UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

Improving time- and cost-efficiency in doping analysis

PhD Thesis

Sara Amalie Solheim

December 2019

ADD
ANTI DOPING DANMARK

UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

Improving time- and cost-efficiency in doping analysis

PhD thesis

Sara Amalie Solheim

This thesis has been submitted to the PhD School of the Faculty of Science, University of Copenhagen

Improving time- and cost-efficiency in doping analysis

Department of Nutrition, Exercise and Sports

Faculty of Science

University of Copenhagen, Copenhagen, Denmark

Supervisors

Associate Professor Nikolai Baastrup Nordsborg

Department of Nutrition, Exercise and Sports,

University of Copenhagen, Copenhagen, Denmark

Dr. Jakob Mørkeberg

Anti Doping Denmark, Brøndby, Denmark

Dr. Yvette Dehnes

Norwegian Doping Control Laboratory,

Oslo University Hospital, Oslo, Norway

Assessment committee

Associate Professor Thomas Elbenhardt Jensen **(chairman)**

Department of Nutrition, Exercise and Sports,

University of Copenhagen, Copenhagen, Denmark

Dr. Jenny Schulze

Swedish Anti-Doping,

Swedish Sports Confederation, Stockholm, Sweden

Professor Per Aaagaard

Department of Sports Science and Clinical Biomechanics

University of Southern Denmark, Odense, Denmark

ISBN 978-87-7209-334-5
Printed by SL grafik, Frederiksberg, Denmark (slgrafik.dk)
Front & back cover design by Ditte Toft Clausen, Anti Doping Denmark

Table of contents

List of papers

I. **Solheim SA**, Mørkeberg J, Dehnes Y, Hullstein I, Juul A, Upners EN, Nordsborg NB. Changes in blood parameters after intramuscular testosterone ester injections – Implications for anti-doping. *Submitted to Drug Testing and Analysis*

II. **Solheim SA**, Levernæs MC, Mørkeberg J, Juul A, Upners EN, Nordsborg NB, Dehnes Y. Dried blood spot analysis of testosterone esters is applicable in doping analysis. *Draft*.

III. **Solheim SA**, Mørkeberg J, Juul A, Freiesleben SY, Upners EN, Dehnes Y, Nordsborg NB. An intramuscular injection of mixed testosterone esters does not acutely enhance strength and power in recreationally active young men. *Submitted to European Journal of Applied Physiology*

IV. **Solheim SA**, Jessen S, Mørkeberg J, Thevis M, Dehnes Y, Eibye K, Hostrup M, Nordsborg NB. Single-dose administration of clenbuterol is detectable in Dried Blood Spots. *Submitted to Drug Testing and Analysis*

Acknowledgements

This PhD project has involved many contributors and collaborators and I wish to express my gratitude to all. Particularly, I would like to acknowledge:

I would like to express my greatest gratitude to Anti Doping Denmark, the Innovation Fund Denmark and the Partnership for Clean Competition, who financially supported this Industrial PhD project and gave me this opportunity.

I would also like to express my sincere gratitude to my supervisors Associate Professor Nikolai Baastrup Nordsborg, Dr. Jakob Mørkeberg and Dr. Yvette Dehnes for the irreplaceable supervision and help in all phases of this PhD project. I could not ask for any better supervisors. – Thank you for always taking your time and for sharing your knowledge. You are inspiring.

To my colleagues at Anti Doping Denmark: It has been a great pleasure to be included in the best team of colleagues. – Thank you for all the support and laughter. I also owe a big thank to my colleagues at Integrative Physiology for good discussions and assistance whenever needed, and to the laboratory personnel at the Norwegian Doping Control Laboratory for welcoming a sports physiologist into their laboratory and for helping out with the projects. Further, I wish to express my gratitude to all the participants in our research projects.

I would also like to thank Dr. Matthias Kamber (former director of Anti-Doping Switzerland) and Dr. Matthew Fedoruk (USADA) for the guidance in the development of the collection and transportation procedures for use in the dried blood spot projects.

Finally, this would never been possible without the support from my beloved family and friends. Thank you for the invaluable support. A special thanks to Waldo for always being there for me and for always understanding. – You are the best. And to Aksel for being a fighter. Last but not least, I would like to thank Bestefar: thank you for always being positive and always supporting me. You were, and will always be, my idol. This thesis is to you.

Copenhagen, December 2019,
Sara Amalie Solheim

Abstract

Testosterone esters and clenbuterol are among the most frequently used doping substances in elite and recreational sports. Direct detection in urine and blood samples is hampered by the costs of collection, transportation and analysis, and the rapid hydrolysis of testosterone esters in blood. Indirect detection of testosterone by the 'Athlete Biological Passport' (ABP) steroidal module is limited by both the associated costs and confounding factors. Therefore, the present thesis aimed to improve the time- and cost-efficiency in doping analysis by evaluating 1) the applicability of dried blood spots (DBS) as a complementary sample matrix and 2) whether the hematological module of the ABP can be used to indicate doping of testosterone and thereby increase detection, given the erythropoietic effect of testosterone, and 3) by determining the most cost-efficient anti-doping testing program based on detection windows and performance-enhancing effects.

In *Paper I-III*, DBS, urine and blood samples from men receiving two intramuscular injections of Sustanon® 250 (n = 9) or placebo (n = 10) in a randomized, placebo-controlled design were analyzed for direct and indirect detection of testosterone esters and assessment of serum levels of reproductive hormones. In *Paper III*, the performances in countermovement jump, 30-s all out cycle sprint and one-arm isometric elbow flexion were measured before and 24 h after the first Sustanon® injection. In *Paper IV*, DBS and urine samples from 6 healthy men receiving a single oral dose of 80 μg clenbuterol were collected and analyzed for detection of clenbuterol.

Paper II and *IV* demonstrated that the DBS assays allow for detection up to 14 days after an intramuscular injection of 250 mg Sustanon®, and for at least 3 days after an oral ingestion of 80 μg clenbuterol, with 100% specificity. Further, preliminary data suggest that DBS-sampling is well accepted by athletes. Additionally, *Paper IV* showed that clenbuterol can be detected for at least 10 days in urine after ingestion of 80 μg of drug. *Paper I* demonstrated that some hematological biomarkers are affected by testosterone administration, and that the largest changes occur 3-10 days after an injection. *Paper III* showed that a single injection of testosterone esters do not enhance human performance acutely in a countermovement jump test, a one-arm isometric elbow flexion test nor a 30-sec cycle sprint test.

In conclusion, the DBS analyses of testosterone esters and clenbuterol appear to have sufficient specificity and sensitivity to be implemented in routine doping control in elite and recreational sports. Given the longer detection windows for clenbuterol in urine, urine is expected to remain as the preferred sample matrix for clenbuterol analysis. However, the implementation of DBS sampling could improve time- and cost-efficiency while reducing intrusiveness, and thereby allow for higher frequency of testing, or testing of a large number of athletes in a short time, with the aim of increasing detection and deterrence. Further, changes in markers in the hematological module could be indicative of testosterone doping, and should be considered an additional tool for targeted follow-up sample collection and confirmatory analysis. Moreover, since testosterone did not have any acute performance-enhancing effects in power/strength exercises, athletes are likely not to have an advantage if administering a single dose of testosterone esters immediately before or during a competition in power/strength sports.

Resumé

Testosteronestere og clenbuterol er blandt de mest anvendte dopingstoffer i elite- og motionsidræt. Direkte detektion i urin- og blodprøver er forbundet med betydelige prøveopsamlingsomkostninger, strenge krav til transport og analyse, samt hurtig nedbrydning af testosteronestere i blod. Denne afhandling havde til formål at forbedre kosteffektiviteten af anti-dopingtestning gennem undersøgelse af 1) anvendeligheden af en komplementær prøveopsamlingsmatrix; dried blood spots (DBS) og 2) hvorvidt det hæmatologiske modul i 'the Athlete Biological Passport' (ABP) kan bruges til at indikere testosterondoping og derved forbedre detekteringen, givet testosterons stimulerende effekt på produktionen af røde blodceller, og 3) ved at give konkrete forslag til testning baseret på sporingstid og præstationsfremmende effekter.

I *Studie I-III* blev DBS, urin- og blodprøver fra mænd, der modtog to intramuskulære injektioner af Sustanon® 250 (n = 9) eller placebo (n = 10) i en randomiseret, placebokontrolleret design, analyseret for direkte og indirekte (ABP) påvisning af testosteronestere og måling af reproduktionshormoner i serum. I *Studie III* blev præstationen i countermovement jump, 30-sekunders cykelsprint og isometrisk albuefleksion målt før og 24 timer efter den første Sustanon®-injektion. I *Studie IV* blev DBS og urinprøver fra 6 raske mænd, der modtog en enkelt oral dosis af 80 µg clenbuterol, opsamlet og analyseret for påvisning af clenbuterol.

Studie II og *IV* viste, at analyser af DBS kan påvise doping op til 14 dage efter en intramuskulær injektion af 250 mg Sustanon® og i mindst 3 dage efter en oral dosis af 80 µg clenbuterol, med 100% specificitet. Endvidere viste *Studie IV*, at clenbuterol kan påvises i mindst 10 dage i urin efter en oral dosis à 80 µg. *Studie I* viste, at de hæmatologiske markører i ABP påvirkes af testosteronadministration, og at de mest markante ændringer finder sted 3-10 dage efter en injektion. *Stude III* viste, at en enkelt injektion af testosteronestere ikke forbedrer præstationen akut i countermovement jump, 30-sekunders cykelsprint eller isometrisk albuefleksion.

Samlet set ser det ud til, at DBS-baserede analyser af testosteronestere og clenbuterol har tilstrækkelig specificitet og sensitivitet til at kunne implementeres i dopingkontrol i elite- og motionsidræt. I betragtning af den længere detektionstid for clenbuterol i urin, forventes urin at fortsat være den foretrukne prøvematrix til at detektere clenbuteroldoping. Dog vil implementering af DBS reducere prøveopsamlingstiden, og derved muliggøre en højere testfrekvens samt testning af mange atleter på kort tid. Endvidere kan ændringer i markører i det hæmatologiske modul indikere testosterondoping, og bør betragtes som et ekstra værktøj til sporing af testosterondoping herunder målrettet testning. Da testosteron ikke havde nogen akut præstationsfremmende effekt i styrke-/kraftøvelser, vil atleter sandsynligvis ikke have en fordel, hvis de administrerer en enkelt dosis testosteronestere umiddelbart før eller under en konkurrence i styrke-/kraftidrætter.

Abbreviations

5α-diol	5α-androstane-3α, 17β-diol
5β-diol	5β-androstane-3α, 17β-diol
A	Androsterone
AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete biological passport
ABPS	Abnormal blood profile score
ATF	Atypical finding
ATPF	Atypical passport finding
cAMP	Cyclic adenosine monophosphate
CMC	Carboxymethyl cellulose
DBS	Dried blood spot
DHT	Dihydrotestosterone
DPS	Dried plasma spot
E	Epitestosterone
ERK	Extracellular signal-regulated kinases
ESA	Erythropoiesis stimulating agent
EtG	Ethyl glucuronide
Etio	Etiocholanolone
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GLUT4	Glucose transporter 4
hCG	Human chorionic gonadotropin
HCT	Hematocrit
HGB	Hemoglobin
IRMS	Gas chromatography-combustion-isotope-ratio mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LH	Luteinizing hormone
LOD	Limit of detection
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MVC	Maximal voluntary contraction
NanoLC-MS/MS	Nano-scale LC-MS/MS
PKA	Protein kinase A
RBC	Red blood cell
RBC#	Red blood cell count
RET%	Reticulocyte percentage
RFD	Rate of force development
rhEPO	Recombinant human erythropoietin
T	Testosterone
TD	Testosterone decanoate
TI	Testosterone isocaproate
TP	Testosterone propionate
TPP	Testosterone phenylpropionate
$\dot{V}O_{2peak}$	Peak oxygen uptake
WADA	World Anti-Doping Agency

1. Introduction

Many of the performance- and body image-enhancing drugs used by athletes and nonathletes fall within the category of lean mass builders that enhance muscle growth, reduce body fat or a combination thereof. Specifically, questionnaires, interviews and anti-doping testing figures show that anabolic agents are some of the most prevalent substances used by athletes[1–3] and people engaging in recreational exercise training.[3–5] In 2017, 44% of all adverse analytical findings in the in World Anti-Doping Agency's (WADA) anti-doping and management system, ADAMS, were substances under Section 1, 'anabolic agents', on the Prohibited List.[2] This includes substances such as the anabolic androgenic steroid (AAS) testosterone and its synthetic analogues, as well as the anabolic agent clenbuterol. Moreover, anecdotal and empirical evidence indicate that doping, particularly with clenbuterol, is an increasing problem among females. In Denmark, 26% of the female fitness customers who delivered a positive doping test between January 2014 and 2019 tested positive for this substance, which is likely to have particular appeal to women because it does not cause the androgenic side effects associated with AAS. Thus, detection of these substances is of high relevance within anti-doping. However, the current methods are challenged by the related costs from sample collection to analysis, as well as the athletes' experience of the sample collection process.[6] Therefore, the present thesis will focus on the further development of both current detection methods and alternative methods, with the aims of improving time-and-cost efficiency within anti-doping, making the process more athlete-friendly and increasing the likelihood of catching dopers.

The following sections will introduce possible ergogenic effects and adverse side-effects of testosterone and clenbuterol, how doping with these substances can be detected, current challenges for doping control sample collection and analysis, as well as future possibilities.

1.1 Performance-enhancing effects of testosterone

Long-term testosterone administration has well-known physiological effects such as promoting skeletal muscle growth,[7] fat loss[8] and increasing red blood cell (RBC) production.[9,10] The underlying mechanisms of testosterone-induced skeletal muscle hypertrophy have been extensively summarized earlier,[11,12] emphasizing the multiple cellular pathways involved, including activation of satellite cells via intercellular androgen receptors, IGF1-mediated stimulation of the anabolic AKT-mTOR pathway and inhibition of muscle breakdown through repressed myostatin activity.

