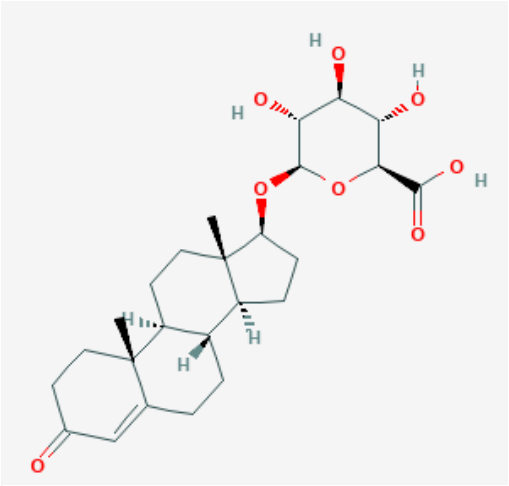


Figure 2-3 Testosterone glucuronide

2.2.4 Genetic Variability

There are large inter-individual variations in the concentrations of steroids in urine, part of which can be explained by differences in serum concentrations but genetic differences in the metabolizing enzymes also play a role [52].

2.2.4.1 CYP17

Cytochrome P450c17α (CYP17) is involved in the hydroxylation of pregnenolone and progesterone producing T (Enzyme 1 in Figure 2-1). CYP17 is also believed to be part of epitestosterone (E) formation [53, 54]. A polymorphism in the promoter region of the CYP17 gene creates a putative Sp1 binding site in silico [55] which however could not be confirmed in vitro [56]. Caucasian (but not Korean) male subjects were shown to have higher urinary E levels if they were heterozygous or homozygous for the C-allele [54].

2.2.4.2 UGT2B17

UGT2B17 is the major enzyme responsible for the phase II metabolism of T [57, 58]. The enzyme catalyzes the reaction to add a glucuronide group to the T molecule making it more water soluble (Figure 2-3). A deletion polymorphism in the *UGT2B17* gene has been shown by Jakobsson et al. to be strongly associated with T excretion [59], explaining the T distribution seen in Figure 2-4.

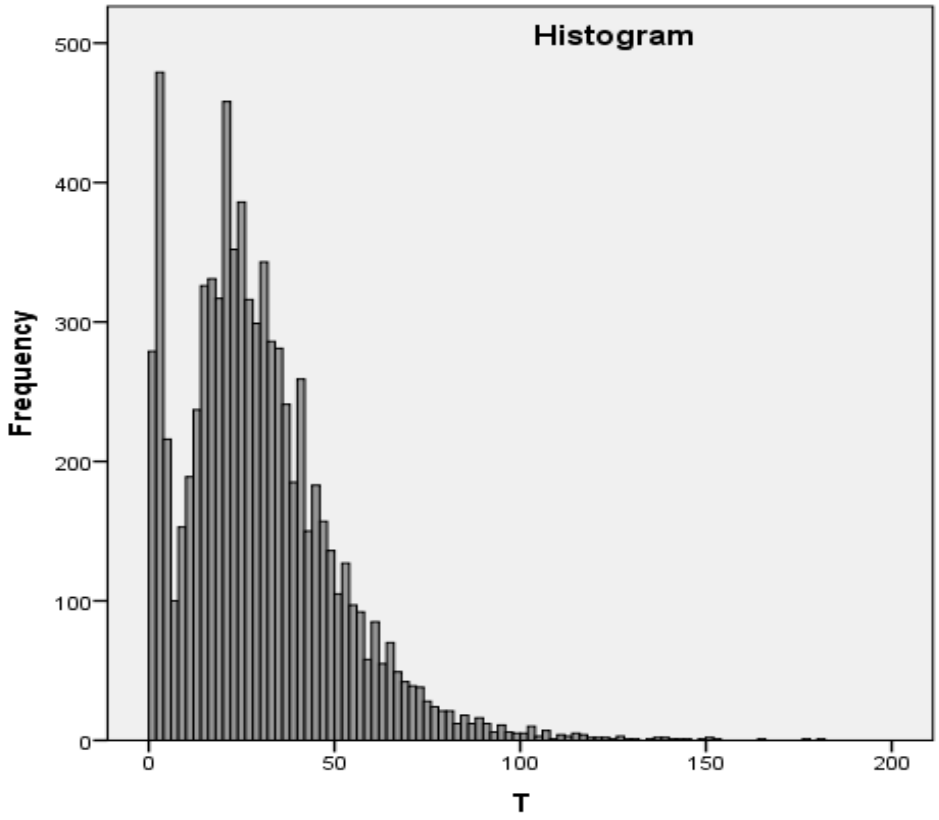


Figure 2-4. Testosterone distribution for Swedish and Norwegian men. A UGT2B17 deletion polymorphism is responsible for the bimodal distribution of urinary T excretion. Individuals with the double deletion of *UGT2B17* excrete low amounts of T and hence make up the first group in the distribution. (This figure was made in IBM SPSS Statistics 24 with data from Study VI.)

Jakobsson et al. also showed, that the *UGT2B17* genotype distribution was significantly different between a Swedish and a Koreans population showing the Koreans were 7.2 times more likely to have the *deletion/deletion* (*del/del*) genotype [59]. All subjects with the *del/del* genotype had negligible amounts of T in the urine, whereas subjects with one or two copies (*ins*) of *UGT2B17* showed up to several hundred times higher T concentrations. Although the different genotypes showed a substantial difference in urinary T concentration, there was no significant difference in serum concentrations of the steroid [60]. Men with the double deletion of UGT2B17 have shown to excrete more Etio-sulfate than insertion carriers [61].

2.2.4.3 UGT2B15

T is also conjugated by UGT2B15 but to a lesser extent. UGT2B15 shows 96% identity with UGT2B17 but has broader substrate specificity [62]. There is a polymorphism in *UGT2B15* resulting in a change of aspartate (D) to tyrosine (Y) at position 85. No association between urinary T metabolite levels and the *UGT2B15* D85Y polymorphism have been found [54, 63]. However, expression of UGT2B15 is increased in men with the double deletion of UGT2B17 [54, 64].

2.2.4.4 UGT2B7

UGT2B17 has affinity for E but the enzyme will not catalyze the glucuronidation of E, rather UGT2B7 is the enzyme conjugating E, at least in vitro [58, 65]. UGT2B7 has poor affinity for T [66]. There is a common missense polymorphism in *UGT2B7* where the histidine (H) amino acid is replaced by tyrosine (Y) at position 268, H268Y. This polymorphism does not have any significant impact on urinary androgen concentrations in Caucasian or Korean men [54].

2.3 DETECTION OF DOPING WITH ENDOGENOUS STEROIDS

As of today, the mass-spectrometric method used in routine doping control cannot distinguish between exogenous and endogenous steroids based on the mass spectrum. Therefore, doping with endogenous steroids presents a major difficulty in traditional doping tests. These hard-to-detect steroids include T, its precursors (e.g. androstenediol, androstenedione and DHEA), DHT as well as E. To detect these banned substances, concentrations of the following steroids are measured in urine: T, E, androsterone (A), etiocholanolone (Etio), 5α-androstan-3α,17β-diol (5αAdiol) and 5β-androstan-3α,17β-diol (5βAdiol). In the sample preparation procedure before injection to the GC-MS(/MS), the urine is hydrolyzed with β-glucuronidase. As a consequence, it is the unconjugated as well as the glucuronidated fractions that are quantified.

Instead of using concentrations of steroids, ratios are used because they have proven to be more stable as well as more sensitive to doping [3]. The use of biomarker ratios rather than individual concentrations also bypasses the problem of fluctuations of steroid concentrations caused by variations in urinary flow rate. Therefore, the steroid concentrations are combined into the ratios T/E, 5αAdiol/5βAdiol, A/Etio, A/T and 5αAdiol/E.

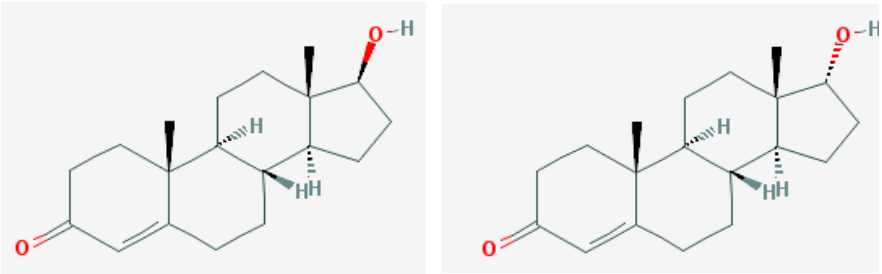
2.3.1 Biomarkers of the Steroid Profile

2.3.1.1 Epitestosterone (E)

Epitestosterone (E), or 17α-hydroxy-4-androsten-3-one, is an isomer of T that differs from T in the configuration at the C17 position (Figure 2-5). It is formed in parallel with T although the exact paths of biosynthesis are unknown [67]. One putative precursor is epiandrostenediol (androst-5-ene-3β,17α-diol) which would be converted to E by 3βHSD [68, 69]. Another possible precursor is androstenedione, but the biosynthesis contribution from androstenedione is believed to be minimal [70, 71]. The interconversion of T and E is negligible, if any [70].

The daily production of E is only 3% of that of T and most of it is likely formed in the testes although part of the production probably occurs in the adrenal gland since administration of ACTH significantly increases urinary E concentration in healthy men [70, 72]. Even though the production is only a few percent of that of T, due to poor phase I metabolism of E in man, the excretion rate is 30-50% of that of T [70].

Unlike T, very little is known about E physiological role but on the basis of animal experiments, it has been suggested to have anti-androgenic effect thus being able to modulate the androgenic effects mediated via the androgen receptor [67]. Nevertheless, E has a central role in doping testing since the administration of T suppress the excretion rate of E [73, 74]. E regarded as a masking agent and is prohibited in sports as its administration in combination with T at a dose ratio of approximately 1:30 results in a stable T/E ratio [75].



Figur 2-5. Chemical structure of testosterone (to the left) and epitestosterone (to the right).

2.3.1.2 T/E ratio

The T/E (testosterone to epitestosterone) ratio is the most sensitive ratio currently used to detect administration of T and the most important parameter of the steroid profile. The T/E ratio is sensitive to doping since T increases with administration of T, and E decreases. When this ratio was implemented as a biomarker in 1983, an upper limit of 6 was set based on population studies, but the cut off ratio was lowered to 4 in 2004. Using a population-based threshold for T/E has shown to be ineffective since some people naturally have higher T/E than 4 (resulting in increased risk of false positives) whereas others have such low T/E values that they never can reach the cut-off value even after T injections (resulting in false negatives) [62, 76].