1.1.1 Ergogenic effects of long-term and short-term testosterone administration

While the physiological effects of testosterone are well-explored, the number of well-designed placebo-controlled studies investigating the ergogenic effects of testosterone administration on human exercise performance is limited.[13–16] When controlling for the potentially confounding variables nutrition and training volume, Bhasin and co-workers showed that supraphysiological doses (600 mg/wk) of intramuscular testosterone enanthate administered for 10 weeks increased muscle size in arms and legs and maximal strength in bench press and squat in healthy eugonadal men with weight-lifting experience, compared with placebo.[13] Moreover, the combination of testosterone administration and strength training lead to greater strength improvement in bench press and squat than testosterone administration or strength training alone. Further, 12 weeks with weekly intramuscular injections of around 300 mg testosterone enanthate, combined with a supervised strength training program, improved maximal bench press strength[14,17] and 30-m running sprint performance[17] in 11 weight-trained men compared with the strength-training placebo group (n = 10). Interestingly, the majority of strength gain took place in the initial 6 weeks of the 12 weeks administration period (14% at week 6 and 22% at week 12), suggesting that short-term testosterone administration can have ergogenic effects.[14] This was supported by the finding that three weeks with intramuscular injections of 200-300 mg/wk testosterone enanthate, but not placebo, combined with heavy strength training was sufficient to increase maximal bench press strength and total work in a 10-s cycle sprint in nine healthy, weight-trained, young men.[15] Although neural adaptations account for the major proportion of initial strength gains, skeletal muscle hypertrophy has been reported to occur already after three weeks of resistance training in recreationally active individuals.[18] This early hypertrophic response is likely to be potentiated by testosterone administration. Therefore, the reported strength gains following short-term testosterone administration combined with strength training may be attributed to both neural adaptations and muscle hypertrophy.

Interestingly, 100 mg/week of testosterone propionate for three weeks was insufficient to induce consistent administration effects on lower and upper body isokinetic strength, in nine weight-trained adults (8 men, 1 woman). However, this might be explained by the administered dose, because the effects of testosterone administration on muscle strength, leg power,[19,20] muscle size[20,21] in healthy, young men are dose- and concentration dependent, evident by larger changes in the 300 and 600 mg/wk groups than in the groups receiving weekly injections of either 25, 50 or 125 mg of testosterone enanthate for 20 weeks. In this regard, it should be mentioned that there are ethical concerns related to the dose and number of doses when administering testosterone to healthy subjects, due to the suppression of the subjects' reproductive hormone production.[22]

Further, one study has investigated the effect of testosterone on running performance and recovery. Baume and co-workers hypothesized that multiple doses of AAS would enhance the recovery and thereby the response to endurance training, leading to improved endurance performance.[16] No consistent treatment effects were observed on running speed at anaerobic threshold or on biomarkers of physical stress in healthy, recreationally active men following four weeks of combined endurance training and orally administered placebo (n = 9), testosterone undecanoate (80 mg, n = 8) or 19-norandrostendione (100 mg, n = 8) three times per week.[16] Noteworthy, serum testosterone concentration remained unchanged during the intervention, which suggests that the administered dosage was insufficient to induce any measurable changes in recovery and physical adaptation to training. Therefore, this single, relatively small and short study preclude firm conclusions, especially on testosterone's effects on endurance performance. Interestingly, testosterone administration has been found to stimulate erythropoiesis[9,10,23] in a dose-dependent manner,[24] possibly through a combination of direct and indirect androgen effects on the bone marrow erythropoiesis. This includes increased number of erythropoietin-responsive progenitor cells in the bone marrow,[25] increased renal secretion of erythropoietin[10,26] and increased iron availability through suppressed hepcidin.[9,10] Thus, theoretically, it is possible that the testosterone administration can enhance endurance performance through increased arterial oxygen content, and thereby maximal oxygen uptake relative to body weight ($\dot{V}O_{2max}$ or $\dot{V}O_{2peak}$), which is an important determinant of endurance performance.[27,28] Noteworthy, data suggest that four weeks administration of the erythropoiesis stimulating agent (ESA) recombinant human erythropoietin (rhEPO) is sufficient to increase hemoglobin concentration (HGB), arterial oxygen content, $\dot{V}O_{2peak}$ and time trial performance in healthy non-athletes.[29] Therefore, more studies addressing the effect of testosterone administration on endurance performance are needed.

Based on the existing literature, supraphysiological doses of testosterone seem to induce measurable increases in strength and power, but not in endurance performance, however more empirical evidence on the latter is needed. Further, ergogenic effects have been reported after both long-term (months) and short-term (weeks) testosterone administration.

1.1.2 Acute performance-enhancing effects of testosterone

Noteworthy, limited data are available on whether testosterone administration has acute (seconds to hours) ergogenic effects on human exercise performance. In healthy men, an intramuscular injection of 5000 IU human chorionic gonadotropin (hCG) increased the plasma testosterone levels and reduced the

cortical motor threshold to evoke a muscular response 48 hours post-injection.[30] Therefore, increased plasma levels of testosterone can potentially facilitate the corticospinal pathway and thereby influence muscle activity. Further, stimulation with supraphysiological testosterone levels rapidly and transiently (seconds to minutes) increased the intracellular calcium concentration in cultured rat myotubes.[31] This response was mediated trough the inositol triphosphate pathway, and followed by increased phosphorylation of extracellular signal-regulated kinases (ERK) 1/2. In another study, stimulation with dihydrotestosterone (DHT), the most potent testosterone metabolite, increased the force production by more than 20% in intact isolated mice skeletal muscle fibres, accompanied by increased phosphorylation of mitogen-activated protein kinase/ERK 1/2.[32] Further, in addition to the rapid activation of the Akt, ERK and mTOR pathways, testosterone stimulation of incubated human skeletal muscle cells has been found to increase glucose transporter (GLUT) 4 mRNA expression and GLUT4 protein translocation,[33] with the potential to promote glucose uptake and recovery in skeletal muscle cells. These results suggest that testosterone administration, either directly or via DHT, rapidly can affect skeletal muscle contractions, energy metabolism and recovery.

Testosterone therapy rapidly causes vasodilatation *in vitro*[34] and mediates vasodilatation and augments cardiac output in heart failure patients.[35] Further, high testosterone levels, either naturally or by administration, increases the competitive drive and aggressive behaviour in some individuals.[36–39] For example, Carré and co-workers demonstrated a rapid increase in aggressive behaviour within an hour following a single testosterone gel administration in men with dominant or impulsive personality styles.[38] Based on these studies, acute testosterone administration can potentially increase the cardiorespiratory capacity, as well as provide psychological advantages in sports.

The ability to deduce human effects from rodent studies and *in vitro* studies is unclear. Likewise, testosterone-induced physiological changes may not always result in measurable alterations of human performance. Hence, until further research on humans is conducted, the effects of single-dose administration on human exercise performance remain speculative. Nevertheless, based on the existing literature, it is possible that testosterone administration induces acute performance-enhancing effects, which may provide testosterone dopers with an acute competitive edge if administering testosterone right before or during a competition. Because resting testosterone levels are positively related to vertical jump height[40,41] and sprinting performance,[41] the biological effects of such single-dose administration may depend on the initial serum testosterone level, causing a blunted biological effect in individuals with naturally high testosterone levels. Studies evaluating these questions are of high relevance both for a

physiological understanding of testosterone and for anti-doping authorities aiming to determine the most cost-efficient testing programs.

1.2 Performance-enhancing effects of clenbuterol

Clenbuterol is a sympathomimetic amine classified as a β_2-adrenergic receptor agonist. The drug was originally marketed as a bronchodilator for asthmatic treatment, but is banned by WADA due to the anabolic, anti-catabolic and lipolytic effects observed after long-term clenbuterol administration in several rodent studies.[42–44]

Upon binding to the β_2-receptor, several intracellular pathways are activated, including the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway and the Akt/mTOR pathway.[45] The cAMP/PKA-pathway targets several proteins involved in the regulation of glycolytic activity, Ca^{2+} handling, and Na^+/K^+-ATPase activity in skeletal muscles.[46–48] β_2-adrenergic stimulation can, thus, acutely alter skeletal muscle metabolism, contractions and fatigue prevention. In the case of long-term stimulation, β_2-agonists have been shown to induce skeletal muscle hypertrophy through a combination of accelerated protein synthesis and reduced protein degradation, as extensively described in a recent review by Hostrup and co-workers.[49]

Noteworthy, no studies have examined the performance-enhancing effect of clenbuterol administration in healthy, non-hospitalized humans. Thus, clenbuterol's potential ergogenic effects on human performance are based on studies on animals and humans with muscle-wasting conditions, as well as human studies applying other β_2-agonists, and the assumption that these effects are class-specific and not limited to the applied drug.

1.2.1 Ergogenic effects of long-term clenbuterol administration

In humans, long-term administration of β_2-agonists have been shown to enhance muscle strength and power output during maximal cycling. Hostrup and co-workers observed increased maximal voluntary contraction (MVC) of *m. quadriceps*, as well as increases in both peak power and mean power during a 30-s all-out cycle sprint following high-dose oral β_2-agonist administration (5 mg/30 kg body weight of terbutaline) twice daily for 4 weeks in in 9 trained men.[50] The accompanied increase in lean body mass, but similar fiber type distribution, suggest that skeletal muscle hypertrophy, rather than changes in fiber type distribution, is the primary underlying mechanism for enhanced muscle strength and power observed following long-term β_2-agonist administration.[50] Further, prolonged cycling time to

exhaustion at 80-85% of $\dot{V}O_{2peak}$ was observed after three weeks of 12 mg oral salbutamol (4 mg three times daily) administration in 8 trained men compared with placebo,[51] whilst, on the contrary, two weeks of 8 mg/day oral salbutamol induced no significant effect on cycling time to exhaustion at 110% of $\dot{V}O_{2peak}$ in 10 endurance-trained athletes.[52] These results suggest that long-term administration of β_2-agonists has the potential to enhanced endurance performance at submaximal intensities.

1.2.2 Ergogenic effects of acute clenbuterol administration

β_2-agonists can also induce ergogenic effects if administered acutely, i.e. right before or during physical exercise. These effects appear dose-dependent, as studies applying lower doses (e.g. 8 mg terbutaline) have reported no effect on MVC[53,54] nor running sprint performance[55] in moderately trained to well-trained men, while the administration of higher dose (15-20 mg terbutaline) or combined inhalation of several β_2-agonists have shown to be sufficient to enhance quadriceps isometric muscle strength and maximal arm ergometer and bike ergometer sprint performance in recreationally active men and endurance athletes.[47,56]

When excluding the four subjects experiencing side effects, van Baak and co-workers observed an acute increase in cycling time to exhaustion at 70% of maximal Watts post-administration of 4 mg oral salbutamol in 16 recreationally active men.[57] Likewise, cycling time to exhaustion at 80-85% of $\dot{V}O_{2peak}$ was prolonged post-administration of 6 mg oral salbutamol in 9 moderately trained men.[58] On the other hand, 6 mg oral salbutamol induced no significant differences in cycling time to exhaustion at 90% of $\dot{V}O_{2peak}$ in 8 moderately trained men.[59] Thus, as with long-term β_2-agonist administration, the potential performance-enhancing effect of acute β_2-agonist administration on endurance seems dependent on the exercise undertaken and whether the individual experiences side effects.

1.2.3 Summary

Based on the existing literature, both long-term and acute administration of β_2-agonists can, depending on dosage and exercise modality, enhance performance in muscle strength,[50,57,60] maximal sprinting[50,60,61] and endurance[51,57,58] exercises in healthy non-asthmatic, moderately trained to well-trained individuals. Because β_2-agonists act via the β_2-adrenoceptors, it seems likely that clenbuterol-stimulation can induce similar effects. However, studies evaluating the ergogenic effects of clenbuterol on exercise performance in healthy humans are needed.

1.3 Actual or potential health risk

In the long term, testosterone usage for nontherapeutic reasons have been reported to have adverse health consequences such as cardiomyopathy, dyslipidemia and hypogonadism,[62] while clenbuterol, depending on dosage, can induce adverse effects such as headache, muscle tremor, palpitation, hypokalemia, hyperglycemia, rhabdomyolysis and myocardial ischemia.[63] Most of the evidence about the adverse side-effects are based on case reports and cross-sectional studies, from which causality cannot be directly determined. However, based on the potential health risk, detection of testosterone, testosterone analogs and clenbuterol appears important not only to protect clean athletes, but also the public health. The following sections discusses the existing state-of-the-art methods to detect testosterone and its synthetic analogues, as well as clenbuterol.

1.4 Indirect detection of doping with testosterone and its synthetic analogues

1.4.1 Steroid profile

Molecular similarities between the endogenous hormone and the exogenously administered substance makes the direct detection of testosterone challenging. Therefore, state-of-the-art detection of AAS administration is based on evaluation of so-called 'urinary steroid profiles', by mass spectrometry coupled to gas chromatography (GC-MS/MS).[64] With the steroid profile, biomarkers for doping with testosterone and prohormones are monitored. This includes the concentrations and ratios of glucuroconjugated and free urinary compounds related to testosterone (T) and its metabolism (Figure 1), of which the primary marker is the T to epitestosterone (E) ratio. Additionally, four secondary urine parameters are included: 5α-androstane-3α, 17β-diol (5α-diol)/5β-androstane-3α, 17β-diol (5β-diol), 5α-diol/E, androsterone (A)/etiocholanolone (Etio) and A/T. Table 1 illustrates the typical behavior of the biomarkers following testosterone administration.

Table 1. The influence of testosterone administration and common confounding factors on urine biomarkers.