2.3.1.3 5αAdiol/5βAdiol

5αAdiol and 5βAdiol are Phase I metabolites of T and are formed from DHT and 5β-DHT, respectively (Figure 2-2). After DHT, 5αAdiol is the most potent metabolite, but not quite as potent as T, whereas 5βAdiol shows no androgenic activity [77]. When administering T, the levels of both diols increase but depending on the administration route or steroid administered the diols change differently leading to changes in the 5αAdiol/5βAdiol ratio. This ratio is especially sensitive to transdermal T administration due to high levels of 5α-reductase in the skin, favoring the 5α-route of metabolism [78, 79]. Also, DHT administration leads to an increase in 5αAdiol/5βAdiol [80], since DHT is a precursor of 5αAdiol (Figure 2-1).

2.3.1.4 A/Etio

A and Etio are end products of the Phase I metabolism of T and hence, can be detected in high amounts in urine. A is produced from 5αAdiol and has some androgenic activity, whereas Etio is produced from 5βAdiol and has no androgenic activity [77]. The ratio between these steroids is just like 5αAdiol/5βAdiol sensitive to detect application of DHT and transdermal T.

2.3.1.5 A/T

The A/T ratio was earlier used as T/A, switching the numerator and denominator lead to easier values (not as many decimals needed). However, when an A/T ratio shows suspicious signs of doping it is low as compared to the other ratios that are higher than normal after intake of steroids. The usefulness of this ratio has been discussed.

2.3.1.6 5αAdiol/E

5αAdiol/E is the newest addition to the steroid profile and was introduced after Geyer et al. found this ratio to be the most sensitive for T gel detection [79]. It is believed to be useful for detection of all transdermal T preparations as well as DHT.

2.3.2 Traditional Testing

Here the meaning of traditional testing refers to the way of analyzing the results before the steroidal module of the ABP was established. This is still the method used for athletes tested only once. Note that these thresholds were set by WADA and therefore only refer to doping control regulated by WADA, i.e. for athletes. In traditional testing, each athlete's steroid concentrations and ratios are compared to population-based reference ranges. According to traditional testing, the sample's steroid profile is suspicious if any of the following criteria are met [2]:

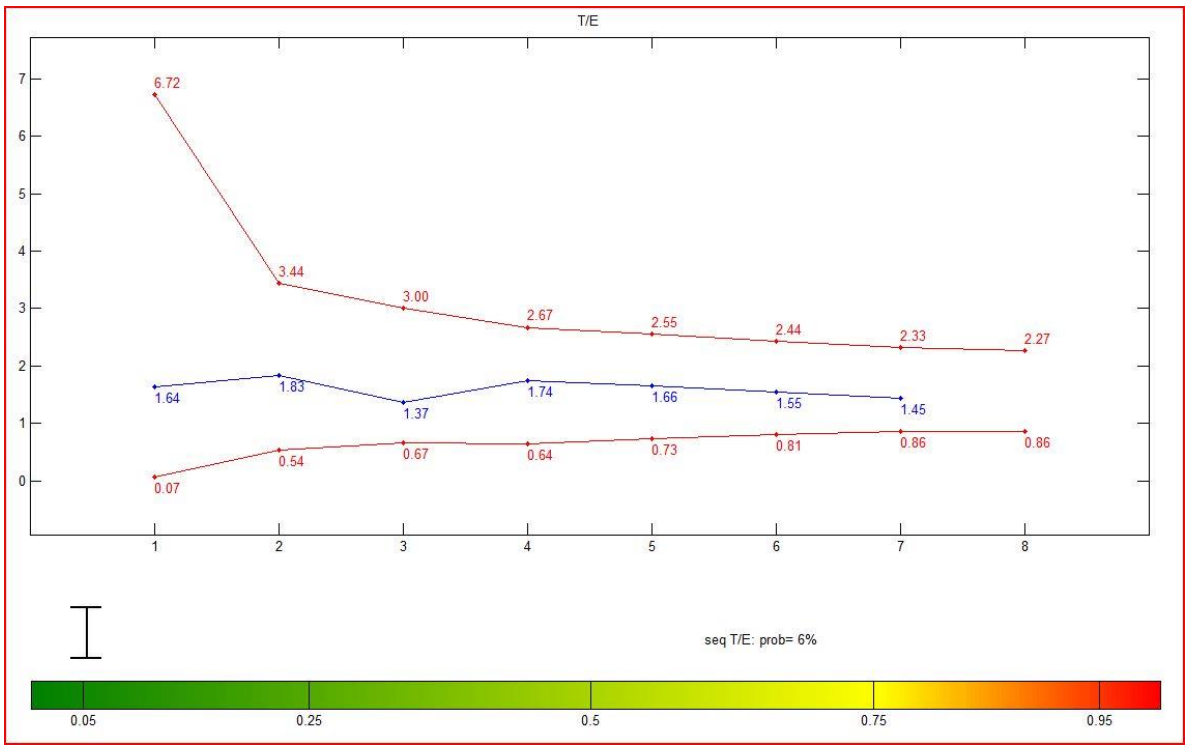
Positive criteria according to traditional testing [2]

i. T/E > 4.0
ii. A/T < 20
iii. 5αAdiol/5βAdiol > 2.4
iv. Concentration of T or E (adjusted for SG) > 200 ng/mL in males or > 50 ng/mL in females
v. Concentration of A or Etio (adjusted for SG) > 10,000 ng/mL
vi. Concentration of 5αAdiol (adjusted for SG) > 250 ng/mL in males or > 150 ng/mL in females, combined with a 5αAdiol/E > 10 in either sex.

This method of looking at the steroid profile is complicated since for example, some individuals have a natural T/E ratio higher than 4 and some have shown not to reach a T/E above 4 even after an injection of a large doses of T [62].

2.3.3 The Athlete Biological Passport (ABP)

Since there are no one-size-fits-all reference ranges for steroids, Donike et al. proposed the application of individual steroid profiling already in 1994 [81]. In 2007, Sottas et al. improved this method by suggesting a Bayesian screening test for the detection of abnormal values in longitudinal biomarkers [82]. This resulted in the steroidal module of the Athlete Biological Passport (ABP). With an algorithm that calculates reference ranges based on the athlete's previous results, every individual gets his or her own reference ranges [83]. The more samples each athlete adds to his or her passport the narrower the individual reference range gets, an example is shown in Figure 2-6. The goal of using Bayesian theory is to evaluate how likely the passport data are assuming a normal physiological condition. An atypical passport finding (ATPF) is obtained when a sample in a passport goes outside the individually calculated reference ranges.



Figur 2-6. The T/E profile of the ABP for an individual tested seven times. The blue dots on the center blue line represents the T/E ratios measured at each test point. The outer red lines are the individually calculated reference range that gets more specific with each test.

The introduction of the steroidal module of the ABP has greatly improved the detectability of endogenous steroids. One study showed a 41% false positive risk when using traditional testing as compared to 4% when using the ABP [84]. The sensitivity of the steroidal module has been tested for a variety of T formulas and administration routes, all studies showing a vast improvement with longitudinal monitoring [78, 79, 85-87].

There are factors other than doping that can influence the steroid profile and give ATFPs. It is the role of the Athlete Passport Management Units (APMUs) to evaluate each passport and draw conclusions on the likelihood of doping. To make correct interpretations the APMUs need information about other factors and how they influence the steroid profile. Because of the difficulty of this interpretation as well as the collection of reliable baseline values, T detection still remains one of the most difficult challenges in doping control analysis.

2.3.4 Confirmation with IRMS

When the T/E passport has generated an automatic ATPF or raised any other suspicion, the sample will proceed to confirmation with gas chromatography-combustion-isotope-ratio mass spectrometry (IRMS) [1]. In the confirmation, the isotopic composition of five target compounds (TC) are measured with IRMS and compared to an endogenous reference compound (ERC). The carbon isotopic composition is presented as δ^{13}C-value which is $^{13}C/^{12}C$ in the unit of per mille (‰) compared to a virtual carbon isotopic international standard (called Vienna Pee-Dee Belemnite) [88, 89]. The TCs are compared to the ERC which gives $\Delta\delta^{13}$C-values. For a test to be positive it has to meet one of the following sets of criteria:

IRMS positivity criteria [1]

i. The $\Delta\delta^{13}C$-value of ERC-T > 3‰ and either ERC-5αAdiol or ERC-5βAdiol > 3 ‰

ii. The $\Delta\delta^{13}C$ -values of ERC-5αAdiol and ERC-5βAdiol pairs are both > 3 ‰

iii. E > 50 ng/mL in females or > 200 ng/mL in males (SG-adjusted) and the $\Delta\delta^{13}C$ -value of ERC-E > 4 ‰

iv. The $\Delta\delta^{13}C$ -value of ERC-A > 3 ‰ or ERC-Etio > 4 ‰

v. The $\Delta\delta^{13}C$ -value of ERC-A is between 2-3 ‰ or ERC-Etio is between 3-4 ‰, and one of ERC-5αAdiol or ERC-5βAdiol > 3 ‰

vi. The $\Delta\delta^{13}C$ -value of ERC-5αAdiol > 4 ‰ and 5αAdiol ≤ -27 ‰

This method is based on the principle that biomarkers excreted after external administration of steroids have a different isotopic composition of their carbon atoms than if the steroids were produced by the body. The carbons in steroids produced by our body come from our diet which is based on two groups of higher plants, C3 and C4 plants [90]. C3 plants have less ^{13}C and hence a lower δ13C-value than C4 plants. Our diet is a mix of these two types of plants and therefore the normal range, of δ13C of -26 to -16 ‰, falls in between that of C3 and C4 plants, depending on which plant source make up the bigger part of our diet [91-93]. One big drawback with this method is that illicit testosterone preparations with isotopic compositions similar to or even within the normal range exist (Figure 2-6) [94-96]. In these cases, IRMS will not be able to confirm exogenous use. Since IRMS is used as the ultimate confirmation of doping with endogenous steroids, the sensitivity of this method has to be further evaluated.