	T	E	5α-diol	5β-diol	A	Etio	T/E	A/T	5α-diol/5β-diol	A/Etio	5α-diol/E
Oral/injection of testosterone[65,66]	↑	↓	↑	↑	↑	↑	↑	↓		↑	↑
Testosterone gel[67–69]	↑	↓	↑		↑	↑	↑	↓	↑	↑	↑
Hormonal contraceptives[70]		↓					↑				↑
Pregnancy[71]		↑	↑	↓	↑	↓	↓		↑	↑	↓
Ethanol consumption[72–74]	↑						↑	↓			
Homozygote deletion of UGT2B17[65,75,76]	↓		↓	↓			↓	↑	↑		

As illustrated in Table 1, elevated urinary concentrations of T, and, thus, a rise in the ratio of T to E is the main indicator of testosterone administration.[65–68] Moreover, decreased excretion of E may as well occur,[65] as a result of testosterone-induced suppression of gonadotropin-releasing hormone and/or luteinizing hormone (LH) through the hypothalamic-pituitary-adrenal axis, and consequently reduced endogenous production of T and E.[22] Interestingly, studies on the administration of topical or intranasal testosterone gel have demonstrated augmented 5α-diol/5β-diol, 5α-diol/E and A/Etio, probably due to high levels of 5α-reductase in the skin.[67–69]

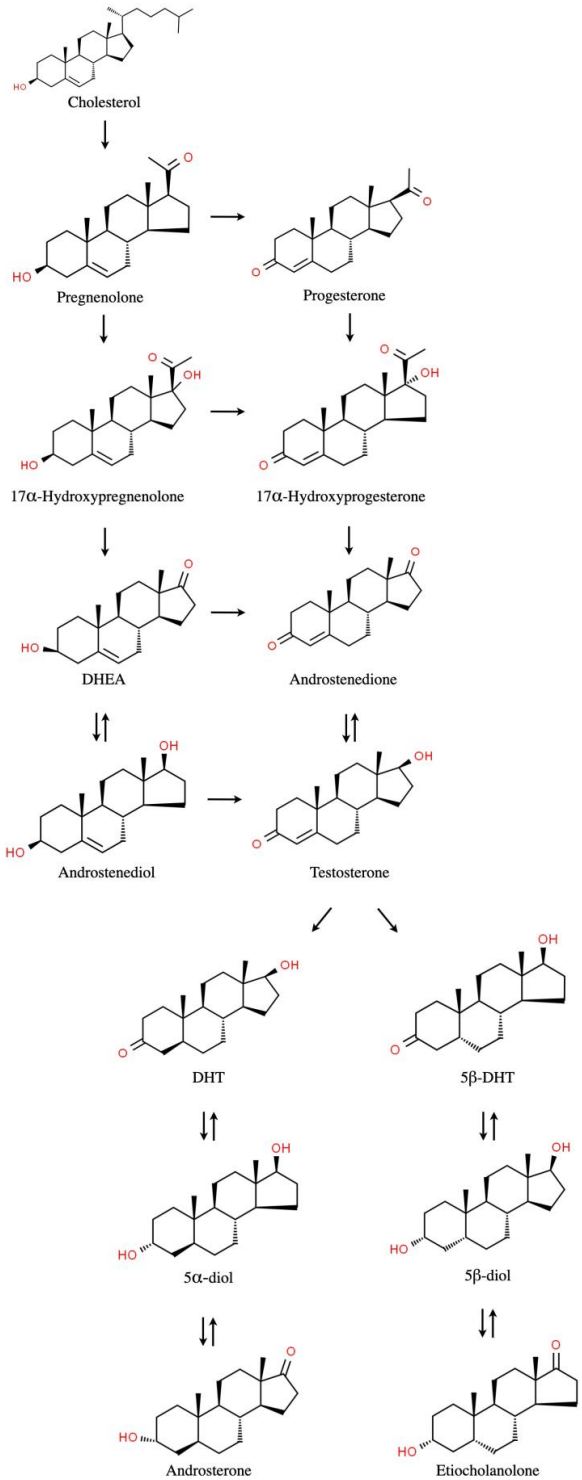

Figure 1. Pathways of synthesis and metabolism of testosterone. The figure has been modified from Mareck et al., 2008.[77]

1.4.2 Evaluation of the steroid profile

The T/E ratio was introduced already in 1983 to indirectly detect doping with AAS, with a population-based upper limit set at 6:1.Today, the T/E ratio is still the most sensitive marker for testosterone administration, but the threshold has been reduced to 4:1.[64] The use of population-based reference ranges for the sample's ratios and concentrations, i.e. the sample's urinary steroid profile, is still the screening method used for individuals tested only once; the first ABP sample in an athlete's steroidal passport.[64] However, some markers show high inter-individual variability, and, thus, the screening method has been found to lack specificity (*specificity* = the proportion of clean samples not resulting in a positive finding) and sensitivity (*sensitivity* = the proportion of doped samples resulting in a positive finding) in some cases.[78] For example, people with homozygous deletions of the UGT2B17 gene, the major enzyme for testosterone glucuronidation, show reduced urinary excretion of testosterone glucuronide (Table 1).[65,75,76] Thus, blunted increases in the T/E urinary ratios can occur when testosterone is administered,[65,75] which increases the risk of false-negative results. Moreover, data indicate that some individuals have naturally elevated T/E ratios, resulting in resources wasted on subsequent negative confirmatory analyses.[78]

Based on this large inter-individual variability, an individual-based approach was proposed,[78] and the 'Athlete Biological Passport' (ABP) steroidal module was introduced in 2014 to longitudinally monitor the urinary biomarkers T/E, A/T, A/Etio, 5α-diol/5β-diol, and 5α-diol/E within an athlete.[79] Initially, before a passport is established, the biomarkers are compared with population-derived thresholds. As samples from an individual are collected and incorporated in the 'adaptive model', the ABP uses Bayesian inference to calculate more narrow individual reference ranges based on the individual's own previous test results.[80] A value, or a sequence of values, outside the individual thresholds is termed an 'atypical passport finding' (ATPF).[81] In case of an abnormally high T/E ratio, the adaptive model automatically requests a confirmatory IRMS analysis. While in case of an abnormality in any of the secondary markers, the Athlete Passport Management Unit evaluates the likelihood of doping, and advices the testing authority on follow-up testing strategies and/or confirmatory analysis.[79] Subsequently, a confirmatory gas chromatography-combustion-isotope-ratio mass spectrometry (IRMS) analysis is needed to unequivocally establish the origin (exogenous or endogenous) of a steroid and its metabolites, as described in WADA's technical document for IRMS analysis in force at the time of analysis, e.g. TDIRMS2019.

1.4.3 Future possibilities

The implementation of the steroidal module of the ABP has improved the sensitivity and specificity for screening for testosterone doping. The superiority of using individualized thresholds was illustrated in a study analyzing 432 urine samples from 28 control subjects and 88 samples following the administration of 80 mg testosterone undecanoate. The use of a population-based limit of 4.0 for T/E resulted in 24 incorrect identifications of suspicious samples (i.e. 94% specificity) and 34 correct identifications of suspicious samples (i.e. 39% sensitivity). While the use of ABP, on the other hand, lead to two incorrect identifications of suspicious samples (i.e. 99% specificity) and 51 correct identifications of suspicious samples (i.e. 58% sensitivity).[78] Moreover, the adaptive model in the ABP steroidal module has shown promising results for doses as low as 125 mg intramuscular testosterone enanthate[65] and 100 mg testosterone gel[67] in healthy men. However, the passport evaluation is still a difficult task due to confounding factors such as pregnancy,[71] the use of hormonal contraceptives[70] and alcohol consumption,[72–74] (Table 1) along with other pharmaceutical, technical/analytical and biological factors that have been extensively reviewed previously.[77,82] For example, both small and large doses of ethanol have been found to increase the T/E ratio and reduce the A/T ratio in healthy volunteers.[72–74] Moreover, the reliance on collection of 'clean' baseline samples, from which the intra-individual thresholds are calculated, further complicates the passport evaluation. Therefore, more research should be conducted to improve the performance of the ABP.

Because testosterone stimulates erythropoiesis and alters iron homeostasis,[9,10,23] one approach might be to look at the already existing hematological module of the ABP, which collects information on variables that can indicate blood doping by e.g. ESAs or blood transfusions. The two primary physiological markers are the HGB and reticulocyte percentage (RET%), which also are combined in the OFF-Score (OFF-Score = HGB (g/L) – 60*(√[RET%])).[83] The module also consists of a multiparametric marker named the 'abnormal blood profile score' (ABPS), which is calculated from seven hematological parameters: hematocrit (HCT), HGB, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), RBC count (RET#) and RET%.[80,84] Abnormal fluctuations in either of the primary markers HGB or OFF-Score can trigger an ATPF, while the RET% and the ABPS, among others, are regarded as secondary markers.[79] Interestingly, studies indicate that RET% and ABPS have great potential in identifying doping with ESAs, and in some cases even superior sensitivity compared with the primary markers HGB and OFF-Score.[84,85]

Recent studies have established that HGB, RET% and RET#, markers which are incorporated in the ABP hematological module, can increase already within one to four days following 100 mg testosterone gel[67] or an intramuscular injection of 500 mg testosterone enanthate.[26] Hence, it is possible that combining information from the blood passport with that in the steroid passport can help evaluating the likelihood of doping in steroidal passport cases, for example in the presence of confounding factors that do not affect the hematological module. However, the mentioned studies[26,67] did not apply the adaptive model in the ABP hematological module, repeated administration nor a placebo-controlled design to evaluate the biomarkers' sensitivity over a period of time. Such studies are needed to evaluate the potential of the hematological profile as a screening tool to indicate testosterone doping and for the selection of suspicious samples for follow-up testing.

1.5 Direct detection of doping with testosterone and its synthetic analogues

Following a suspicious single urine sample or an ATPF in the steroidal module, a urine analysis by IRMS is needed to directly confirm the origin of the steroids. The principle behind the method is that the carbon isotopic ratio ($^{13}C/^{12}C$) of the endogenously produced steroids, which is determined by the $^{13}C/^{12}C$ ratio of the food in our diet (e.g. grains, corns), differ from that of exogenous anabolic steroids (Figure 2). Thus, in IRMS analysis, the $^{13}C/^{12}C$ of the steroids in the urine sample, expressed in units per thousand (‰) relative to the international standard Vienna Pee Dee Belemnite ($\delta^{13}C_{VPDB}$), are compared to the $\delta^{13}C_{VPDB}$ of endogenous reference compounds.[81] However, studies have reported that testosterone preparations with isotopic compositions close to or within the range for endogenous urinary steroids exist (Figure 2).[86,87] The use of these products will not be confirmed according to the sets of criteria described in the WADA technical document for IRMS analysis. Furthermore, the use of IRMS analysis could confirm doping in only two out of five subjects between 12 and 24 hours after a single dose of 100 mg testosterone gel.[67] Therefore, further improvement of the IRMS sensitivity, as well as the development of supplementary confirmation methodologies to detect testosterone doping, are warranted. Because IRMS is a very laborious and expensive technique, especially the latter is of high relevance.

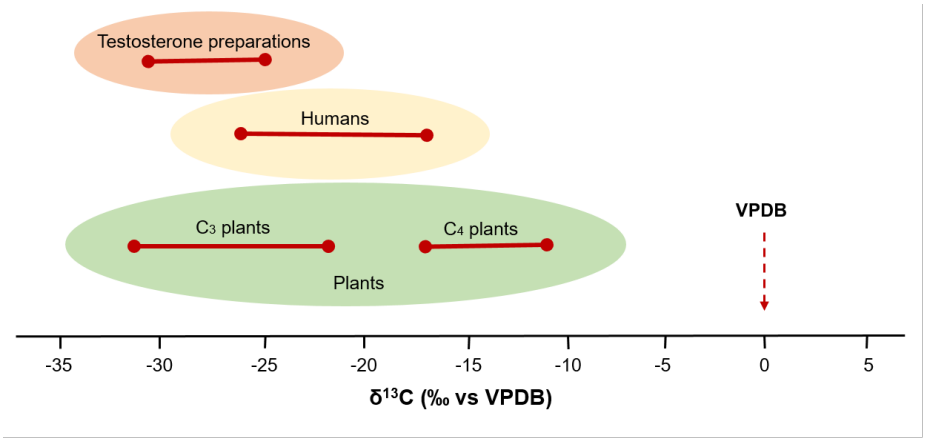

Figure 2. Carbon isotopic composition $\delta^{13}C$ in testosterone preparations,[86,87] humans[86,88] and plants.[89] The figure has been modified from Brand, 1996.[89] VPDB = the international standard Vienna Pee Dee Belemnite. C_3 plants = vegetables, fruits, grains, C_4 plants = corn, sugarcane.

Due to the rapid hepatic metabolism of pure testosterone, many testosterone products are provided as testosterone esters, with esterification of the 17-hydroxygroup, which makes the molecule more hydrophobic in proportion to the length of the side chain. This modification slows down the release of testosterone ester from the injection site into the circulation, and, thus, prolongs the duration of action.[90] Despite rapid hydrolysis into active testosterone in the bloodstream, intact testosterone esters can still be present in blood.[91–93] Based on characteristic retention times and diagnostic mass-to-charge ratios for the precursor ions and characteristic product ions, Forsdahl and coworkers were able to separate steroid esters from endogenously produced testosterone by the use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).[91] The assay was proofed applicable for confirmatory doping analysis by means of an administration study where three volunteers received an intramuscular injection of Sustanon® 250, a blend of four testosterone esters. It was reported that intact esters could be detected up to 18 days post-injection, depending on the length of the ester sidechain.[92] Therefore, when both blood and urine samples have been collected, confirmatory analysis for steroid ester abuse can be considered done in serum or plasma.[64]

1.6 Detection of doping with clenbuterol

Detection of clenbuterol (4-amino-3,5-dichloro-*a-tert*-butylaminomethylbenzyl alcohol hydrochloride) in urine is a part of routine analysis at the WADA-accredited laboratories. The excreted prodrug is detectable in urine, with reliable identification by LC-MS/MS in the low pg/mL range.[94] In contrast to inhaled β_2-agonists such as salbutamol, formoterol and salmeterol, clenbuterol is a non-threshold

substance. Nevertheless, accurate estimation of urinary concentrations is necessary since clenbuterol is used as a growth promoter in livestock industry in countries such as Mexico, China and Guatemala. A positive doping test for clenbuterol at low levels can thus be a result of unintended clenbuterol intake through contaminated meat[94,95] rather than the tail end of the excretion following intended clenbuterol administration. Hence, as of June 2019, findings of < 5 ng/mL clenbuterol in urine should be reported as an 'atypical finding' (ATF), rather than an 'adverse analytical finding' (AAF), and can be investigated as a potential meat contamination case.[96]

Following single oral administration, clenbuterol is bioavailable after 30 minutes, and achieves a peak plasma concentration within 2-3 hours, with a relatively long elimination half-life of 20-35 hours.[97,98] The long half-life is likely to allow for a long window of detection, however, limited data are available on the pharmacokinetics of clenbuterol in human urine. Yamamoto and co-workers[97] studied the cumulative urinary excretion of clenbuterol after the oral intake of either 20 μg (n = 3), 40 μg (n = 3) or 80 μg (n = 3) clenbuterol, which are in the low to medium range of the doping practice reported in questionnaires (20 to 200 μg per administration)[4] and discussed in doping forums on the internet (40 to 120 μg per administration).[49] They found that 10-15% of clenbuterol was excreted within 24 hours, while the cumulative urinary excretion rate was around 20% in all three groups at 72 hours post-administration. Given a common daily urine volume of two liters, the intake of 80 μg clenbuterol could theoretically result in a urinary concentration of 8 ng/mL after three days, which is likely to be detected by today's routine doping analysis. Moreover, Nicoli and co-workers found clenbuterol at low pg/mL levels in urine from healthy volunteers up to six days after a single oral intake of 10 μg clenbuterol.[99] This gives an indication of detection windows for clenbuterol, however, the applied dose was similar to that which can be caused by the intake of contaminated food. Therefore, more proof-of-concept studies applying doses similar to the suspected practice by clenbuterol dopers are needed to establish evidence-based windows of detection for clenbuterol misuse.