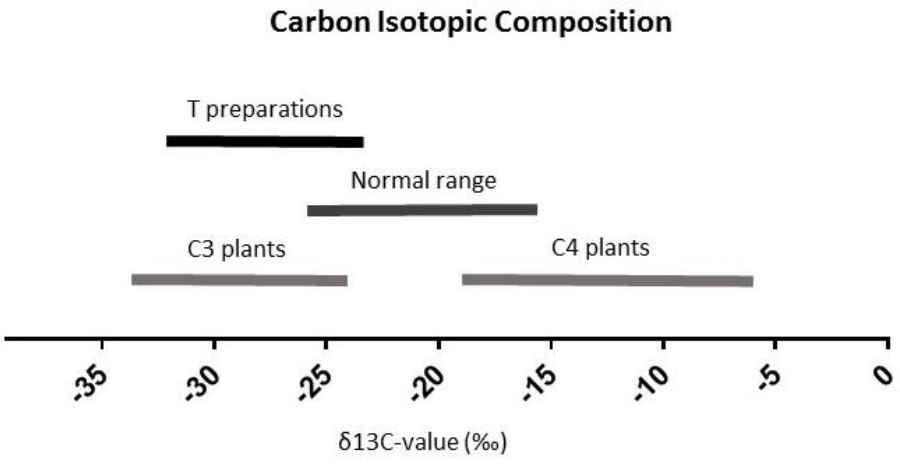


Figure 2-6. Carbon isotopic composition in Δδ13C-value of T preparations [95-97], normal steroid range [92, 93] and of the two types of higher plants that are the sources for our diet (i.e. C3 and C4 plants) [90].

2.4 STABILITY OF THE URINARY STEROID PROFILE

What can be considered normal urinary steroid levels depends on the population in question. It differs depending on gender [64, 98], ethnicity (due to genotype similarities within ethnicities) [54, 62], if the subjects are athletes and also the type of sport they are involved in [32](our unpublished results). As a result, reference ranges should be obtained from an as similar population as possible to those investigated. These reference ranges are important to the ABP algorithm that uses these as a starting point when no previous measurements of an individual are available. Then the algorithm progressively switches from these population-based values to individually calculated reference ranges as the tests number increases.

Although the inter-individual variation is larger than the intra-individual variation, there are still large variations within an individual. What is known about some of the factors affecting steroid levels is explained in this chapter.

2.4.1 Circadian and Annual Rhythm

T is produced with a diurnal variation in men with maximum concentration in blood observed around 6:00-8:00 and a minimum around 18:00-22:00 [99-103]. Also in women, have T been indicated to show circadian variation with highest levels in the early morning hours [30, 32]. This circadian variation has a high inter-individual variation. The diurnal variation of testosterone in normal men is due to a change in secretion rather than in clearance and is largely LH driven [100]. The circadian variation of urinary steroids has only been studied in few individuals and not for all steroids measured in today's doping tests [100, 104, 105]. In ten women and ten men, it was seen that peak A and Etio concentrations in urine occur between 12:00-15:00 for both men and women [106]. The circadian variation seen in women is believed to be more significant than the variation seen in men due to lower concentrations of metabolites [107]. The effect of time of day on the steroid profile needs to be further evaluated in a larger population to rule out significance in the evaluation of passports.

If there are annual fluctuations in serum T levels is controversial with several studies pointing in different directions [108]. In a large population of Norwegian men (N=1548) total serum T showed peak levels in October-November and lowest levels in June [109], but other studies failed to confirm these results but rather reported inconsistent variations [110-112]. Little research has been done on seasonal variations in urinary steroid levels, and this area is therefore mostly unknown. One pilot study of five women showed no annual rhythm in excretion rates of steroids [113].

2.4.2 Changes with Age

Serum total and free T decreases with age in men [114], with maximum concentrations at 20-24 years [115]. The decline in serum T is usually accompanied by increased or unchanging levels of LH and increased levels of FSH [116]. Also in women have T been shown to decrease slowly with age, however, due to a drop in SHBG in women with age, free androgen levels rise [117]. To investigate the changes in urine androgen levels with age Dehenin et al. studied 140

men between 13 and 20 years and concluded that T and E glucuronide increased during the pubertal development with no significant change in the T/E ratio [118]. Similar results were seen by Raynaud et al [119]. In a group of adolescent girls (aged 6–17, n=256), a decreasing T/E ratio during development was seen, due to larger relative increase in E excretion [120].

2.4.3 Variations with Stress

Circulating T and cortisol levels fluctuate during stressors, such as athletic competitions. In a meta-analysis with both male and female soccer players, serum and salivary T was shown to increase after a match win and decrease if the player lost the game [121]. Female T being marginally more affected.

In a study investigating students before and after a big exam, the urinary steroid profile showed significant changes, especially in women [122]. However, the increases seen in A and Etio disappeared when combined into the ratio A/Etio in women and was only slightly higher for men. They saw no difference in T/E ratio, in agreement with Donike et al. results showing that T/E is not affected by competing in the Tour de France [81] nor participating in a pistol shooting competition [123]. In a study with almost 4200 urine samples collected from male soccer players, the steroid concentrations and ratios showed no significant difference if collected in competition (IC) or out of competition (OOC) [26]. However, they did not discriminate between IC samples collected after a win or a loss. Even though there seems to be no clear evidence that steroid profile ratios are affected by IC or OOC testing, it is commonly believed to be the case.

2.4.4 Menstrual Cycle

Serum LH levels change dramatically throughout the menstrual cycle and since androgen secretion from the ovary is under LH control, androgen secretion would be expected to vary throughout the cycle. Indeed, serum levels of A and T are lowest in the early follicular phase and rise to a maximum around the time of ovulation and then gradually fall again [124, 125].

The effect of the menstrual cycle on the urinary steroid profile is unclear. There are contradictory results of the menstrual cycle's effect on the T/E ratio with several studies reporting no common pattern in T/E ratio during the menstrual cycle [104, 107, 113, 126, 127]. However, Longhino et al. measured total T and E in two women during two cycles and saw significant changes in T and E with maximal levels at the end of the cycle (20-25 and 22-23 days respectively) [128]. Catlin et al. collected morning urine every day from three women for five menstrual cycles and saw a T/E peak in the first days of the menstrual cycle [129]. In conclusion, most studies point towards a random or at least individual variation in the T/E ratio with the menstrual cycle. However, no study was large enough to draw any definitive conclusions and the other ratios of the ABP were not considered.

2.4.5 Hormonal Contraceptives

Hormonal contraceptives (HC) consist of a progesterone derivative or a combination of a progesterone and an estrogen derivative. Both progesterone and estrogens are negative regulators of the HPG axis [130, 131] and therefore suggested to affect androgen secretion. In fact, non-HC users have significantly higher serum levels of DHEAS, androstenedione, free testosterone, LH, and FSH values than HC users [32].

The use of HC has been suggested to suppress the production of E and thus lead to an increase in urinary T/E. So far, only a crossover study with four volunteers has been performed to investigate HC effect on the urinary steroid profile [132]. This study showed significant increases in the T/E-ratio during the time the subjects were on contraceptives.

Some hormonal contraceptives contain norethisterone which results in presence of 19-norandrosterone (19-NA) in urine [133, 134]. This steroid is interesting because it is the main metabolite of nandrolone (and other 19-norsteroids) and hence monitored in doping tests [135]. Some of the urinary 19-NA seem to come from impurities of the norethisterone tablets (i.e. 19-norandrostenedione) but most of it is formed as a product of norethisterone metabolism [136-138].

2.4.6 Pregnancy

During pregnancy, the female body encounters dramatic hormonal changes. Pregnancy causes hCG to increase during the first three months of pregnancy. The placenta is able to convert cholesterol into progesterone, which increases continuously throughout pregnancy and is excreted in maternal urine mainly as pregnanediols [139]. T production increases with progressing pregnancy and drops quickly after pregnancy [140, 141].

Controlled studies of urinary steroid profile during pregnancy are scarce. One case study investigating the urinary T/E ratio in three pregnant women showed no difference in T during the different stages of pregnancy but an increase in urinary E causing a significant change in T/E ratio [139]. They saw no change in the 5αAdiol/5βAdiol during pregnancy. The increase in E could possibly be related to the increasing secretion of hCG during pregnancy since hCG administration has shown to increase E excretion in men [142].

Small amounts of 19-NA of natural origin can be present in both men and women [143, 144]. However, during pregnancy, this production is increased and 19-NA levels up to 15 ng/mL have been measured in urine from pregnant women [134, 145, 146]. For this reason, WADA allows 19-NA concentrations to be as high as 15 ng/mL for pregnant women [135]. If the concentration exceeds 15 ng/mL confirmation with IRMS is performed.

2.4.7 Doping with Low Doses of Testosterone

Gel, patches and oral capsules are popular routes of administration of smaller doses of T and intramuscular injections for a slower release of larger doses [147]. Low doses of endogenous steroids are a real challenge to detect since they are hard to distinguish from natural fluctuation when interpreting ABP profiles. Several studies have investigated the detectability of different T formulations and administration routes with the methods used today.

A study administering T gel to 6 men estimated that the detection time for a single dose of 100 mg T gel was about 7 days when longitudinally monitoring with individually set reference ranges were used [83]. In another study, 18 men were administered with 100 mg T gel per day for 6 weeks [79]. This study showed that the administration can be detected with longitudinal follow-up and that the best parameters for detecting T gel are T/E, 5αAdiol/E and A/E but unfortunately, they did not investigate the detection time.

To my knowledge, there is only one study available investigating the effect of T doping on the female steroid profile. The study involved 10 female Japanese women injected with 100 mg T enanthate [76]. T/E did not exceed the traditionally used T/E cut-off ratio of 4 in any of the individual's devoid of the UGT2B17 gene (n=6), in agreement with what has been seen in male del/del subjects [62]. IRMS could successfully confirm the administration in all women. There is no available longitudinal steroid profile of these women, since only one baseline value was taken.

Several groups have proposed the use of other biomarkers than those in use today for better detectability of steroid doping. In a pilot study using extensive steroid profiling and longitudinal monitoring, the ratios 6α-OH-androstenedione/16α-OH-dehydroepi-androstenedione, 4-OH-androstenedione/16α-OH-androstenedione, 7α-OH-testosterone/7β-OH-dehydroepiandrostenedione and DHT/5βAdiol were identified as sensitive urinary biomarkers for T misuse [148]. Also, sulphoconjugated epiandrosterone has been suggested [149]. Apart from other urinary metabolites, other proposed biomarkers include, but not limited to, serum levels of LH, T, DHT, 17-OH progesterone and ratios of those [73, 150-153], and miRNA-122 [154].

3 AIMS

3.1 GENERAL AIMS

The general aims of this thesis were to evaluate natural variations of the steroid profile, including variations during the menstrual cycle and pregnancy, as well as how certain drugs, such as hormonal contraceptives and testosterone, affect the steroid profile. This knowledge can aid the interpretation of the steroidal module of the ABP.