1.7 Biological matrices in doping analysis

Today, anti-doping testing is based on the collection and analysis of two WADA-approved matrices: urine and venous blood, including whole blood and serum. The advantages and limitations of conventional test matrices and possible complimentary matrices have been thoroughly discussed in a review by Thevis and co-workers.[6] As pointed out in the review, existing state-of-the-art methods for doping analyses can efficiently detect a wide variety of doping substances: Urine represents the default specimen with easy and noninvasive collection, and allows for long-term identification of drug

metabolites and prodrugs of mainly lower molecular mass. Blood complements with the evaluation of hematological parameters, identification of higher molecular mass analytes not excreted in urine,[6] as well as the quantification of the biological activity of drugs. However, the collection and transportation of urine and cooled blood samples according to the WADA standards are logistical challenging, expensive and time consuming. Further, the low to moderate analyte stability at room temperature in the current matrices is an issue of concern.[6] Moreover, in a study by Elbe and Overbye, over one third of the 400 elite athletes completing the web-based survey had experienced stress and difficulties urinating during doping control,[100] which illustrates that the urine sample collection in front of a Doping Control Officer can be intimidating. Hence, development and validation of new sample collection, shipment and analytical methods are warranted. Alternative, complementary matrices including saliva, exhaled breath, hair and dried matrices such as dried blood spots (DBS) or dried plasma spots (DPS) have been subject of several studies related to sports drug testing.[6] The next section will focus on the use of dried matrices in doping analysis.

1.7.1 Microvolume dried matrices

The above-mentioned challenges can be addressed by the implementation of microvolume sampling of whole blood (DBS) or plasma (DPS) on filter paper. In current DBS applications, 20-50 µL of capillary blood is collected per spot on a filter paper. DBS has attracted massive research attention due to the minimally invasiveness and the wide application possibilities, including newborn screening,[101] screening of substance abuse[102] and proteome wide association studies,[103] but further development and validation is required for systematic application in specialized fields such as anti-doping. Of special interest for anti-doping efforts are the reduced costs for collection, transportation and storage, including no need for trained phlebotomists, the low invasiveness and discomfort, the possibility for automated system for on-line liquid chromatography-high resolution mass spectrometry analysis,[104,105] as well as a high analyte stability for some compounds compared with liquid matrices.[106–109] For example, the synthetic peptide hormone Synacthen® has been found to be stable in DBS at room temperature for at least ten days,[106] due to the drying process and the corresponding reduction of enzymatic activity. Whilst the analyte is rapidly degraded within hours in urine[110] and plasma/serum.[111] This potential benefit of DBS could simplify sample transportation and allow sample collection in geographically remote areas.

A major limitation of DBS is the limited sample volume available, which influences the assay sensitivity. Another challenge relies on the composition of the analyzed blood, especially when quantification analyses are performed. In this regard the HCT of the blood is of importance, as it

relates to the blood viscosity and consequently the spreading of the sample on the filter card. Data indicate that a partial punch-out from a DBS sample with low HCT contains a lower sample volume than that from a DBS with high HCT, leading to bias in the quantitative analysis of DBS.[112,113] However, this is likely circumvented by analyzing whole, volumetrically applied DBS or by the use of DPS sampling material. In DPS collection, RBCs are trapped on the upper membrane and only plasma is transferred to the filter paper,[114] resulting in a preparation suitable for analyses of other compounds than those possible with a DBS preparation. For example, many drugs do not enter the RBC, and in these situations the RBCs act as diluent.[115] Therefore, the development of DPS cards is expected to have even greater potential than DBS.

While research in the DPS methodology for anti-doping purposes is in its infancy and only covers a limited number of doping substances such as opioids and stimulants,[116,117] the portfolio of promising DBS applications in sports drug testing is growing and includes both WADA prohibited substances and methods (Table 2). However, in the endeavor to have DBS and/or DPS accredited and implemented in doping control and analysis, further development of reliable DBS and DPS methodologies are needed. Moreover, it is required to evaluate the analyte stability on DBS cards from sample collection to analysis, so that the transportation and storage procedures to be used in doping control and analysis can be optimized. Additionally, as illustrated in Table 2, many of the proposed assays lack proof-of-principle data via clinical trials and/or real-life testing to demonstrate the assays' applicability for routine sports drug testing. Indeed, some studies have provided data on drug monitoring in DBS samples from patients, drug abusers or animals for clinical and forensic purposes or due to ethical issues related to human administration studies (Table 2). However, it is unclear whether these samples are representative for a healthy athlete population. Therefore, administration studies with healthy volunteers, reproducing scenarios of cheating athletes, or real-life testing of athletes, are required before the assays can be implemented. Hence, the next step in the development of the DBS and DPS methodologies for anti-doping purposes is to compare the specificity, sensitivity and applicability of DBS and DPS cards for analysis of frequently occurring doping substances, to find the best performing method to be used in routine sport drug testing.

Table 2. Promising dried blood spot assays for WADA prohibited substances and methods			
Reference	**Substance/Method**	**Class**	**Study design**
Antelo-Dominguez et al., 2013[118]	Cocaine	S6 Stimulants	Real-life testing of polydrug abusers (*n* = 13)
	Morphine	S7 Narcotics	
	Codeine	Narcotics included in WADA's Monitoring Program	
Antunes et al., 2015[119]	Tamoxiphen	S4 Hormone and metabolic modulators	Real-life testing of patients (*n* = 91)
Cox et al., 2013; 2014[120,121]	Insulin-like growthfactor-1	S2 Peptide hormones, growth factors, related substances and mimetics	Real-life testing for endogenous IGF-1 (*n* = 8 healthy volunteers)
Cox et al., 2017[122]	Blood doping monitoring (*Indirectly via the biomarkers CD71, Band 3, CD41, CD45*)	M1 Manipulation of blood and blood components S2 Peptide hormones, growth factors, related substances and mimetics	Real-life testing of clean, non-athletes and athletes
Cox et al., 2017[123]	Autologous blood transfusion (*Indirectly via the biomarkers CD71 and Band 3*)	M1 Manipulation of blood and blood components	Placebo-controlled administration study (*n* = 15 blood, *n* = 11 placebo)
Ferro at al., 2017[124]	rhGH (*Indirectly via the biomarker fibronectin-1*)	S2 Peptide hormones, growth factors, related substances and mimetics	Controlled administration study (*n* = 10 rhGH, n = 4 control)
Höppner et al., 2014[125]	Sirtuin-1 activating compounds	S0 Non-approved substances	Administration study with rats (*n* = 9)
Lange et al., 2019[126]	Sotatercept	S2 Peptide hormones, growth factors, related substances and mimetics	Spiked samples Placebo-controlled administration study (Bimagrumab, *n* = n/a)
	Luspatercept		
	Bimagrumab	S4 Hormone and metabolic modulators	
Lawson, Cocks and Tanna, 2012[127]	Atenolol	P1 Beta-blockers	Administration study (*n* = 2 healthy volunteers)
Kojima et al., 2016[128]	Ephedrine	S6 Stimulants	Administration study (*n* = 5 healthy volunteers)
	Methylephedrine		
Mommers et al,. 2013[129]	Morphine	S7 Narcotics	Spiked samples
Möller et al., 2012[130]	Peginesatide	S2 Peptide hormones, growth factors, related substances and mimetics	Spiked samples
Peng et al., 2000[131]	Testoterone undecanoate (*Indirectly via. blood steroid profile*)	S1 Anabolic agents	Administration study (*n* = 6 healty volunteers)
	Testoterone propionate (*Indirectly via. blood steroid profile*)		
	Testosterone enanthate (*Indirectly via. blood steroid profile*)		
Protti et al., 2018[132]	Oxycodone	S7 Narcotics	Real-life testing of patients (*n* = 18)
Reverter-Branchat et al., 2016[133]	rhGH	S2 Peptide hormones, growth factors, related substances and mimetics	Placebo-controlled pilot administration study (*n* = 2 rhGH, *n* = 2 control) Administration study (*n* = 8 healhty volunteers)

Reverter-Branchat et al., 2018[134]	rhEPO	S2 Peptide hormones, growth factors, related substances and mimetics	Administration study with rhEPO (*n* = 2 healthy volunteers)
	NESP		Real-life testing of patients for NESP (*n* = 12)
	CERA		Spiked samples
Rosting et al., 2015[135]	hCG	S2 Peptide hormones, growth factors, related substances and mimetics	Spiked samples
Rosting et al., 2016[107]	Human insulin	S4 Hormone and metabolic modulators	Spiked samples
Salamin et al., 2019[136]	Blood doping (*Indirectly via the biomarker ALAS2*)	M1 Manipulation of blood and blood components S2 Peptide hormones, growth factors, related substances and mimetics	Administration study with rhEPO (*n* = 2 healthy volunteers) Placebo-controlled administration study with blood withdrawal (*n* = 16 healthy volunteers)
Saussereau et al., 2012[137]	Morphine	S7 Narcotics	Spiked samples Real-life testing of drug abusers (*n* = 20)
	Codeine	Narcotics included in WADA's Monitoring Program	
	Cocaine	S6 Stimulants	
	Amphetamine		
	Methamphetamine		
	MDA		
	MDMA		
	MDEA		
Thomas et al., 2011[138]	Salbutamol	S3 Beta2-agonists	Spiked samples Administration study (Pseudoephedrine, *n* = 1 healthy volunteer)
	Cocaine	S6 Stimulants	
	Pseudoephedrine		
	Amphetamine		
	JWH-018	S8 Cannabinoids	
Thomas et al., 2012[139]	Metoprolol	P1 Beta-blockers	Spiked samples
	Bisoprolol		
	Propronalol		
	Clenbuterol	S1 Anabolic agents	
	Metandienone		
	Andarine		
	Stanozolol		
	SARM S1		
	Salbutamol	S3 Beta2-agonists	
	Formoterol		
	Anastrozole	S4 Hormone and metabolic modulators	
	Clomiphene		
	Exemestane		
	Hydrochlorothiazid	S5 Diuretics and masking agents	
	MDA	S6 Stimulants	

	MDMA		
	Strychnine		
	Methylhexaneamine		
	Methylphenidate		
	Cocaine		
	Nikethamide		
	Mesocarb		
	THC	S8 Cannabinoids	
	Dexamethasone	S9 Glucocorticoids	
	Budesonide		
Thomas et al., 2018[140]	Human insulin	S4 Hormone and metabolic modulators	Spiked samples Administration study (insulin aspart, *n* = 1 diabetic patient)
	Insulin lispro		
	Insulin aspart		
	Insulin glulisine		
	Insulin glargine		
	Insulin detemir		
	Insulin tresiba		
	Bovine insulin		
	Porcine insulin		
Tretzel et al., 2014[108]	Testosterone undecanoate	S1 Anabolic agents	Spiked samples Administration study (Testosterone undecanoate, *n* = 1 healthy volunteer)
	Testosterone decanoate		
	Testosterone phenylpropionate		
	Testosterone acetate		
	Testosterone cypionate		
	Testosterone isocaprioate		
	Nandrolone phenylpropionate		
	Trenbolone enanthate		
	Nandrolone		
Tretzel et al., 2015[106]	Synacthen	S2 Peptide hormones, growth factors, related substances and mimetics	Administration study (*n* = 1 healthy volunteer)
Tretzel et al., 2015[141]	Stanozolol	S1 Anabolic agents	Administration study (*n* = 2 healthy volunteers)
	Dehydrochlormethyl-testosterone		
	Pseudoephedrine	S6 Stimulants	Administration study (*n* = 4 healthy volunteers)
Tretzel et al., 2016[105]	Nicotine	Stimulant included in WADA's Monitoring Program	Real-life testing (*n* = 23 smokers/snus users, *n* = 10 control)
Tretzel et al. 2016[142]	Meldonium	S4 Hormone and metabolic modulators	Administration study (*n* = 2 healthy volunteers)
Verplaetse and Henion, 2016[143]	Morphine	S7 Narcotics	Spiked samples Adminsitration study (Hydrocodone, *n* = 1 healthy volunteer)
	Fentanyl		
	Oxycodone		
	Codeine	Narcotics included in WADA's Monitoring Program	
	Hydrocodone		

1.7.2 Detectability of testosterone esters and clenbuterol in DBS/DPS

In the next step, it is of obvious importance to focus on frequently occurring substances such as testosterone esters and clenbuterol. Promising methods to detect clenbuterol and intact anabolic steroid esters from spiked DBS samples have been proposed, with limit of detection (LOD) of 0.05 ng/mL for clenbuterol[139] and 0.1 ng/mL for the testosterone esters testosterone phenylpropionate (TPP), testosterone isocaproate (TI) and testosterone decanoate (TD).[108] However, both methods showed incomplete extraction of the analytes. For clenbuterol the extraction recovery was 70%, while for the testosterone esters TPP, TI and TD only 12.6% to 15.8% were extracted, partly due to the adsorption effect of the esters to the sample material.[108] Increased sensitivity through improved sample preparation and analytical procedures is desirable for prolonged windows of detection, as well as for the possibility to detect low administration doses. This might be achieved using alternative extraction conditions and derivatization agents (i.e. signal enhancement), as well as sensitive instrumentation such as nano-scale LC-MS/MS (NanoLC-MS/MS). Moreover, incomplete elution of the analytes from the sampling card might be circumvented by alternative collection materials. One promising approach is to use water-soluble materials such as carboxymethyl cellulose (CMC), which can be almost completely dissolved in a an aqueous buffer solution, making the entire sample available for further processing.[107,135,144]

Following method development and validation, only the mentioned testosterone ester assay's sensitivity was evaluated in a proof-of-concept study for doping control purposes. Hence, a DBS study on human administration of clenbuterol has yet to be conducted. The testosterone ester assay was proofed reliable for detection of orally administered testosterone undecanoate (80 mg Andriol® Testocaps) in a healthy subject up to eight hours post-administration,[108] despite limited analyte recovery and the low bioavailability of oral testosterone undecanoate.[145] As a proof-of-concept, a sample size of one ($n = 1$) is often used in the field of anti-doping to demonstrate that a method has applicable sensitivity. Notably, due to the large consequences of sanctioning an athlete based on a false adverse analytical finding, high specificity is of great importance. This is particularly important for direct detection methods such as direct detection of testosterone esters and clenbuterol in DBS/DPS, Therefore, and due to the between-subject variability in adsorption/elimination rates,[92] it is required to collect samples from more than one healthy subject undergoing administration resembling the suspected practice by cheating athletes. Clean baseline samples in a clinical trial can be used to evaluate the specificity, however, randomized placebo-controlled studies, with samples from a placebo group undergoing the same intervention as the drug administration group, is desirable to provide extensive information of the assay's specificity for a period of time. These points should be

taken into consideration in the further development and validation of the DBS and DPS methodologies for detection of frequently occurring doping substances and methods.

Before the DBS and DPS methodologies can be implemented in a doping control setting, the transport and storage procedures must be optimized and standardized among doping control agencies and laboratories. In this regard, it is required to evaluate the stability of analytes on DBS cards under varying storage conditions. A 28-76% degradation was observed for anabolic steroid esters on DBS after 4 weeks storage at room temperature,[108] while clenbuterol was found to be stable on DBS card for at least one week when stored at cooled conditions (2-8 °C). Since the long-term stability of clenbuterol and testosterone esters on DBS cards at cooled or frozen conditions has yet to be reported, studies assessing DBS analyte stability at varying storage temperatures are warranted.

1.8 Applicability to anti-doping: objectives

Important elements of an efficient anti-doping system are targeted testing and frequent and unpredictable sample collection to maximize detection and deterrence. However, as highlighted in the previous sections, the system is limited by challenges including time-consuming and expensive collection and shipment, limited analyte stability, labour intensive analysis and, in some cases, insufficient sensitivity and specificity of the existing state-of-the-art analytical methods to detect some doping substances. Thus, continued research to improve the time- and cost-efficiency and increase the likelihood of catching cheating athletes and recreationally active, is warranted.

The minimally invasive, easy and cost-reduced DBS and DPS techniques have the potential to improve the time-and-cost efficiency compared to traditional collection, shipment and analysis. As complementary matrices, DBS and DPS can increase deterrence and the capacity to better reveal doping practices by allowing more frequent out-of-competition testing and large-scale testing, also in geographically remote areas. Therefore, the present PhD project aimed to compare the specificity, sensitivity and applicability of DBS and DPS materials for analysis of frequently occurring doping substances, to find the best performing method for routine doping analysis. Additionally, the project aimed to evaluate the long-term stability of four different testosterone esters on DBS cards after storage at cooled and frozen conditions, to achieve a robust matrix suitable for a doping control setting. Moreover, we aimed to evaluate whether the administration of the four testosterone esters TD, TI, TPP and testosterone propionate (TP) (Sustanon® 250) or clenbuterol to healthy, male volunteers could be detected in DBS/DPS samples.

Knowledge about the pharmacokinetics and pharmacodynamics of a doping substance is important for targeted testing. Therefore, the present project aimed to evaluate detection windows for testosterone esters and clenbuterol in DBS and/or urine samples collected from humans subjected to administration resembling the suspected practice by cheating athletes. Moreover, we aimed to examine the acute ergogenic effects of a mixed testosterone ester injection (250 mg Sustanon®) on human physical performance, and whether the basal serum testosterone concentration influences the performance in strength and power exercises, to help anti-doping authorities determine the most cost-efficient testing programs.

To further develop already existing detection methods is a cost-efficient way to improve the likelihood of revealing doping practices. Based on the erythropoietic-stimulating effect of testosterone, the project aimed to evaluate the potential of the adaptive model in the ABP hematological module to infer testosterone doping and as a screening tool to identify suspicious samples for subsequent IRMS analysis.

The following hypotheses were investigated:

I. Single and repeated intramuscular injections of 250 mg testosterone esters (Sustanon® 250) affect biomarkers in the hematological module of the Athlete Biological Passport.

II. A mix of testosterone esters administered to healthy participants is detectable by application of a DBS-based analytical method with sufficient specificity and sensitivity to complement existing analytical strategies while increasing time-cost efficiency. Further, long-term stability of four different testosterone esters on DBS cards can be achieved by storage at frozen conditions.

III. One intramuscular injection of 250 mixed testosterone esters enhances physical performance in strength and power exercises acutely 24 h after injection. Additionally, the basal serum testosterone concentration influences the performance in countermovement jump, 30-s all out cycle sprint and one-arm isometric elbow flexion.

IV. Clenbuterol can be detected in DBS and urine samples following single-dose ingestion of 80 μg oral clenbuterol.

The present PhD project was a part of the Innovation Fund Denmark's Industrial Researcher Program. The Industrial PhD project was a collaboration between Anti Doping Denmark and the University of Copenhagen, with the WADA-accredited Norwegian Doping Control Laboratory as a

third party. The present Industrial PhD thesis is based on two of the Industrial PhD's studies, from which four papers have been formed –three submitted and one draft. The papers are referred to as *Paper I, II, III* and *IV*, corresponding to those listed in 'List of papers'. Data collection took place in Copenhagen, Denmark, under the guidance of Associate Professor Nikolai Baastrup Nordsborg, at the University Copenhagen, and Dr. Jakob Mørkeberg, the Senior Science Manager at Anti Doping Denmark. The analytical work for *Paper I-III* was performed at the Norwegian Doping Control Laboratory in Oslo, Norway, by the PhD student and laboratory personnel, under guidance of the Laboratory Director Dr. Yvette Dehnes. The analytical work for *Paper IV* was performed at the WADA-accredited laboratory in Cologne, Germany.

2. Results and discussion

The main results from *Paper I-IV* are described and discussed in the following subchapters.

2.1 Acute performance-enhancing effects of testosterone in humans

As outlined in the introduction, findings indicate that testosterone administration rapidly can affect skeletal muscle contractions,[30–32] energy metabolism and recovery,[33] as well as increase the cardiorespiratory capacity[34,35] and aggressive behavior in some individuals.[38] These physiological and psychological effects could potentially provide testosterone dopers with an advantage if administering testosterone right before or during a competition, but studies on the acute performance-enhancing effect of testosterone administration in humans are lacking. The following sections address potential mechanisms by which acute testosterone administration can enhance muscular strength, sprint performance and vertical jump performance, and relate the current knowledge to the results of *Paper III*.

2.1.1 Acute ergogenic effects of testosterone administration on muscular strength

It is possible that acute testosterone administration can enhance muscular strength. Intracellular calcium concentration has been reported to increase within seconds to minutes in cultured rat myotubes after testosterone stimulation.[31] In skeletal muscles, intracellular calcium regulates contraction via binding to troponin C.[146] Further, stimulation by the testosterone metabolite DHT has been found to rapidly increase force production via phosphorylation of the regulatory myosin light chain kinase in fast-twitch mouse muscle fibres.[32] In skeletal muscles, phosphorylated myosin light chain kinase can increase the sensitivity of the contractile proteins to calcium.[147] Thus, acute testosterone administration can potentially stimulate increased maximal force production (e.g. measured as MVC) in humans by both augmented intracellular calcium release and calcium sensitivity for cross-bridge formation.

Noteworthy, the ability to deduce human effects from rodent studies and *in vitro* studies is unclear. For example, human skeletal muscles appear to have low activity of 5α-reductase, the enzyme that converts testosterone to DHT.[148] The conversion of testosterone to DHT, and thereby the potential DHT-mediated effect of testosterone on human skeletal muscle contractility, might thus be limited. In *Paper III*, we were the first to evaluate the acute effects of testosterone administration on human physical performance. We found that the MVC in a one-arm isometric elbow flexion test remained

unchanged 24 hours after an intramuscular injection of 250 mg mixed testosterone esters (Sustanon®), as well as compared with placebo administration. Thus, it appears that the potential testosterone-induced, acute alterations in intracellular calcium release and calcium sensitivity for cross-bridge formation,[31,32] were not considerably affected by the testosterone administration in *Paper III*. This is in line with a study on mice: based on *in vitro* findings in rodents, the authors hypothesized that acute androgen administration would enhance muscle performance in mice. However, no effects were observed on maximal force production, power or fatigue of the tibialis anterior muscles 30, 60 and 90 minutes following testosterone or DHT administration.[149]

Findings suggest that testosterone administration can act acutely on the central nervous system and increase the central descending motor drive,[30] as well as augment the aggression and competitiveness in humans.[38,39] This could in theory lead to more rapid muscle activation, and increased motivation to perform the task. An elevation in plasma testosterone levels 48 hours after the administration of 5000 IU hCG reduced the cortical motor thresholds to evoke a muscle response in *m. interosseus dorsalis I* in six healthy men.[30] In this respect, it must be noted that no accompanying increase in muscular response was observed, evident by unchanged slope of the input-output relationship between the transcranial magnetic stimulation intensity and the size of the motor-evoked potential.[30] Therefore, it is unknown whether this acute effect of testosterone will transfer into faster muscle activation and thereby contribute to augmented rate of force development (RFD) in voluntary movements. Notably, we observed no acute effect of the administration of 250 mg Sustanon® on RFD in a one-arm isometric elbow flexion test. This suggests that the potential improvements in central descending motor drive,[30] following testosterone administration has no meaningful impact on explosive strength in humans.

2.1.2 Acute ergogenic effects of testosterone administration on sprint performance

Sprint performances such as 30-sec all-out sprinting has been shown to require a high (~80%) anaerobic energy contribution from phosphagens and glycolysis.[150] The accompanying accumulation of metabolites and ions disturbs the cellular homeostasis, which impairs the excitation–contraction coupling of skeletal muscles and can lead to skeletal muscle fatigue. Thus, increased capacity to regulate ion homeostasis and thereby sustain power output and attenuate fatigue, is a potential mechanism to enhance sprint performance.[151] Acute testosterone administration has been found to have a vasodilatory effect *in vitro* and mediate vasodilatation and augment cardiac output in heart failure patients.[34,35,152] This could potentially increase the O_2 delivery to the working muscles as well as the removal of metabolites. However, given the small aerobic energy contribution during maximal sprinting,[150] and that peripheral vasodilation can elevate blood flow to other tissues and organs than

the exercising muscles, it is questionable whether these effects are likely to provide any beneficial effects on sprint performance. In support of this notion, mean power and fatigue index in a 30-s all-out cycle sprint remained unchanged after an intramuscular injection of 250 mg mixed testosterone esters in recreationally active men (*Paper III*).

Further, it is possible that the rapid rise in aggression after testosterone gel administration,[38] combined with the potential effect of testosterone on skeletal muscle contractility,[30,31] acutely can improve peak power output during a 30-s all-out sprint. However, testosterone administration had no effect on peak power in *Paper III*, which is in line with concomitantly unchanged elbow flexion MVC and RFD (*Paper III*). In this respect, it must be noted that testosterone-induced potentiation in aggressive behavior, evaluated in a well-validated decision-making game, was only observed in individuals scoring high on trait dominance or low on trait self-control.[38] Therefore, it appears that a potential psychological effect on sprint performance only would be apparent in individuals with dominant or impulsive personality styles. Collectively, our findings suggest that a single testosterone ester injection has no acute ergogenic effects on human sprint performance.

2.1.3 Acute ergogenic effects of testosterone administration on vertical jump performance

Positive relationships have been found between testosterone levels and vertical jump height [40,41] which may be due to both acute and long-term effects of testosterone. Therefore, one can speculate that increasing serum testosterone levels by testosterone administration acutely will enhance vertical jump performance.

Lower-body rate of force development and maximal force production have previously been reported to be the main contributors to vertical jump performance.[153,154] Thus, testosterone administration could potentially increase vertical jump height acutely by increasing maximal muscle force production, given the rapid increases in myoplasmic calcium concentration,[31] and myofibrillar calcium sensitivity,[32] observed after testosterone or DHT stimulation in rodent studies. Combined with the potential of testosterone to increase central descending motor drive,[30] this could lead to more rapid muscle activation. Thus, acute testosterone administration could theoretically augment RFD. Nonetheless, until now it has been unexplored whether these potential effects result in enhanced vertical jump performance in humans. In *Paper III*, we were the first to evaluate the acute effect of a single testosterone ester injection on the vertical jump height in a countermovement jump test. Interestingly, the vertical jump height remained unchanged in both the testosterone ester group and the placebo group after administration. These results imply that the potential testosterone-induced changes in

muscle contractility[31,32] and muscle activation[30] do not meaningfully impact human vertical jump performance.

2.1.4 Serum testosterone and performance capacities

If the basal serum testosterone concentration influences the performance capacities, it could be speculated that the biological effect of a testosterone ester injection would be blunted in individuals with naturally high serum testosterone levels. In *Paper III*, we combined the pre-administration data for the placebo group and the testosterone ester group and evaluated the relationships between the subjects' serum testosterone levels and performance outcomes in strength and power exercises. No correlations were observed (*Paper III*). This contradicts the observed positive relationships between serum testosterone and vertical jump height[40,41] and sprinting performance[41] in elite athletes of various sports. It must be noted that we included a mixed group of untrained and moderately trained individuals (*Paper III*). Hence, it can be speculated that there is a stronger linkage between testosterone and performance in a homogenous group of elite-trained individuals than in a group of recreationally active. On the one hand, this is supported by the finding of correlations between testosterone levels in saliva and leg strength and sprint performance in individuals with high strength levels but not in less strong individuals.[155] On the other hand, when evaluating the correlations between salivary testosterone concentration and neuromuscular performance in a group of elite rugby players and in a group of recreationally active men, no correlations were observed in either cohort[156] In line with these latter findings, resting plasma testosterone concentrations could not account for the performance differences when dividing the subjects in *Paper III* into two groups based on their MVC measurements at baseline. Notably, differences in lean body mass were sufficient to account for the sex differences in the performance of 693 elite athletes in various sports, and it was postulated that serum testosterone does not determine performance.[157] Collectively, it appears that human performance in strength and power exercises cannot be predicted solely on testosterone levels.

2.1.5 Implications for anti-doping

Information about the pharmacodynamics of a substance is important when anti-doping authorities plan cost-efficient testing programs. It is clear that long-term (months) and short-term (weeks) testosterone administration have ergogenic effects,[13–16] while testosterone dopers, independent on baseline testosterone levels, are likely not to have an advantage if administering a single dose of testosterone esters immediately before or during a competition in strength and power sports (*Paper III*). Collectively, these results suggest that in case of testosterone doping in strength and power sports,

anti-doping authorities should focus on out-of-competition testing. If testing in-competition, it is noteworthy that there seem to be no performance-enhancing effects of Sustanon® at a time point where it is detectable in DBS (*Paper II*), plasma[92] and urine (*Paper I*).