3.2 STUDY SPECIFIC AIMS

Study I. The Dose Study

To investigate if injections with T enanthate could be detected using the ABP and IRMS analysis as well as the *UGT2B17* deletion polymorphisms role in this detection.

Study II. The Testosterone Gel Study

To investigate if a single dose of T gel could be detected using the ABP and IRMS analysis and if some blood parameters were sensitive as biomarkers for this detection.

Study III. The Hormonal Contraceptives Study

To investigate if hormonal contraceptives affect the steroid profile and to study three polymorphisms relation to the steroid profile.

Study IV. The Menstrual Cycle and Emergency Contraceptive Study

To investigate if and in that case how the steroid profile varies during a menstrual cycle including both glucuronide and sulfate conjugated fractions. Additionally, to study what happens to the steroidal passport after administration of an emergency contraceptive pill.

Study V. The Pregnancy Study

To investigate what happens to the steroid profile and 19-NA during pregnancy. In addition, androgen disposition in relation to two polymorphism were investigated.

Study VI. Inter-Individual Variation of the Steroid Profile

To evaluate natural variations in the steroidal profile and factors that influence the profile, including but not limited to gender, age, time of day, time of year and in competition or out of competition testing.

4 METHODOLOGICAL CONSIDERATIONS

The methods used in this work are either methods used for doping control purposes accredited by WADA, genotyping assays or standard methods accredited by a hospital for medical use. Paper I, IV and V describe new or partly new methods developed for the paper or just previously unpublished. The methods are described in detail in these papers, whereas this chapter gives a broader overview of the methods with changes made during the way as well as limitations.

4.1 STUDY SUBJECTS

Table 4.1 gives a summary of the study subjects used for each study.

Study	Subjects	Recruited from	Analyzed for
I. The dose study	25 male volunteers	Sweden	✓ Urinary steroids ✓ Serum steroids ✓ UGT2B17 genotype ✓ Carbon isotopic composition
II. The testosterone gel study	8 male volunteers	Sweden	✓ Urinary steroids ✓ Serum steroids ✓ UGT2B17 genotype ✓ Carbon isotopic composition
III. The HC study	79 female elite athletes	Sweden	✓ Urinary steroids ✓ UGT2B17, UGT2B7, CYP17A1 genotype
IV. The menstrual cycle + EC study	6 female volunteers	Sweden	✓ Urinary steroids ✓ Sulfate and glucuronide fractions
V. The pregnancy study	69 female volunteers	Canada	✓ Urinary steroids ✓ 19-NA ✓ UGT2B17, CYP17 genotype
VI. Inter-individual variation of the steroid profile	5473 athletes (4180 men and 1293 women)	Sweden and Norway	✓ Urinary steroids

4.1.1 Limitations

As always there are limitations with the study populations recruited. The aim of this thesis was to investigate factors, internal and external, that can affect the steroid profile to aid evaluation of steroid passports. Steroid passports are only used for athletes and hence, athletes are the target population. Of the studies performed, only two were performed on elite athletes (Study III and VI), the rest being performed on healthy volunteers. Even though androgen levels in athletes are similar to that of the general population, androgen levels are known to be induced by physical exercise [155].

With subject recruitment comes selection bias. The only representative group of those tested would be Study VI where all steroid profiles collected in Sweden and Norway during a time period of a few years were included. However, this population may only be representative of athletes from Sweden and Norway since for example, steroid excretion depends on genetic disposition and that varies in different geographical areas. Additionally, individuals taking medications are not excluded from this study unless this was discovered during the analyses (e.g. corticosteroids) and it is likely that at least some were doped. The study population in Study III was also based on elite athletes and controlled for hormonal contraceptive use. However, this recruitment was made before a summer Olympics games so mainly subjects from summer sports were included.

In study I, II, IV and V the subjects were recruited by different methods. For example, in study V, all pregnant women were recruited from one clinic and hence all from the same geographical area. However, for being from one geographical area the spread of ethnicities was large. In addition, all women recruited had normal pregnancies and therefore the results are only valid for normal pregnancies.

4.2 URINE AS SAMPLE MATRIX

The level of an androgen in urine does not always closely reflect that in blood because of genetic differences in metabolism of steroids [60] and factors that affect renal function in extreme stress situation [156]. However, since a large portion of the T for women is produced by peripheral conversion of weaker precursor androgens, studying serum levels of T is not always representative of the androgenic load of the body [157]. Instead, the urinary excretion of androgen metabolites is of interest as it may reflect the androgen exposure [158]. Also, urine sampling has been preferred since it is cheaper to transport and less invasive to collect.

4.2.1 Storing Urine Samples

For some of the studies, the urine samples were stored in the freezer for up to two years leading up to the analysis. The urine samples were stored at -18°C and sometimes subject to several thaw cycles. The major concerns storing urine samples for a longer time is bacterial contamination and possible degradation of steroids. Bacterial degradation is measured by studying bacterial markers and the samples are excluded if there were signs of bacterial degradation (see section 4.3.2.3). TG and EG have been shown to be stable at 4˚C, -20˚C for

22 months as well as for up to three freeze-thaw cycles [159]. However, that study used sterile urine to start with. Van Eenoo et al. had a seemingly better approach by looking at measurement uncertainty data in real urine samples after storing [160]. However, he did not study the steroids but rather other doping agents. He did conclude that 19-NA is stable at 4˚C and -20˚C for 9 months and up to 6 freeze-thaw cycles. The isotopic composition of steroids have been shown to be stable at 37˚C for up to 5 days even when there were signs of bacterial growth in the sample [161].

4.2.2 Specific gravity

Since concentrations of steroids in urine is dependent on the amount of fluids excreted, the measured steroid concentrations have to be corrected for the dilution of the urine. Otherwise, concentrated urine will give an overestimation of the absolute concentration whereas overly diluted urine will give an underestimation of the concentration. To adjust for urine dilution in doping control, specific gravity (SG) is used [2]. SG is the ratio of the weight of a volume of urine to the weight of the same volume of pure water at the same temperature [162]. Since it is the ratio of densities, it is unitless. The correction formula corrects each sample to a specific gravity of 1.020 according to:

$$C_{corrected} = C_{measured} * \frac{1.020 - 1}{SG - 1}$$

There is some controversy on how good this formula for correction is, especially at low (such as <1.005) and high (> 1.035) SG. An alternative way to correct for urine dilution is to use creatinine, this method is generally used in medicine. However, creatinine is a byproduct of muscle metabolism and therefore not an option for athletes. The use of ratios in the steroid profile circumvents the need for dilution correction.

4.3 GC-MS AND GC-MS/MS ANALYSES

The urinary steroid profile was obtained with the standard method used at the Doping Control Laboratory in Stockholm (Division of Clinical Pharmacology, Karolinska University Hospital in Huddinge), at the time of each study. For this reason, the method used for analyzing steroid profiles changed from GC-MS in Study I and III to GC-MS/MS in study II, IV, V and VI. The GC-MS method is essentially described by Chung et al. [163], with minor modifications according to Garle et al. [164], whereas the GC-MS/MS method was described in paper IV.

4.3.1 GC-MS vs GC-MS/MS

The concentrations of the steroid profile obtained by the GC-MS have been proven to yield very similar results to GC-MS/MS [165]. The T/E ratio did show some discrepancies likely due to the higher specificity of the GC-MS/MS method as well as the different ways for the two methods to calculate this ratio (corrected area or concentration based). Even if the methods do not give identical results, this does not affect the results obtained in this thesis because within

one study only one of these methods have been used and hence, only compared to values obtained from the same method. Each batch contained at least one calibration standard and quantification was achieved by comparing the signal to the corresponding isotope labeled internal standard. The limit of quantification (LOQ) and linear range of the two methods can be found in Table 4-2.

Table 4-2. Limit of quantification (LOQ) and linear range for the two GC-MS methods used

Steroid	**GC-MS**		**GC-MS/MS**	
	LOQ (ng/mL)	Linear range (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)
T	2	2-200	0.5	0.5-200
E	2	2-200	0.5	0.5-200
5αAdiol	5	5-500	1.5	1.5-600
5βAdiol	9	9-900	1.5	1.5-600
A	100	100-10000	100	100-10000
Etio	100	100-10000	100	100-10000

4.3.2 Sample preparation controls

In addition to the following internal standards, there are controls samples such as a buffer blank, negative urine and a certified reference material as external control.

4.3.2.1 Hydrolysis control

In steroid screening with GC-MS(/MS), it is the unconjugated as well as the glucuronide fraction that is measured after hydrolysis of the glucuronide group by β-glucuronidase from *E.coli*. The time it takes to hydrolyze each steroid glucuronide varies, and consequently, incomplete hydrolyzation results in characteristic patterns of the steroid profile [3]. T- and E-glucuronide are cleaved completely within several minutes while A- and Etio-glucuronide require extended hydrolysis times. The hydrolysis of Etio-glucuronide is completed faster than the cleavage of A-glucuronide. Consequently, an incomplete hydrolysis leads to decreased A/T and A/Etio ratios [3]. In order to test for the completeness of hydrolysis, the ratio between d4-A-glucuronide and d5-Etio from the internal standard is used (Figure 4-1). If the hydrolysis is not complete, the urine sample has to be prepared again possibly with more β-glucuronidase or extended time for hydrolysis.