2.1.6 Study limitations

One can argue that the applied dose in *Paper III* was too low to induce marked physiological effects, because findings indicate that testosterone-induced effects on muscle strength, leg power[19,20] and muscle size[20] in healthy, young men are dependent on dose. However, we observed a threefold increase in mean serum testosterone level from pre to post-injection in the testosterone group, while it remained unchanged in the placebo group (*Paper III*). Moreover, maximal circulatory concentrations of the testosterone esters, measured in DBS, occurred during the first one to three days post-injection (*Paper II*). These findings indicate that the performance measurements were carried out in the period where acute effects can be expected and that the applied dose was sufficient to elevate serum testosterone to supraphysiological levels.

Worth mentioning is the ethical concerns when conducting testosterone administration studies. We chose 250 mg Sustanon® because this has previously been safely administered to males in controlled studies.[92,158] This dose is in the lower range of the doping practice reported by athletes and recreationally active in questionnaires and interviews (70 mg to more than 2500 mg per week).[5,159,160] Yet, serum concentrations of LH were reduced post-injection in the testosterone ester group (*Paper III*), which illustrates that the testosterone administration caused a feedback inhibition of endogenous testosterone production. As an important note with regards to the safety of participants, one subject experienced suppressed testosterone levels compared to baseline at the medical follow-up three weeks after the second injection, but the levels had returned to baseline at the additional medical follow-up three months later. No additional severe side-effects were reported (*Paper III*).

Additionally, it can be speculated that a performance-enhancement would have been observed if other exercise modalities were applied. Therefore, further research with the use of other exercise modalitiesare needed.

2.2 Indirect detection of testosterone esters

2.2.1 The steroid profile

As highlighted in the introduction, the implementation of the individual-based steroidal module of the ABP has improved the sensitivity and specificity for screening for testosterone doping. In *Paper I*, a total of 133 clean urine samples, including all placebo samples and the baseline samples for the testosterone ester group, were analyzed. When using population-based cut-off values, the specificity was 82% (i.e. 24 incorrect identifications of suspicious samples), while the adaptive model of the ABP steroidal module, on the other hand, had a specificity of 87% (i.e. 17 incorrect identifications of suspicious samples). On the day following the first and second testosterone ester injection, the T/E ratio was suspicious in 5/9 and 6/9 subjects, respectively, when using population-based reference ranges. When using the adaptive model, the T/E ratio identified all subjects in the Sustanon® group on the first day after both injections. This confirms the superiority of using individualized thresholds.

Similar to the application of transdermal[67,69] and intranasal[68] testosterone gel in healthy young volunteers, T/E and 5α-diol/E were the most sensitive steroidal markers for intramuscular Sustanon® injections (*Paper I*). These markers remained increased compared with both baseline levels and levels in the placebo group for ten and fourteen days after the first and second testosterone ester injection, respectively. However, the steroidal module has many confounding factors that challenge the passport evaluation.[77,82] For example, alcohol consumption, evident by the presence of ethyl glucuronide (EtG) in the urine sample,[73] likely caused the atypical T/E ratio in one placebo sample as this sample had the highest measured EtG concentration (318.3 μg/mL). Similarly, alcohol consumption might have contributed to the atypical T/E ratios in the ten Sustanon® samples with EtG concentrations above 3 ng/m, but since the samples simultaneously showed atypical 5α-diol/E ratios they were still evaluated as suspicious.

In the presence of confounding factors, the Athlete Passport Management Unit evaluates the sample validity and possible ATPFs in the context of the remaining urinary markers. In this regard, it could be speculated that combining information from the hematological profile with the steroid profile could be helpful in the passport evaluation, as previously suggested by others.[26,67]

2.2.2 The hematological profile

Based on the proven erythropoietic-stimulating effect of testosterone,[9,10,23,26,67] we evaluated the potential of the adaptive model in the ABP hematological module to infer testosterone doping e.g. to identify suspicious samples for subsequent IRMS analysis (*Paper I*). Recent studies have established

that 100 mg testosterone gel can elevate RET% by 0.1 percentage point and RET# by ~13% within 24 hours[67] and that an intramuscular injection of 500 mg testosterone enanthate can increase the HGB by ~3% within four days.[26] In contrast to *Paper I*, these studies did not apply a placebo-controlled design. Such design is of importance to exclude the possibility that other factors than the testosterone administration caused the observed fluctuations. In *Paper I*, we demonstrated that both single and repeated intramuscular injections of 250 mg mixed testosterone esters elevated the RET% and ABPS and decreased the OFF-score three to ten days after the first and/or second injections. Contrary to the finding of a 3% increase in HGB four days post-injection of 500 mg testosterone enanthate in 24 healthy males,[26] HGB remained unchanged throughout the intervention in *Paper I*. This might be explained by a lower dose in *Paper I,* because the testosterone-induced erythropoiesis has been reported to be dose-dependent.[24] Thus, the relatively low RET% response (0.6 percentage point) might have been too small to induce a measurable change in HGB. Secondly, the subjects did not get iron supplementation, thus, insufficient iron availability for HGB synthesis cannot be excluded.

Paper I was the first study to use WADA's ABP software and a placebo-controlled design to evaluate the sensitivity and specificity of the ABP hematological module to infer single and repeated testosterone ester injections. In agreement with the hypothesis, we showed that intramuscular testosterone ester injections affect not only the steroidal module but also some of the markers in the ABP hematological module. Overall, the hematological module identified suspect samples in 6/9 Sustanon®-treated subjects. The RET% and ABPS identified a larger proportion of doped subjects (2/9 and 5/9, respectively) than the HGB and OFF-Score, which both identified 1/9 Sustanon®-treated subjects. Noteworthy, in the placebo group, ABPS was atypical in 30% of the subjects and was elevated from baseline ten days after the second injection. An increase in the ABPS has previously been reported as indication of blood doping,[161,162] hence, our results show that the ABPS lacks specificity in some cases. This is possibly because the score contains blood variables that are influenced by pre-analytical conditions such as prolonged storage,[163] although, the stable MCV in the placebo group indicates that the pre-analytical conditions in *Paper I* were not sub-optimal. In summary, the RET% seemed to be the best marker in the hematological module for indicating testosterone doping.

As a proof-of-concept, we conducted IRMS analyses based on atypical hematological profiles in *Paper I*. We found that the RET%, ABPS and OFF-Score could, in addition to the T/E ratio, help identifying suspicious samples for more targeted IRMS testing. Indeed, the steroidal profile is more sensitive to testosterone doping than the hematological module. Nonetheless, the use of these markers could be beneficial in cases where confounding factors affect the steroid profile. Furthermore, sometimes no

urine samples but only ABP blood samples are collected to save the costs related to urinary analysis and/or to save time when testing many athletes. In cases of suspicious blood profiles with elevated RET% values but stable HGB values, and where subsequent ESA-analysis have shown negative results, the ABP hematological module could be used to target follow-up urine sample collection and IRMS analysis. This is supported by *Paper I*, where 5 out of 9 Sustanon®-treated subjects had an atypical T/E ratio fourteen days after the second injection, i.e. four days after the RET% was elevated compared with baseline and placebo. This implies that the T/E ratio could still be elevated if a follow-up urine sample was collected 3-5 days after an elevated RET%.

2.3 Direct detection of testosterone esters

Today, doping with testosterone esters is directly detected in urine by IRMS analysis or in serum/plasma by steroid ester analysis.[64] However, as addressed in the introduction, these test methods have some limitations. For example, low administration doses[67] or the use of testosterone preparations with $\delta^{13}C_{VPDB}$ close to or within the range of those for endogenous urinary steroids[86,87] might be difficult to detect by IRMS. Additionally, direct steroid ester analysis is limited by rapid, temperature-dependent degradation of esters in serum/plasma, especially in blood collection tubes not stabilized with the esterase inhibitor sodium fluoride.[92] Further, collection and transportation of urine and venous blood samples are subject to strict criteria as described in WADA's standards and can be time-consuming, logistically challenging and require substantial resources.

2.3.1 Further development of DBS analysis of testosterone esters

DBS and DPS are alternative, low-cost sample matrices which might be applicable for detection of doping with testosterone esters, especially because inactivation of the hydrolase enzymes occurs when the blood is dried on the sampling material.[106,109] In support of this, a promising method to detect intact anabolic steroid esters from spiked DBS samples, with a LOD of 0.l ng/mL, has been proposed. Nevertheless, the method showed incomplete extraction of the analytes and resulted in double chromatographic peaks.[108] Thus, we aimed to further develop the method for analysis of the testosterone esters TD, TI, TPP and TP by improving sample preparation and analytical procedures (*Paper II*).

Various alternative extraction conditions were tested, including different solvents, temperatures (RT and 40°C), incubation periods (15-60 min), sonification and shaking, in order to find the most efficient

extraction procedures for TD, TI, TPP and TP. The use of strong organic solvents was necessary because of the adsorption effect of some esters to the cellulose-based DBS card. Since this resulted in particles from the sampling material in the supernatant, a filtering step was added to remove unwanted particles and protect the instrument. The mixture of acetonitrile and methanol (50:50, v:v) provided the best recoveries, without dissolving the spin filters (*Paper II*).

The final, optimized extraction conditions resulted in recoveries between 15 and 22% (*Paper II*), which is comparable to those previously reported.[108] This suggests that cellulose-based DBS materials are not optimal for DBS analysis of testosterone esters. Therefore, we tested if incomplete elution could be circumvented by the use of a water-soluble CMC material (Aquacel® Hydrofiber™, ConvaTec, Deeside, UK), which has shown great potential for other analytes.[107,135,144] Additionally, a DPS card (Noviplex™ Duo, Novilytic, North Webster, IN, USA) [114] was evaluated due to the potential influence of hematocrit on extraction efficiency, at least at high hematocrit levels.[120,130,141] The recoveries of TD, TI, TPP and TP from the pure cellulose material were comparable to those from CMC and the DPS card, despite the use of an aqueous buffer solution optimized for CMC. Since the DBS card allows for collection of multiple spots and is commercially available as a product suited for clinical trials and doping control, we went forward with this material.

The previously reported method for detection of testosterone esters in DBS used methoxime derivatization as a mean to enhance ionization and thereby chromatographic signal and assay sensitivity.[108] However, methoxime derivatization can lead to the formation of two chromatographic peaks for some testosterone esters,[164] which likely reduces the signal strength compared to a single peak. Single chromatographic peaks were obtained by oxime derivatization by hydroxylamine in pyridine in the method for testosterone ester analysis in serum/plasma.[91] But this derivatization reagent can be corrosive and harm the instrument, and thus, requires sample cleanup by online-SPE.[91] The use of 2-hydrazinopyridine improved the signal strength and resulted in single peaks for the testosterone esters, directly in line with previously findings for steroid hormones,[165] and was therefore selected as derivatization reagent in the final method protocol (*Paper II*).

Initially, LC-MS/MS analysis was performed by conventional flow ultra-high pressure LC system interfaced to a Q Exactive mass spectrometer. This resulted in LODs of 0.5 ng/mL for TP, 3 ng/mL for TPP and 1 ng/mL for TI and TD. Noteworthy, the use of nanoflow LC system, with reduced mobile phase flow and inner column diameter, combined with a Q Exactive Plus mass spectrometer enhanced the method's sensitivity markedly and resulted in LODs of 0.1 ng/mL for TP and TI, 0.05

ng/mL for TPP, 0.2 ng/mL TD (*Paper II*). This was considered sufficient based on the higher plasma concentrations of intact esters following an intramuscular injection of 250 mg Sustanon®,[92] which is an administration dose well within the suspected and purported doses used by dopers.[5,159,160]

Further, the method validation showed that the final method (*Paper II*) was specific, with appropriate precision for qualitative analysis and no notable carry-over effect. Thus, the developed method appeared suitable for analysis of testosterone esters.

2.3.3 Detection of testosterone ester administration in DBS

The previously reported DBS method was proven reliable for detection of orally administered testosterone undecanoate (80 mg Andriol® Testocaps) between two and eight hours post-administration in a $n = 1$ trial.[108] In this respect, it must be stressed that the pharmacokinetics seem to differ between subjects after testosterone ester administration.[92] Thus, given the importance of high specificity in a direct detection method, it is necessary to evaluate the DBS assay's sensitivity and specificity in an administration study with more than one subject. A randomized placebo-controlled design, with a setup mimicking a doping control setting, would provide the most applicable information of the DBS method's specificity and sensitivity. Therefore, we analyzed DBS samples from nine men receiving 250 mg Sustanon® and ten men receiving placebo, in a randomized, double-blinded, placebo-controlled design, using the developed nanoLC-MS/MS-based method (*Paper II*). To mimic a doping control setting, the samples were transported from the sample collection site in Denmark to the laboratory in Oslo within 48 hours.

Importantly, the method showed 100% specificity (i.e. no false adverse analytical findings). Further, TD, TI and TPP could be detected with 100% sensitivity for five days after injection, and for up to 14 days in one subject (Subject 16). The shortest chained ester, TP, showed the shortest windows of detection (max five days) and was only detectable in all subjects for one day after injection. Moreover, the individuals with the highest observed relative abundances were also among the individuals with the longest detection times. Hence, the varying detection windows appeared to be due to both the length of the ester sidechain and inter-individual variation in absorption/elimination rates. Noteworthy, a shorter detection period (2-8 hours) was reported for the long-chained testosterone undecanoate in DBS after a single oral ingestion of 80 mg of the drug.[108] However, the lower detectability was probably a result of the extensive first-pass metabolism and consequently low (7%) bioavailability of orally administered testosterone undecanoate.[145]

Our findings are in agreement with the detection windows reported for TD, TI, TPP and TP in human serum/plasma after an injection of Sustanon® 250.[92] This is of interest since the sample volume in DBS is only ~2% of that used in plasma analysis. The explanation is likely the level of enzymatic activity, and thus, degradation of testosterone esters in plasma[92] compared with dried blood, evident by increased stability of testosterone esters when stored on DBS cards, as reported previously[108] and in *Paper II*. In this regard, it is of note that the increased analyte stability in DBS likely makes cooled transportation redundant, which will reduce the costs compared with shipping of standard blood. Collectively, these findings suggest that DBS could be a favorable sample matrix for testosterone ester analysis in both elite and recreational sports.