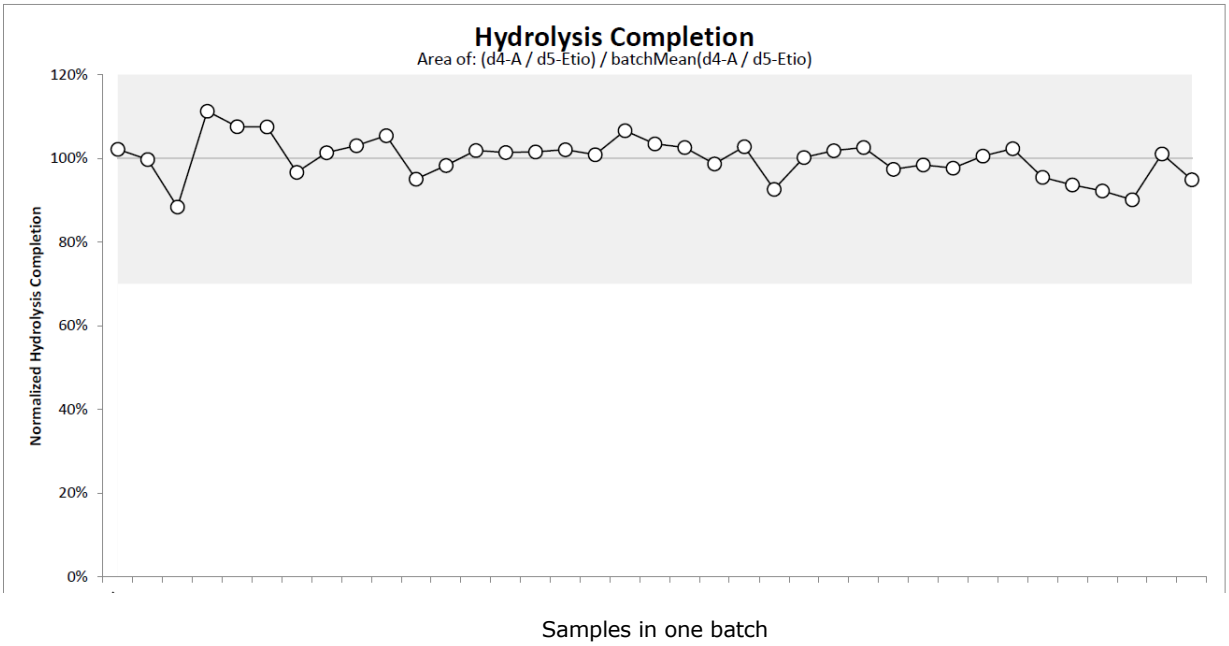


Figure 4-1. Hydrolysis completion results from a batch showing normal variation in hydrolysis between samples. The hydrolysis completion is calculated as d4-A/d5-Etio and normalized to the batch mean. The d4-A comes from the hydrolyzed d4-A-glucuronide in the internal standard.

4.3.2.2 Derivatization control

The last step of sample preparation is derivatization with N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) to form trimethylsilyl (TMS) derivatives. This is done to increase volatility by covering hydrophilic groups which in turn improves GC separation [166]. An incomplete derivatization leads to altered steroid profile ratios, e.g. low A/T [3], as bis-TMS ions are accounted for in GC/MS measurements of endogenous steroids but not those with one TMS group. The completeness of derivatization is controlled by monitoring the Androsterone-mono-TMS, which should be low compared to bis-TMS i.e mono-TMS-A/(mono-TMS-A + bis-TMS-A) < 1%. If the derivatization is incomplete, more derivatization reagent can be added.

4.3.2.3 Bacterial markers

Urine contaminated with bacteria can affect the steroid profile [167], therefore bacterial degradation has to be accounted for. This is done by looking for metabolites bacteria but not humans can produce i.e. 5αAND (5α-androstanedione) and 5βAND (5β-androstanedione) [168]. According to WADA TD2016EAAS, the sample is not valid due to extensive degradation when 5αAND/A ≥ 0.1 and/or 5βAND/Etio ≥ 0.1, values between 0.05 to 0.1 are reported although the sample is still valid [2]. However, not all bacteria can produce 5αAND and 5βAND and therefore, contamination with these bacteria will go unnoticed [168]. An unconjugated fraction higher than 10% of the total amount of testosterone is a useful indicator of urine sample contamination but the unconjugated fraction of T is only measured if suspicion of bacterial contamination is raised.

4.4 UHPLC-HRMS ANALYSES

While GC-MS(/MS) techniques have been used to obtain all steroid profiles in accordance with the WADA guidelines, UHPLC-HRMS was used to study phase II metabolites in Study IV. The benefit of using LC instead of GC is that no hydrolyzation is needed and therefore you can study several phase II modification routes simultaneously, such as the glucuronide, sulfate as well as free fraction. Although the endogenous steroids can be measured with LC-MS, this analytical approach is not used in doping testing, but initial analysis and confirmation should be based on GC separation [2].

4.5 IRMS ANALYSES

During the gas chromatography- combustion- isotope ratio mass spectrometry (GC-C-IRMS) analysis the steroids in the sample are combusted following GC-separation and prior to detection. It is, therefore, necessary that the samples injected are properly cleaned from possible contaminants. Consequently, the sample preparation of the urine before IRMS analysis is extensive. This procedure is explained in detail in paper I. The method had improved before publication of Study II with more separation accomplished by a second HPLC analysis for some of the steroids. The second HPLC was added to decrease the background and further separate some simultaneously eluting endogenous steroids.

The purity of each steroid peak was checked by spectral analysis using GC-MS with the same chromatographic conditions as in the GC-C-IRMS method. If the purity of the peak was less than 95% as compared to a reference spectrum of the compound, the steroid result was rejected and the sample was prepared all over again.

Pregnanediol (PD) was used as ERC in all studies because it is the principal ERC according to the WADA technical document [1]. However, other ERCs such as 5α-androst-16-en-3α-ol (16-en), 11β-hydroxyandrosterone (11-OH-A) or 11-keto-etiocholanolone (11-oxo-Etio) can be used and according to WADA shall replace PD if the PD signal is suppressed, affected by poor chromatography or by administration of a precursor e.g. pregnenolone. We accoutered no problem with PD and hence used it for all samples.

4.6 GENOTYPING

All genotyping was performed using genotyping assays and real-time polymerase chain reaction (rt-PCR). The copy number variation analysis (e.g. *UGT2B17*) is based on $\Delta\Delta C_T$-method using cycle threshold values and comparing to a reference gene [169]. The reference gene used was Albumin in Study I, II and III and changed to RNaseP in Study V. To distinguish between those without the *UGT2B17* gene (del/del) and those with one copy is unproblematic since they will show signals for the reference gene but not UGT2B17. However, to distinguish between those with one or two copies can be more difficult since there may be an overlap in their signals. For this reason, the *UGT2B17 ins/ins* and *ins/del* groups have been combined. Whenever possible, whole blood was used for DNA extraction. However, in Study V, DNA was isolated from saliva.

4.7 THE ATHLETE BIOLOGICAL PASSPORT SOFTWARE

The ABP software used was a research version from the Swiss Laboratory for Doping Analyses, Epalinges, Switzerland, that came before the steroidal module of the ABP was implemented. The algorithm used in the ABP as well as the current software is held secret by WADA and not accessible for researchers.

Since the ABP version used was an older version, the newest ratio in the steroidal module, i.e. 5αAdiol/E, is not included. Instead, we as well as others [78, 87, 170] used average ± 3SD of the baseline values to calculate the subjects individual cut-off ranges. How well this corresponds to the actual algorithm remains unknown because of the secrecy of the algorithm. The ABP algorithm starts by using population-based values and progressively switches to individually calculated thresholds. Using average ± 3SD does not take the population-based values into account and hence will differ from the ABP software.

4.8 STATISTICS

As proven before [171] and in Study VI, steroids do not show a normal distribution but rather a log-normal distribution. The only exception is T, and therefore also the ratios including T (e.i T/E and A/T), which are bimodal due to a deletion polymorphism in *UGT2B17* [59]. Consequently, non-parametric tests are used in this thesis.

The software used has been GraphPad Prism version 4-7 for Windows, GraphPad Software, La Jolla, California, USA; Mplus24 version 5.2 for windows, Muthén & Muthén, Los Angeles, CA; R version 3.3.2 and IBM SPSS Statistics for Windows, Version 24.0, IBM Corp. Armonk, NY.

5 MAIN RESULTS

5.1 NATURAL VARIATIONS IN THE URINARY STEROID PROFILE

Based on the coefficient of variations (CVs) calculated from 11000 steroid profiles, we conclude that using steroid ratios is almost always superior to absolute concentrations (Paper VI). Additionally, all concentrations and ratios show larger between-subject than within-subject variation. Women generally show larger variations in their steroid profiles than men, both inter- and intra-individually. The most stable ratio, e.i. the ratio showing the least intraindividual variation was T/E for men (CV of 16.4%) and A/Etio for women (CV of 22.7%). The greatest variation was seen in 5αAdiol/E for both sexes (intraindividual CV of 26.7% and 40.2%, for men and women respectively).

Based on the bimodal T distribution it was calculated that 13.6% of the men belonged to the group excreting low amounts of T and therefore believed to be homozygous for the deletion of *UGT2B17*. The population-based threshold set to T/E of 4 was exceeded by 4.3% of the men and 2.0% of the women. 0.7% and 0.2% of the men and women, respectively, showed T/E ratios above 6 (Figure 5-1).

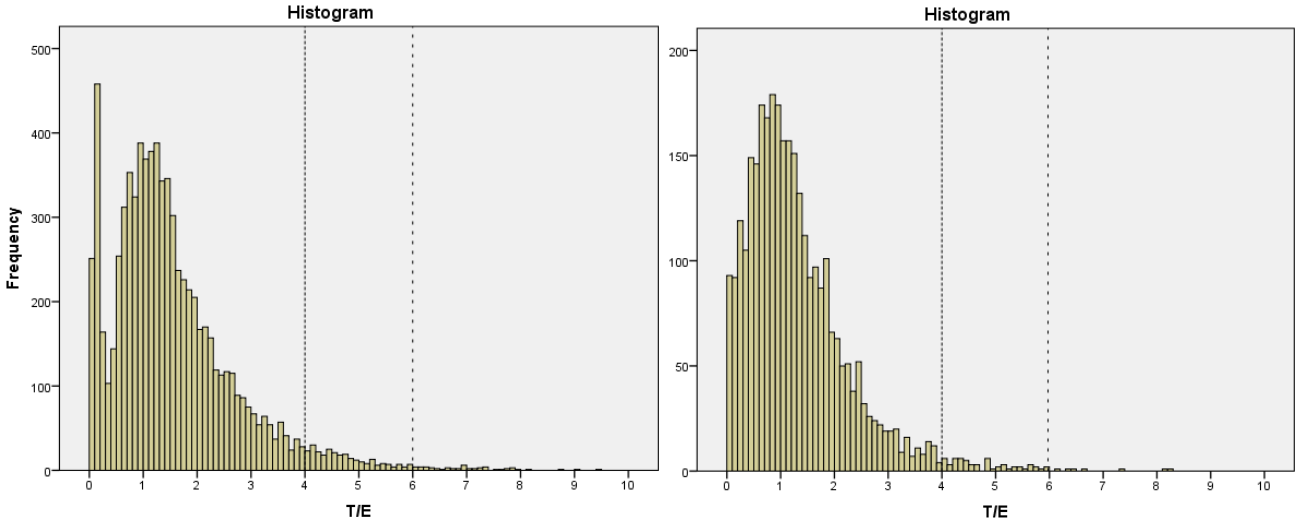


Figure 5-1. T/E ratio distribution for men (to the left) and women (to the right) based on 11000 steroid profiles in Study VI showing part of the distribution outside of WADAs current population threshold of 4 and the previously set one at 6. The absence of bimodality for the female T/E distribution is probably due to the large numbers of missing values because of concentrations below LOQ (N=608).

Some of the inter-individual variation seen in steroid concentrations and ratios could be explained by factors reported on the doping control forms. Such factors included what sport the athlete was participating in (data partly presented in Paper VI), the age of the athlete, if the urine was collected at a competition or not, as well as what time, both of day and year, the sample was collected. The most influential to the steroid profile of these factors was the sports classification belonging (detailed in the appendix to Paper VI) as well as if the sample was collected in competition or not.

5.1.1 Variations with time

The steroid profiles vary over the day for both sexes but women's steroid profiles vary more than men's do. Generally, the ratios are higher in the afternoon than in the morning but especially for women, there are large variations over the day. The largest variation could be seen in 5αAdiol/5βAdiol where the ratio was 48% higher in the afternoon as compared to morning. For men, 5αAdiol/E showed the largest time of day variation with 43% higher values at night as compared to the morning.

Women also show greater variation in their steroid profiles during the year, with significant changes in all five steroid ratios (Paper VI). The T/E ratio varied most with 30% higher median in September as compared to December. Men showed no more than 16% difference of medians in steroid ratios where the months differed significantly.

Men show peak urinary T concentration and T/E ratio at approximately 20 years of age. Women's T, on the other hand, is stable with age but T/E drops due to an increase in E.

5.1.2 In Competition vs. Out of Competition

Women show higher concentrations of all steroids but 5βAdiol when the urine is collected in competition (IC) as compared to out of competition (OOC) (PaperVI). The net effect on the ratios is increases of T/E, A/Etio, and 5αAdiol/5βAdiol as well as decreases of A/T and 5αAdiol/E. Men's steroid levels do not differ as much between IC and OOC testing, however, all ratios but A/T show increased levels IC. Interestingly, women showed 65% higher T excretion IC whereas men T was almost unchanged.

5.1.3 Menstrual cycle

E was the only metabolite that significantly changed during the menstrual cycle, being at its highest at the end of the cycle (Paper IV). This leads to significant decreases in T/E and 5αAdiol/E at the end of the cycle as compared to the first day of the menses.

5.1.4 Pregnancy

Pregnancy affects urinary steroids and the ratios of the steroidal module even in the first trimester (Paper V). E was higher and consequently, the T/E and 5αAdiol/E ratios were lower in the pregnant women. A/Etio, on the other hand, was increased during pregnancy. Depending on what group we compered the pregnant women to (postpartum or non-pregnant women) both ratios 5αAdiol/5βAdiol and A/T showed significant differences (Figure 5-2).

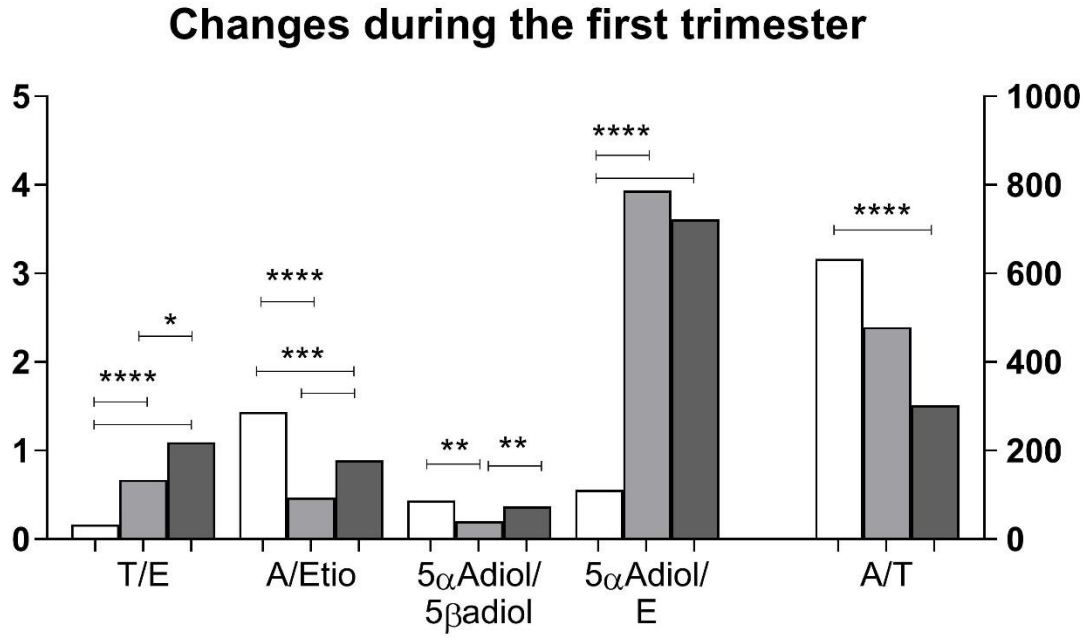


Figure 5-2. Median ratios of the ABP during the first trimester (white boxes, n= 24), postpartum (light grey, n= 20) and in the ABP study population of women (dark grey, n= 3229). A/T values are shown on the right y-axis whereas the others ratios on the left.

5.2 THE URINARY STEROID PROFILE AFTER DRUG USE

5.2.1 Hormonal Contraceptives

Female athletes taking hormonal contraceptives showed 40% lower urinary E than non-users, whereas T was the same. After removing individuals homozygous for the deletion of UGT2B17, the T/E ratio was 29% higher among the HC users (Paper III). A/Etio and 5αAdiol/5βAdiol showed no significant changes. Not used at the time of the publication of paper III was the A/T and 5αAdiol/E ratio, the results from there ratios are shown in Figure 5-3.

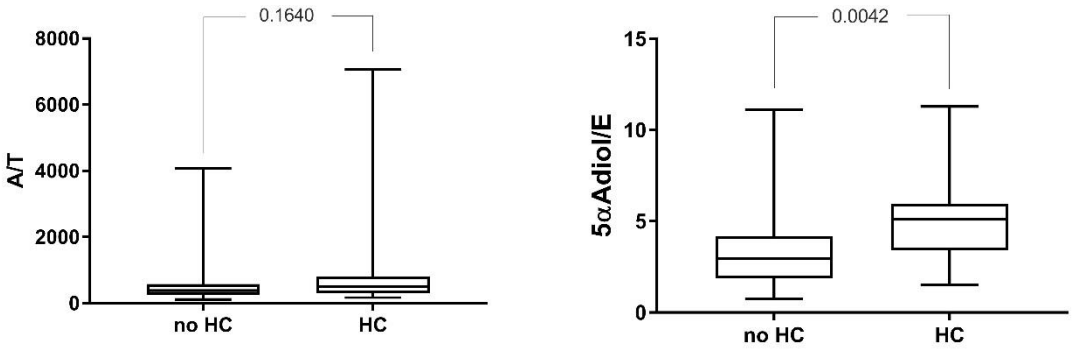


Figure 5-3. A/T and 5αAdiol/E in elite female athletes not taking hormonal contraceptives (no HC) as compared to those taking HC.

There was no significant difference in A/T between the HC users and non-users (Mann-Whitney, p = 0.1640). However, the 5αAdiol/E ratio was 1.7-fold higher in the women using HC (p=0.0042).

Twenty-four hours after a single dose of an emergency contraceptive pill E had decreased 3-fold and A, Etio and 5βAdiol decreased 2-fold (Paper IV). Within 48 hours the levels were back to normal. Despite the changes in concentrations, no ratio changed significantly. However, one woman did show an ATPF in her T/E ratio 12 hours post administration. The research version of the ABP used in this study did not include the 5αAdiol/E, so this ratio was not studied longitudinally. However, if we were to use the mean value of the baseline ± 3SD as a way to calculate the individual reference range, one individual showed ATPF 12 and 72 hours after administration of the emergency contraceptive (Figure 5-4). This was the same individual who showed an ATPF for T/E at 12 h.

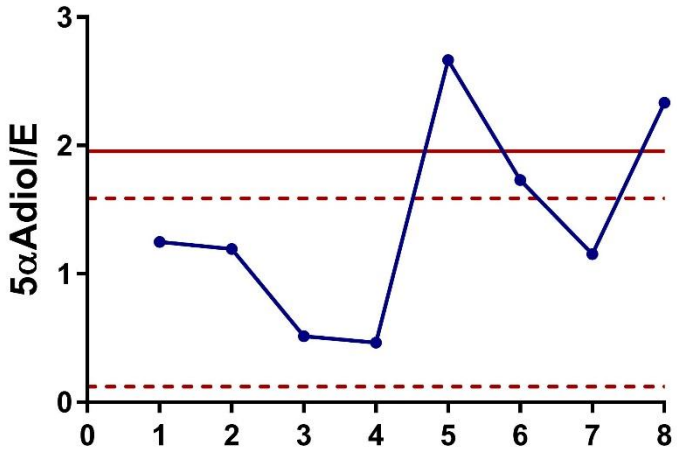


Figure 5-4. Eight 5αAdiol/E values for a woman during a menstrual cycle (1-4, i.e. baseline) and 12, 24, 48 and 72 h after an emergency contraceptive pill. The reference ranges are calculated as mean ± 2SD (dotted line) and ± 3SD (solid line). The lower limit of the reference range (-3SD) is below 0 and therefore not shown.

5.2.2 Testosterone administration

T enanthate injections as low as 125 mg and a single dose of 100 mg T gel could be detected with current methods of doping testing, at least one time point after administration. In Paper I, we saw that T and its metabolites increase in a dose-dependent manner after T enanthate injections, whereas E decreased but not dose-dependently. The suppression of E could still be seen 6-8 weeks after injection of 500 mg T enanthate. The most sensitive marker in the ABP for this detection was T/E followed by 5αAdiol/E, A/T only sporadically went outside the individual's threshold. The other ratios were not affected enough to give ATPFs. 5αAdiol/5βAdiol and A/Etio did not change even if the included concentrations did because of an equal increase of the numerator and denominator of these ratios. 5αAdiol/E was not tested in this study, but when mean of baseline ± 3SD was used all individuals with three baseline values showed atypical profiles for the two higher doses (500 and 250 mg) and all but three individuals for the lowest dose (125 mg).

After T gel administration the major changes in the steroid profile were increases in T and 5αAdiol as well as a decrease in E (Paper II). These changes lead to ATPFs in primarily 5αAdiol/E, T/E and 5αAdiol/5βAdiol, but the other ratios also showed abnormalities. The steroid profile was abnormal for at least 48 hours in half of the individuals, mostly 5αAdiol/E contributing to this longer detection window. T/E, on the other hand, was only atypical for 24 hours for half of the subjects and shorter for the rest.