Interestingly, the detection windows in DBS (*Paper II*) are comparable to the indirect detection periods observed in the ABP steroidal module after single and repeated injections of 250 mg Sustanon® (*Paper I*). In *Paper I*, we observed that the urinary T/E and 5α-diol/E ratios could indicate testosterone doping with 100% sensitivity for at least five days, and in some individuals up to 14 days after the injections. This suggests that DBS analysis potentially could be used for unambiguous confirmation of doping with testosterone esters based on atypical steroid profiles.

2.4 Detection of clenbuterol

Clenbuterol is a widely used doping substance by bodybuilders and athletes seeking to increase lean body mass. Its anabolic and lipolytic effects have been observed in several mammalian species,[42–44] but until now, it is unknown whether clenbuterol has hypertrophic and metabolic effects in healthy humans. In a parallel investigation to *Paper IV*, we were the first to evaluate the effects of acute clenbuterol administration on resting metabolism and skeletal muscle signaling in healthy, young men. We observed increased energy expenditure, fat and carbohydrate oxidation, as well as skeletal muscle signaling of mTOR and PKA around 2.5 hours after the ingestion of 80 μg of clenbuterol in six healthy men (Jessen et al., unpublished data, December 2019). The observed effects can potentially translate into increased fat free mass. The study was limited by a small sample size and the lack of a placebo group. Notwithstanding, the observed changes were of relatively great magnitude, with low inter-individual variation, and are thus unlikely to be results of random variation. Therefore, this study indicates that the detection of doping with clenbuterol, even a single dose, is of relevance. Further, based on the potential health risk associated with clenbuterol misuse,[63] detection of clenbuterol doping is also important for the protection of the health of athletes and recreationally active.

2.4.1 Detection of clenbuterol in urine

Today, urine analysis for clenbuterol is a part of routine doping analysis. However, only a few studies have evaluated the detection windows for clenbuterol in urine.[97,99] Yamoamoto and colleagues used an enzyme immunoassay to evaluate the cumulative urinary excretion after a single administration of 80 μg oral clenbuterol, but only for three days post-administration.[97] While Nicoli and co-workers applied a longer sample collection period following administration (6 days), the administered dose was only 10 μg of clenbuterol.[99] Ten μg clenbuterol is lower than the suspected and purported doses (20 to 200 μg per administration) used by dopers.[4] Therefore, in *Paper IV*, we evaluated whether clenbuterol could be detected in DBS samples collected up to 72 hours following single-dose administration of 80 μg oral clenbuterol.

As previously reported,[166] we observed a large inter-individual variability in urinary concentration profiles, likely due to differences in absorption and urine flow rates. Notwithstanding, clenbuterol was detected in all samples for at least three days and with 67% sensitivity seven days after administration. The longest detection period observed in a subject was ten days. Our results show that clenbuterol can be detected in urine with high specificity for at least 7-10 days when administered as a dose within the range of the expected use by dopers.

Worth mentioning is that fourteen out of 36 clenbuterol findings in urine were below 5 ng/mL. Of these, two samples were collected only 24 hours after ingestion. According to the recent amendment to Article 7.4 of the World Anti-Doping Code, these findings should be reported as ATF instead of AAF.[96] This is because low urinary clenbuterol levels have been observed following the ingestion of meat in countries where clenbuterol is used to induce muscle growth in livestock.[94,95] Our findings indicate that in some subjects, urinary clenbuterol concentrations below 5 ng/mL can occur rapidly after administration of 80 μg clenbuterol, which is a higher dose than what is expected to be inadvertently ingested through contaminated meat.[95] It must be noted that the drug administration in *Paper IV* was witnessed by research personnel, thus, the possibility that the substance was not received in its entire dose is non-existing. Therefore, an ATF for clenbuterol necessitates careful investigation by the Result Management Authority to evaluate the likelihood of meat contamination vs intentional clenbuterol administration.

2.4.2 Detection of clenbuterol in DBS

A sensitive method has been developed for detection of clenbuterol in DBS samples,[139] but the detectability of clenbuterol in DBS samples from humans after clenbuterol ingestion has not been evaluated. Such clinical trials are needed to demonstrate the method's applicability in doping analysis.

Paper IV was the first study to evaluate the detectability of clenbuterol in DBS samples collected from healthy males after clenbuterol ingestion. We observed that clenbuterol could be detected with 100% sensitivity for 24 hours and with 50% sensitivity three days after a single oral ingestion of 80 μg clenbuterol. Further, the analytical method was 100% specific, which is crucial given the large consequences of sanctioning an individual based on a false adverse analytical finding.

The concurrent evaluation of DBS samples and urine samples in *Paper IV* allowed for direct comparison between detectability of clenbuterol in the two matrices. While the urine matrix allows for longer windows of detection for clenbuterol than DBS (*Paper IV*), the fast DBS sample collection, on the other hand, makes frequent and large-scale testing possible, which potentially can increase detection and deterrence. Furthermore, DBS seem to be suited for long-term storage, illustrated by detectable concentration levels of clenbuterol after storage at 2-8 °C for one week[139] and at -80 °C for eight months (*Paper IV*).

It must be noted that although clenbuterol is a non-threshold substance, accurately estimated concentrations are necessary given the risk of inadvertent clenbuterol doping.[94,95] In *Paper IV*, we observed substantial differences in the estimated concentrations of clenbuterol in DBS between subjects. It could be speculated that the differences in DBS concentration profiles were because all subjects received the same absolute dose of clenbuterol. However, the results were the same when the DBS concentrations were normalized to body weight (data not shown). Further, the between-subject variations were larger than those observed for the urine clenbuterol concentrations (*Paper IV*). Therefore, it is likely that the differences in DBS concentration profiles were mainly attributed to reasons other than inter-individual variation in absorption and elimination rates. Based on the fact that the blood was non-volumetrically spotted on the DBS cards in Paper IV, and that a whole spot was used for analysis, it is reasonable to believe that the main explanation was heterogeneity in the spotted volumes of blood. In support of this notion, high accuracy was obtained when analyzing fixed-volume DBS enriched with clenbuterol.[139] This suggests that clenbuterol concentrations in DBS can be accurately estimated, but that a correction strategy for hematocrit or a fixed-volume collection device should be used.

Collectively, our findings imply that DBS is a suitable matrix for long-term storage and routine doping analysis of clenbuterol, even after a single-dose administration. However, studies evaluating different collection and transportation procedures would be valuable to optimize the procedures for use in doping control and analysis. Given the longer detection windows for clenbuterol in urine, urine is expected to remain as the preferred sample matrix for clenbuterol analysis, while DBS sampling could be useful e.g. when testing a large number of athletes in a short time.

2.5 Implementation of DBS in doping control

For DBS to be implemented by WADA in routine doping analysis, standardized and optimized protocols for collection, transportation and storage procedures are required. In the following sections, possible procedures and benefits of DBS collection are discussed.

2.5.1 Sample collection procedures

One of the benefits with DBS is the minimally invasive collection of capillary blood, e.g. from a finger prick. In this respect, it must be noted that while finger prick blood sampling is a fast and minimally invasive sample collection procedure, it appears to have some limitations. For example, in the work with *Paper II* and *Paper IV*, we experienced difficulties collecting DBS samples from individuals with poor blood circulations in the hands. Excessive squeezing or 'milking' of the site to force blood out of the finger may cause hemolysis and/or squeeze tissue fluid into the blood drop and affect the content of the collected sample.[167] Thus, sufficient training of the doping control officers and written guidelines, including pre-sampling techniques to stimulate blood flow (e.g. hand rubbing, commercial hand warmers or running water) might improve DBS collection. Further, athletes who use their fingers in performing their sport, such as in swimming, handball or archery, may be reluctant to provide a finger prick blood samples. Alternatively, collection devices and collection of capillary blood from other anatomical sites may be used to facilitate the DBS collection. One example is the TAP™ push-button blood collection device (SeventhSense Biosystems, Medford, MA, USA), which uses microneedles and vacuum to collect 100 μL capillary blood e.g. from the upper arm.[168] This circumvents the potential problem of poor blood flow in the hands. Subsequently, the blood can be deposited on the DBS card. In a recent pilot study with ten healthy volunteers, a strong correlation was observed between the expression levels of ALAS2, a specific biomarker for heme biosynthesis,[169,170] in DBS collected by the TAP™ system and in DBS collected by finger prick.[136] This illustrates the potential of TAP™ in DBS sampling. Nevertheless, other possible collection devices should be evaluated in future studies.

In general, analyte concentrations measured in DBS correlate well with those measured in venous blood.[119,121,132,137] Hence, it appears that DBS can be accurately used for quantitative purposes. In this regard, it must be noted that when a partial punch-out from a DBS is analyzed, different hematocrit levels, and thereby the distribution of the spot onto the card, can lead to incorrect quantification.[112] Further, inconsistent DBS volumes, particularly when spotting directly from the fingertip, complicates the estimation of analyte concentrations (*Paper IV*) or relative abundances (*Paper II*) when analyzing the entire spot. Accurate determination of analyte concentrations is particularly important in quantitative analysis of threshold substances. As illustrated by Tretzel and co-workers, these challenges can be overcome by whole-spot analysis of known spot volumes.[141] Therefore, it appears that fixed-volume DBS sampling should be the procedure of choice in doping control when analyzing for threshold substances. When whole blood is not needed for analysis, an alternative is the use of dried plasma, i.e. DPS. During the method development preceding *Paper II*, we observed no differences in the performance of the tested materials (i.e. CMC, commercial DBS and DPS), and continued with the product best suited for an administration study and doping control. However, DPS might be the best performing sampling material for other compounds. Therefore, studies comparing different collection materials for dried matrix analysis of other substances are needed.

In this thesis, we collected two DBS cards with four spots each (i.e. A/B samples) and the collected DBS samples dried for a minimum of two hours at room temperature before being packed in individual watertight, sealable plastic bags containing two desiccant packets (*Paper II* and *Paper IV*). This appears preferable in many aspects. The collection of several spots makes it possible to combine 2-3 spots for one analysis if sensitivity requires, or the possibility to develop substance-specific pre-analytical purification protocols, if necessary, and use one spot for each designated analysis. Further, A/B sample collection makes B confirmation analysis possible, while individual, sealable bags with humidity absorbents likely enhances analyte stability and reduces the likelihood of sample manipulation. Contrary, two hours of drying is unsuitable in a doping control setting when trying to improve the time- and cost-efficiency. Thus, studies evaluating shorter drying, as well as alternative procedures for packing and sealing of DBS samples, are needed.

2.5.2 Transportation and storage procedures

Information about analyte stability at varying temperatures is important when optimizing the transportation and storage procedures to be used in routine doping analysis. When stored at room temperature for four weeks, a degradation of 28-76% was observed for anabolic steroid esters on

DBS,[108] while we observed less degradation following storage at -20 °C (*Paper II*). Concerning clenbuterol, the analyte has been reported to be stable for at least one week when stored refrigerated (2-8 °C).[139] Interestingly, we found that testosterone esters and clenbuterol are highly stable (i.e. >18 and >8 months, respectively) when stored at -80 °C (*Paper II* and *Paper IV*). Based on these observations, it appears that DBS samples for analysis of steroid esters and clenbuterol can be transported at room temperature but should be stored frozen if subsequent re-analysis is considered.

2.5.3 Possibilities and benefits with DBS

Based on the small sample volume in DBS, which may limit the assay sensitivity, and the fact that the prodrugs are only momentarily in the systemic circulation, urine will likely continue to be the backbone for doping analysis. Notwithstanding, there are many possibilities with DBS sampling in anti-doping testing. As highlighted in *Paper II*, *Paper IV* and by others,[141] the fast collection and simplified logistics with DBS sampling make more frequent and/or large-scale testing possible, which may improve detection and render deterrence from doping. Further, the improved analyte stability in DBS compared with liquid matrices, as reported by others[106–109] and in *Paper II*, as well as the possibility for long-term storage (*Paper II* and *Paper IV*), could allow for testing in more remote areas and storage of samples for future analyses. Moreover, another benefit with DBS sampling is the gender aspect of sample collection. Opposed to urine sample collection, where the doping control officer must be of the same gender as the athlete, DBS sampling can be performed independent of gender. In addition to the easy setup, i.e. no toilet needed, this will likely make doping controls more feasible.

In a pilot project, we tested the use of DBS collection by a TAP™ device (SeventhSense Biosystems, Medford, MA, USA) in real-life doping control with Anti Doping Denmark. Nineteen athletes were selected for in-competition testing and provided a pair of A/B DBS samples each, as well as responded to a questionnaire concerning their experiences of DBS sample collection. Of the tested athletes, 13 had been tested before. In total, 17 would prefer the DBS sampling procedure compared with urine sampling. Similarly, 17/19 would prefer this sampling procedure compared with collection of venous blood. The average pain score when grading pain sensation from 0 to 9 (i.e. 9 is most painful) was 0.4 ± 0.9. Based on these preliminary data, it appears that DBS sampling is very well accepted by athletes. Because DBS sampling seems more athlete-friendly than conventional sampling methods, DBS could potentially be the preferred sample matrix in anti-doping education programs, e.g. when young athletes are introduced to the doping control process.

Of interest for anti-doping is also the possibility of automating the entire analytical workflow from sample extraction to LC-MS/MS analysis.[6,143] This can reduce the manual laboratory workload, analyte loss during sample transfer and risk of contamination, as well as increase the sample throughput in routine analysis.

3. Conclusion and perspectives

This thesis demonstrated that DBS analysis allows for detection up to 14 days after an intramuscular injection of 250 mg Sustanon®, and for at least three days after an oral ingestion of 80 μg clenbuterol, with no false adverse analytical findings. Additionally, preliminary data indicate that DBS sampling is very well accepted by athletes and regarded as more athlete-friendly than existing matrices. Thus, DBS-based analytical methods for detection of doping with testosterone esters and clenbuterol seem to have sufficient specificity and sensitivity to complement existing analytical strategies while improving time- and cost-efficiency and reducing intrusiveness. This can potentially allow for more frequent and/or large-scale testing to increase detection and deterrence in both elite and recreational athletes. Further, the high analyte stability on DBS likely makes cooled shipping redundant and makes DBS a suitable sample matrix for long-term storage for future analysis. However, future studies should evaluate different collection procedures and shorter drying and packing procedures suited for use in doping control.