IRMS was very sensitive to detect doping with T enanthate, one subject being positive for 6-8 weeks after 500 mg injection. IRMS was positive in all four individuals studied after T enanthate injection whereas only two of five tested after T gel met a full criteria for a positive test. The most sensitive metabolite for detection of T injection was T and 5βAdiol whereas for T gel it was 5αAdiol.

5.3 UGT2B17 AND DOPING TESTS

A double deletion polymorphism in *UGT2B17* has previously been associated with T excretion in men, in Study III we show this to be true even for women. We also found that women homozygous for the deletion had higher 5αAdiol/5βAdiol ratios. In Study I and II we saw that none of the *UGT2B17 del/del* subjects reached the population-based thresholds used in traditional testing (Table 5-1). However, using individual reference ranges with three baseline values, seem to be almost as effective for *UGT2B17 del/del* subject as for insertion carriers. IRMS results do not seem to be affected by the *UGT2B17* deletion polymorphism other than that T usually cannot be used as a target compound due to low concentration.

Table 5-1. Sensitivity of the three methods used to detect T doping after administration of three doses of T enanthate injections and one dose T gel. The table shows how many percent of the subjects tested positive at some point after administration according to the positivity criteria of the different tests. Note that in the T gel study there was only 1 del/del subject.

		Traditional testing		ABP		IRMS	
	Dose	*Ins*	*del/del*	*Ins*	*Del/del*	*Ins*	*Del/del*
T enanthate	500 mg	Positive	Negative	Positive	Positive	Positive	Positive
	250 mg	Positive	Negative	Positive	Positive	Positive	Positive
	125 mg	80% positive	Negative	Positive	88% positive	Positive	Positive
T gel	100 mg	14% positive	Negative	Positive	Positive	25% positive	Positive

5.4 SPECIFIC GRAVITY CORRECTION (PRELIMINARY RESULTS)

To adjust for different dilutions, the specific gravity of the urine samples is used. The median specific gravity of the 11 000 urine samples in Study VI were 1.016 for women and 1.019 for men ($p < 0.001$) with a range from 1.002 to 1.050. To investigate if the correction formula for specific gravity is accurate, A concentrations (not corrected for SG) among men were used. Assuming that the median A concentrations were not dependent on SG, but rather the other way around, the correction formula was compared to median A concentrations. The dotted line Figure 5-5 is the correction formula for SG:

$$C_{corrected} = C_{measured} * \frac{1.020 - 1}{SG - 1}$$

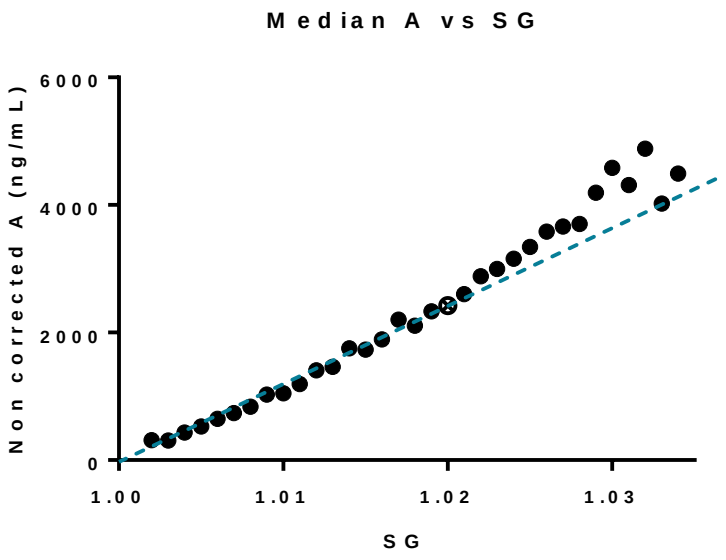


Figure 5-5. Median Androsterone concentration not corrected for specific gravity plotted against specific gravity for men. The correction formula for SG is illustrated as the dotted line.

6 DISCUSSION

Implementation of the steroid module of the ABP has improved detectability of doping with endogenous steroids as evidenced by the results in Study I and II. However, evaluating steroid profiles is a difficult task since the steroid profile is affected by many factors other than doping. In this thesis, factors such as season, time of day, age, IC/OOC testing, menstrual cycle, hormonal contraceptives, and pregnancy have been studied in relation to their effect on the urinary steroid profile used in doping control.

We have seen both **annual** and **diurnal** variations in steroid profiles when looking at a large population of athletes, with time of day having a larger impact on markers of the steroid profile. For example, the women in this population showed almost 50% higher median 5αAdiol/5βAdiol in the afternoon as compared to the morning. The relevance of this diurnal variation when evaluating passports has to be evaluated on an individual basis. However, these results show that variations over the day, and maybe even year, might be factors to consider when interpreting steroid passports. Ideally, in order to minimize the intra-individual variability due to diurnal variation, the urine would always be collected at the same time of the day. However beneficial for the ABP, this would not be a favorable approach for doping testing in general. Some doping substances have short detection windows and would be easy to get away with if the surprising momentum disappeared from doping testing.

Steroid profiles collected **in competition** (IC) can differ from those collected **out of competition** (OOC). Samples from women collected IC showed on average 65% higher T levels than those collected OOC whereas men showed almost no change. This is likely due to the fact that a great part of women's androgen production occurs in the adrenal gland which is under control of the stress hormone ACTH [30]. Almost all male T production, on the other hand, occurs in the testes under control of LH. However, serum T levels have previously been shown to increase after a match win in soccer and decrease after a loss in both men and women [121]. It is possible that larger changes between IC and OOC can be seen if the performance in the competition is taken into account.

The variations women show during the **menstrual cycle** further complicates interpretation of female steroid passports. Women show a larger variation in almost all markers of the steroid profile as compared to men and part of this is due to the menstrual cycle. The results from our pilot study involving 6 women were in agreement with those of Longhino et al. showing maximal E levels by the end of the cycle [128]. Our results in combination with what has previously been reported [129] seem to strengthen the theory of a decrease in T/E with progressing menstrual cycle. However, the menstrual cycle will likely just be one factor increasing the intra-individual variability that is not taken into account in the evaluation of passports. The effect of the menstrual cycle on the steroid profile is not enough to justify having athletes give out as personal information as the status of their menstrual cycle. In addition, a lot of athletes have irregular menses, making this information unreliable.

The use of **hormonal contraceptives**, however, can give patterns on the steroid passports similar to micro-doping with T and should be taken into account when evaluating passports. When a woman starts taking HC, her E excretion is suppressed leading to an increase in the T/E ratio. On the doping control forms, the athletes have to give information about all medications taken and hence also contraceptive use. This should be checked by the APMUs before suspicion of micro-doping is raised in a female athlete.

Pregnant women show great differences in their steroid profiles as compared to non-pregnant women. Athletes can compete when pregnant and be subject to a doping test, especially in the first trimester. The APMUs evaluating the passports do generally not know if the passport they are studying belongs to a pregnant woman. However, pregnancy gives a clear pattern to the steroid profile and suspicion should be raised by the evaluator. In these cases, the urine should be tested for hCG to confirm pregnancy and invalidate this sample for future use in the passport if the athlete continues being tested after the pregnancy.

We have seen that **doping** with as low as 125 mg T enanthate (i.e. 90 mg bioavailable T) and 100 mg T gel (approximately 10 mg absorbed), can be detected with the ABP. However, this detectability might be an overestimation since the detection required three baseline values which are easy to collect for a clinical study but harder in doping testing of athletes. Our baseline values were relatively stable, however, in real doping testing, this may not be the case. The reasons for this might be that all our baseline samples were collected in the morning and the study did not go on for very long, minimizing the effect of diurnal and annual variation. In addition, the test subjects were not (as far as we know) subject to any large stressors like competitions, and use of drugs was restricted. It is possible that these administrations might not have been detected because of larger variation of baseline values in samples from athletes, or explained away as natural variation by those evaluating the passports.

If suspicion of doping is raised from a passport, this alone can be enough to convict someone of doping, but only after three independent experts have evaluated the passport as "likely doping". For this to happen, the dose taken has to be large enough to make changes in the passports that cannot be explained by any other factors than doping. In reality, most of the samples are instead sent to further analysis with **IRMS** that is used as ultimate proof of doping. This method was in our studies relatively sensitive at detecting doping with T. However, we used T preparations with a different isotopic composition than what can be seen naturally. IRMS is inadequate if the T preparation has an isotopic composition similar to the athlete's endogenous production and preparations like this are known to exist [94-96]. In addition, isotopic composition is affected by HC [89, 172], since estrogens and progestogen in oral contraceptive pills have delta values around -30‰ [173]. However, the decrease is only around −0.70‰ so not enough to give a positive IRMS result.

One of the many benefits of the individual approach utilized in the ABP is that the reference ranges corrects for excretion differences between individuals after a number of tests. This is important as subjects with the double deletion of ***UGT2B17*** excrete much lower T than individuals with one or two copies of the gene. The *UGT2B17 del/del* subject will normally not reach a population-based threshold in T or ratios including T, even after doping with T [62]. However, as seen in Study VI the vast majority of athletes (at least in Sweden and Norway) are only tested once. For evaluation of these results, population-based reference ranges have to be used because there is no other option. In these cases, genotyping for *UGT2B17* would lead to great improvement for detection of EAAS doping [174].

In doping, **specific gravity** is used to correct for different dilutions of the urine samples. Preliminary results show that this formula is relatively good in the interval mainly used (1.005-1.035). However, median A concentrations, plotted in Figure 5-5, are not corrected for time of day. Time of day can very well be a confounder in this analysis since we have shown that there are diurnal variations in steroid levels and urine is normally more concentrated in the morning (i.e. higher specific gravity). Until the median concentrations have been corrected for time of day, these results should be viewed cautiously. On the other hand, the use of ratios in the ABP circumvent the problem of dilution since no adjustment is necessary.

7 CONCLUSIONS AND FUTURE CONSIDERATIONS

Interpretation of steroid passports is a difficult task. We have seen that doping with low doses of T enanthate as well as T gel gives atypical passports. However, the steroid profile is sensitive to other factors than doping and even natural variations can behave very similar to doping with small doses of endogenous steroids. There are many factors to consider when interpreting steroid passports and most of them we do not know enough about to say exactly how they affect the steroid profile. The articles in this thesis give some answers but far from all. There is a great need for more information for those evaluating profiles.

Due to the influence of confounding factors, some of them described here, and others still unknown, it is difficult to undeniably evaluate a profile as "likely doping" without any additional proof or intelligence. To date, the steroid module of the ABP has not been brought forward as the only evidence in a case (whereas for the hematological module > 100 such cases exist). Therefore, the steroid module is so far mainly used for target testing and increased accuracy when selecting samples for IRMS analyses. IRMS really is a remarkable method where you can find out the isotopic composition of the molecule and distinguish if it could have been produced by the body. It is a great and sensitive method if the isotopic composition of the EAAS taken differs from the steroids produced by the body. The problem is that many of them do not have a different isotopic composition. Athletes can use these "IRMS-proof" substances without fear of testing positive as long as they do not take too much.

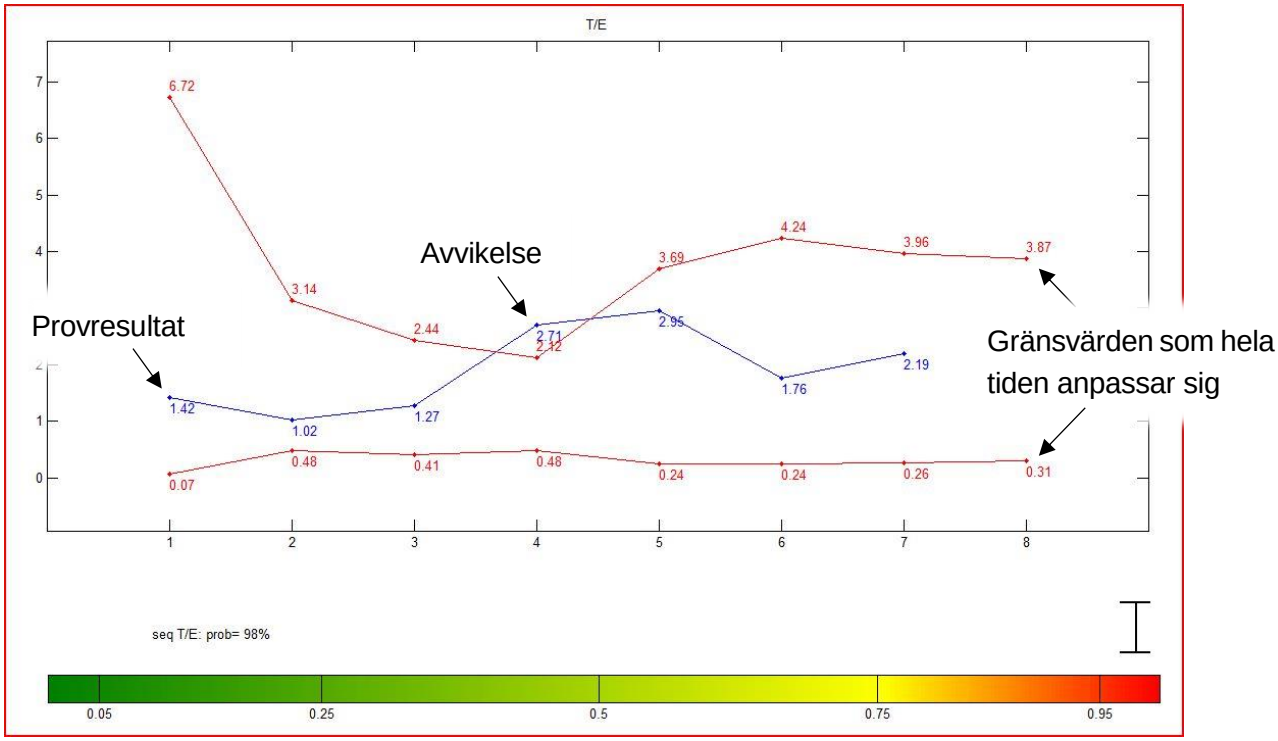
There is a need for more tools to be able to catch doping with endogenous steroids. These tools could be new analytical methods – preferably an "uncheatable" confirmation method, addition of new sensitive biomarkers, complete mapping of all factors that affect the profile or better, all of those things. Some new methods have been proposed, like hydrogen-IRMS [175] and direct detection of T esters in blood [176]. We have seen that other biomarkers used in the hematological module of the ABP change when EAAS are used, so studying the two modules together might improve detectability. In addition, if markers like serum LH and DHT is added to a third module, the endocrine module, this can further improve the sensitivity for EAAS detection. Additionally, this thesis begins to evaluate some factors affecting the steroid profile which can be used for evaluation of steroid passports.

In spite of the difficulties interpreting steroid passports, the steroidal module has greatly improved the detection of EAAS. The purpose of the ban of T is to promote health, fairness, and equality for athletes. Even if T doping still likely exists, the doses they can get away with are lower than before the steroidal module was implemented. If the implementation of the ABP has resulted in lower doses used, this means fewer side effects and therefore better health of the athletes, as well as less advantage for those taking EAAS and hence more fairness and equality. The ABP has a lot of potential for improvement but is a great step in the right direction to fight doping with endogenous steroids.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Doping med kroppsegna substanser är svårare att upptäcka än substanser som normalt sett inte finns i kroppen. En sådan substans som kan användas i prestationshöjande syfte är det manliga könshormonet testosteron. För att upptäcka testosterondoping mäts koncentrationen av testosteron och några andra relaterade steroider i urin, dessa värden utgör en steroidprofil. Det finns generella gränsvärden som man jämför denna steroidprofil med som är framtagna med populationsbaserade studier. Alla är olika och dessa populationsbaserade gränsvärden är väldigt generöst tilltagna vilket gör att det är svårt att upptäcka doping med små mängder testosteron.

Skillnaden mellan individer är dock stor och det är därför bättre att använda individuella gränsvärden. Så istället har man börjat räkna ut egna gränsvärden för varje utövare baserat på tidigare tester och för att gränsvärdena ska bli så bra som möjligt behöver idrottaren testas minst tre gånger. Sedan 2014 läggs en idrottares alla steroidprofiler in i ett biologiskt pass med vilka ett datorprogram gör grafer med individuella gränsvärden som programmet har räknat ut. Om en steroidprofil går utanför de individuella gränsvärdena tolkas resultatet av en expert som avgör om doping kan vara anledningen till avvikelsen.



Bilden ovan visar ett exempel på hur en graf i det biologiska passet kan se ut. Grafen beskriver den viktigaste markören för detektion av testosteron, T/E kvoten. Den här personen har testats 7 gånger och i början (prov 1-3 på x-axeln) är värdena stabila, sen händer det något och individens provresultat går utanför gränsvärdena och det ger en avvikelse. I det här fallet avviker provet för att vi, i en studie, gett denna person en dos testosterongel. Gelen smörjdes in mellan det tredje och fjärde urinprovet och prov 4-7 samlades in under nästföljande två dagar.

Det biologiska passet är dock inte helt lätt att tolka för de finns stora spridningar i steroidprofilen även inom en individ och anledningar andra än doping som kan ge avvikelser i passet. I den här avhandlingen har vi studerat hur steroidprofilen varierar normalt och efter intag av läkemedel, både dopingklassade och andra.

Doping med två olika testosteronpreparat, ett som injektion och ett i gel-form, gick att upptäcka hos män med det biologiska passet. När vi istället jämförde med de populationsbaserade gränsvärdena var det många som inte gick utanför gränserna. De här studierna visar att införandet av det biologiska passet har förbättrat möjligheterna att upptäcka doping med testosteron.

Kvinnors steroidpass är mer svårtolkade än mäns. Kvinnors nivåer av testosteron är ibland för låga för att kunna mätas med dagens analysmetoder, vilket inte nödvändigtvis betyder att de inte har dopat sig. Kvinnor har också generellt större naturlig variation av steroidnivåer än män. Bland annat så fann vi att en del av variationen hos kvinnor beror på menscykeln. Kvinnor hade också större inverkan av stress (om provet är taget t.ex. under tävling), tid på dygnet och årstid. När en kvinna blir gravid så ändras också steroidprofilen ordentligt, men sådana uppgifter är idag inte tillgängliga för dem som tolkar passen.

En annat viktigt fynd vi gjorde i våra studier var att p-piller påverkar en av de viktigaste markörerna i steroidpasset och gör det på samma sätt som doping med låga doser testosteron. Vidare analyser av provet kommer att visa att avvikelser på grund av p-piller inte beror på intag av steroider, så ingen kommer att bli tagen för doping efter intag av p-piller men de vidare analyserna är dyra och tidskrävande, resurser som borde spenderas på annat.

Ett avvikande steroidpass kan vara tillräckligt med bevis för doping men nästan alltid krävs det att man skickar urinprovet på vidare analyser för att samla tillräckligt med bevis. Den metod som används för att bevisa intag av testosteron förkortas IRMS och jämför sammansättningen av isotoper i testosteronmolekylen. I våra studier visade sig IRMS vara en bra metod men inte lika känslig som steroidpasset. Tyvärr finns det idag dopingpreparat som är ”IRMS-säkra” där testosteron har framställts på ett sätt som liknar det vi producerar själva. Dessa preparat kommer inte att ge utslag i IRMS testerna och därför skulle det vara bra om steroidpasset självt kunde utgöra tillräckligt med bevis.

Sammanfattningsvis så är steroidpasset den bästa metoden för att upptäcka doping med testosteron, men det är svårt att bevisa doping enbart med steroidpasset då det är svårt att se skillnad på naturliga variationer och doping med låga doser. Det finns dessutom flera andra faktorer som kan påverka steroidpasset och det skulle behövas en ordentlig kartläggning av alla dessa faktorer för att kunna underlätta för dem som utvärderar passen.

9 ACKNOWLEDGEMENTS

When I got accepted to my PhD program I was congratulated upon getting the best main supervisor. Today I could not agree more. The supervision I have received over the last five years from **Lena Ekström** has been incredible. Her broad knowledge and memory for details and her constant willingness to help has made her all I could have wanted in a supervisor. Thank you Lena!

Not only was I lucky with my main supervisor but my three co-supervisors together cover all the expertise I needed. **Jenny Schulze** (co-supervisor), my double with whom I share more than the name in common, knowing everything about genetics and steroid profiling. It has been great to have someone who knows steroid profiling in real doping control and the difficulties therein, putting my research in perspective. I am happy to continue in your footsteps.

Magnus Ericsson (co-supervisor), for always making me feel welcome in the lab. I am thankful for the time you and the rest of your staff have invested in me, teaching me the methods used today for EAAS detection.

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