In the present thesis, it was shown that single and repeated intramuscular injections of 250 mg mixed testosterone esters affect markers in the hematological module of the ABP, with the largest detectable effects three to ten days after an injection. The RET% seemed to be the most selective marker. Thus, the hematological module could help targeting follow-up sample collection and confirmatory analysis by IRMS if only ABP blood samples have been collected, or initiate the analysis for testosterone ester in DBS, if such samples have been collected. Further, the evaluation of markers in the steroid module and hematological module together might improve detectability of testosterone doping, especially in cases when steroid markers are affected by confounding factors.

In contrast to the hypothesis, an intramuscular injection of 250 mg mixed testosterone esters did not enhance performance acutely in a countermovement jump test, a one-arm isometric elbow flexion test nor a 30-sec cycle sprint test. Further, the serum testosterone levels appeared not to influence performance in strength and power exercises at baseline. Thus, when determining the most cost-efficient

testing programs in power and strength sports, it appears that anti-doping authorities should focus on collecting samples out-of-competition when testing for testosterone esters.

The present thesis demonstrated that a single oral ingestion of 80 μg clenbuterol can be detected for at least 10 days in urine. The longer detection windows in urine compared to in DBS suggest that urine is still the preferred sample matrix for clenbuterol analysis, while DBS sampling could be useful e.g. when testing a many athletes in a short time. Further, low (< 5 ng/mL) urinary concentrations of clenbuterol can occur rapidly (≤24 h) after ingestion of 80 μg of drug in some individuals. Thus, an atypical finding for clenbuterol necessitates careful investigation to determine the likelihood of food contamination.

4. References

1. USADA. *USADA Annual Report 2017*. Colorado Springs, Colorado; 2018. https://www.usada.org/wp-content/uploads/2017_annual_report.pdf.
2. World Anti-Doping Agency. *2017 Anti-Doping Testing Figures*. Montreal, Quebec: World Anti-Doping Agency; 2018. https://www.wada-ama.org/sites/default/files/resources/files/2017_anti-doping_testing_figures_en_0.pdf.
3. Sagoe D, Molde H, Andreassen CS, Torsheim T, Pallesen S. The global epidemiology of anabolic-androgenic steroid use: A meta-analysis and meta-regression analysis. *Ann Epidemiol*. 2014;24(5):383-398. doi:10.1016/j.annepidem.2014.01.009
4. Parkinson AB, Evans NA. Anabolic androgenic steroids: A survey of 500 users. *Med Sci Sports Exerc*. 2006;38(4):644-651. doi:10.1249/01.mss.0000210194.56834.5d
5. Evans NA. Gym and tonic: a profile of 100 male steroid users. *Br J Sports Med*. 1997;31(1):54-58. doi:10.1136/bjsm.31.1.54
6. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: Advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230. doi:10.1016/j.jpba.2016.03.055
7. Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D. Effect of testosterone on muscle mass and muscle protein synthesis. *J Appl Physiol*. 1989;66(1):498-503. doi:10.1152/jappl.1989.66.1.498
8. Rebuffé-Scrive M, Mårin P, Björntorp P. Effect of testosterone on abdominal adipose tissue in men. *Int J Obes*. 1991;15(11):791-795. doi:10.2337/dc07-0337.S.D.
9. Beggs LA, Yarrow JF, Conover CF, et al. Testosterone alters iron metabolism and stimulates red blood cell production independently of dihydrotestosterone. *Am J Physiol Endocrinol Metab*. 2014;307(5):E456-61. doi:10.1152/ajpendo.00184.2014
10. Bachman E, Travison TG, Basaria S, et al. Testosterone induces erythrocytosis via increased erythropoietin and suppressed hepcidin: Evidence for a new erythropoietin/hemoglobin set point. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2014;69(6):725-735. doi:10.1093/gerona/glt154
11. de Rooy C, Grossmann M, Zajac JD, Cheung AS. Targeting muscle signaling pathways to minimize adverse effects of androgen deprivation. *Endocr Relat Cancer*. 2016;23(1):R15-R26. doi:10.1530/ERC-15-0232
12. Cheung AS, Grossmann M. Physiological basis behind ergogenic effects of anabolic androgens. *Mol Cell Endocrinol*. 2018;464(November 2016):14-20. doi:10.1016/j.mce.2017.01.047
13. Bhasin S, Storer T, Berman N, et al. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N Engl J Med*. 1996;335(1):1-7. doi:10.1056/NEJM199607043350101
14. Giorgi A, Weatherby RP, Murphy PW. Muscular strength, body composition and health responses to the use of testosterone enanthate: a double blind study. *J Sci Med Sport*. 1999;2(4):341-355. doi:10.1016/S1440-2440(99)80007-3

15. Rogerson S, Weatherby RP, Deakin GB, et al. The effect of short-term use of testosterone enanthate on muscular strength and power in healthy young men. *J Strength Cond Res*. 2007;21(2):354. doi:10.1519/R-18385.1
16. Baume N, Schumacher YO, Sottas PE, et al. Effect of multiple oral doses of androgenic anabolic steroids on endurance performance and serum indices of physical stress in healthy male subjects. *Eur J Appl Physiol*. 2006;98(4):329-340. doi:10.1007/s00421-006-0271-0
17. Weatherby RP, Giorgi A, Kazlauskas R. Retention of performance gains following testosterone enanthate administration. *Med Sci Sport Exerc*. 2002;34(5):90. doi:10.1097/00005768-200205001-01788
18. Seynnes OR, de Boer M, Narici M V. Early skeletal muscle hypertrophy and architectural changes in response to high-intensity resistance training. *J Appl Physiol*. 2006;102(1):368-373. doi:10.1152/japplphysiol.00789.2006
19. Storer TW, Magliano L, Woodhouse L, et al. Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. *J Clin Endocrinol Metab*. 2003;88(4):1478-1485. doi:10.1210/jc.2002-021231
20. Bhasin S, Woodhouse L, Casaburi R, et al. Testosterone dose-response relationships in healthy young men. *Am J Physiol Metab*. 2001;281(6):E1172-E1181. doi:10.1152/ajpendo.2001.281.6.E1172
21. Sinha-Hikim I, Roth SM, Lee MI, Bhasin S. Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *Am J Physiol - Endocrinol Metab*. 2003;285(1):E197-E205. doi:10.1152/ajpendo.00370.2002
22. Schulte-Beerbühl M, Nieschlag E. Comparison of testosterone, dihydrotestosterone, luteinizing hormone, and follicle-stimulating hormone in serum afterinjection of testosterone enanthate or testosterone cypionate. *Fertil Steril*. 1980;33(2):201-203. doi:10.1016/S0015-0282(16)44543-7
23. Bachman E, Feng R, Travison T, et al. Testosterone suppresses hepcidin in men : A potential mechanism for testosterone-induced erythrocytosis. 2010;95(October):4743-4747. doi:10.1210/jc.2010-0864
24. Coviello AD, Kaplan B, Lakshman KM, Chen T, Singh AB, Bhasin S. Effects of graded doses of testosterone on erythropoiesis in healthy young and older men. *J Clin Endocrinol Metab*. 2008;93(3):914-919. doi:10.1210/jc.2007-1692
25. Moriyama Y, Fisher JW. Effects of testosterone and erythropoietin on erythroid colony formation in human bone marrow cultures. *Blood*. 1975;45(5):665-670.
26. Mullen JE, Gårevik N, Schulze JJ, Rane A, Björkhem Bergman L, Ekström L. Perturbation of the hematopoietic profile by anabolic androgenic steroids. *J Horm*. 2014;2014:1-7. doi:10.1155/2014/510257
27. Bassett DR, Howley ET. Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Med Sci Sports Exerc*. 2000;32(1):70-84. doi:10.1097/00005768-200001000-00012
28. Coyle EF, Coggan AR, Hopper MK, Walters TJ. Determinants of endurance in well-trained cyclists. *J Appl Physiol*. 1988;64(6):2622-2630. doi:10.1152/jappl.1988.64.6.2622

29. Thomsen JJ, Rentsch RL, Robach P, et al. Prolonged administration of recombinant human erythropoietin increases submaximal performance more than maximal aerobic capacity. *Eur J Appl Physiol*. 2007;101(4):481-486. doi:10.1007/s00421-007-0522-8
30. Bonifazi M, Ginanneschi F, della Volpe R, Rossi A. Effects of gonadal steroids on the input-output relationship of the corticospinal pathway in humans. *Brain Res*. 2004;1011(2):187-194. doi:10.1016/j.brainres.2004.03.022
31. Estrada M, Espinosa A, Müller M, Jaimovich E. Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology*. 2003;144(8):3586-3597. doi:10.1210/en.2002-0164
32. Hamdi MM, Mutungi G. Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. *J Physiol*. 2010;588(3):511-525. doi:10.1113/jphysiol.2009.182162
33. Antinozzi C, Marampon F, Corinaldesi C, et al. Testosterone insulin-like effects: an in vitro study on the short-term metabolic effects of testosterone in human skeletal muscle cells. *J Endocrinol Invest*. 2017;40(10):1133-1143. doi:10.1007/s40618-017-0686-y
34. Smith A. Characterization of the vasodilatory action of testosterone in the human pulmonary circulation. *Vasc Health Risk Manag*. 2008;Volume 4(6):1459-1466. doi:10.2147/VHRM.S3995
35. Pugh P. Acute haemodynamic effects of testosterone in men with chronic heart failure. *Eur Heart J*. 2003;24(10):909-915. doi:10.1016/S0195-668X(03)00083-6
36. Pope HG, Kouri EM, Hudson JI. Effects of supraphysiologic doses of testosterone on mood and aggression in normal men. *Arch Gen Psychiatry*. 2000;57(2):133. doi:10.1001/archpsyc.57.2.133
37. Kouri EM, Lukas SE, Pope HG, Oliva PS. Increased aggressive responding in male volunteers following the administration of gradually increasing doses of testosterone cypionate. *Drug Alcohol Depend*. 1995;40(1):73-79. doi:10.1016/0376-8716(95)01192-7
38. Carré JM, Geniole SN, Ortiz TL, Bird BM, Videto A, Bonin PL. Exogenous testosterone rapidly increases aggressive behavior in dominant and impulsive men. *Biol Psychiatry*. 2017;82(4):249-256. doi:10.1016/j.biopsych.2016.06.009
39. Carré JM, McCormick CM. Aggressive behavior and change in salivary testosterone concentrations predict willingness to engage in a competitive task. *Horm Behav*. 2008;54(3):403-409. doi:10.1016/j.yhbeh.2008.04.008
40. Cardinale M, Stone MH. Is testosterone influencing explosive performance? *J Strength Cond Res*. 2006;20(1):103. doi:10.1519/R-16864.1
41. Bosco C, Tihanyit J, Viru A. Relationships between field fitness test and basal serum testosterone and Cortisol levels in soccer players. *Clin Physiol*. 1996;16(3):317-322. doi:10.1111/j.1475-097X.1996.tb00577.x
42. Hinkle RT, Hodge KMB, Cody DB, Sheldon RJ, Kobilka BK, Isfort RJ. Skeletal muscle hypertrophy and anti-atrophy effects of clenbuterol are mediated by the ?2-adrenergic receptor. *Muscle Nerve*. 2002;25(5):729-734. doi:10.1002/mus.10092

43. MacLennan PA, Edwards RH. Effects of clenbuterol and propranolol on muscle mass. Evidence that clenbuterol stimulates muscle β-adrenoceptors to induce hypertrophy. *Biochem J*. 1989;264(2):573-579. doi:10.1042/bj2640573
44. Petrou M, Wynne DG, Boheler KR, Yacoub MH. Clenbuterol induces hypertrophy of the latissimus dorsi muscle and heart in the rat with molecular and phenotypic changes. *Circulation*. 1995;92(9):483-489. doi:10.1161/01.CIR.92.9.483
45. Lynch GS, Ryall JG. Role of β-adrenoceptor signaling in skeletal muscle: Implications for muscle wasting and disease. *Physiol Rev*. 2008;88(2):729-767. doi:10.1152/physrev.00028.2007
46. Hostrup M, Kalsen A, Ørtenblad N, et al. β2-Adrenergic stimulation enhances Ca2+ release and contractile properties of skeletal muscles, and counteracts exercise-induced reductions in Na+-K+-ATPase Vmax in trained men. *J Physiol*. 2014;592(24):5445-5459. doi:10.1113/jphysiol.2014.277095
47. Hostrup M, Kalsen A, Bangsbo J, Hemmersbach P, Karlsson S, Backer V. High-dose inhaled terbutaline increases muscle strength and enhances maximal sprint performance in trained men. *Eur J Appl Physiol*. 2014;114(12):2499-2508. doi:10.1007/s00421-014-2970-2
48. Juel C, Hostrup M, Bangsbo J. The effect of exercise and beta2-adrenergic stimulation on glutathionylation and function of the Na,K-ATPase in human skeletal muscle. *Physiol Rep*. 2015;3(8):1-11. doi:10.14814/phy2.12515
49. Hostrup M, Jacobson GA, Jessen S, Lemminger AK. Anabolic and lipolytic actions of beta2-agonists in humans and anti-doping challenges. *Drug Test Anal*.:In press.
50. Hostrup M, Kalsen A, Onslev J, et al. Mechanisms underlying enhancements in muscle force and power output during maximal cycle ergometer exercise induced by chronic B2-adrenergic stimulation in men. *J Appl Physiol*. 2015;119(5):475-486. doi:10.1152/japplphysiol.00319.2015
51. Collomp K, Candau R, Lasne F, Labsy Z, Préfaut C, De Ceaurriz J. Effects of short-term oral salbutamol administration on exercise endurance and metabolism. *J Appl Physiol*. 2000;89(2):430-436.
52. Hostrup M, Kalsen A, Auchenberg M, Bangsbo J, Backer V. Effects of acute and 2-week administration of oral salbutamol on exercise performance and muscle strength in athletes. *Scand J Med Sci Sport*. 2016;26(1):8-16. doi:10.1111/sms.12298
53. Crivelli G, Maffiuletti NA. Actions of β2-adrenoceptor agonist drug on neuromuscular function after fatigue. *Med Sci Sports Exerc*. 2014;46(2):247-256. doi:10.1249/MSS.0b013e3182a54ee3
54. Crivelli G, Borrani F, Capt R, Gremion G, Maffiuletti NA. Actions of β 2-adrenoceptor agonist drug on human soleus muscle contraction. *Med Sci Sports Exerc*. 2013;45(7):1252-1260. doi:10.1249/MSS.0b013e318284706a
55. Sanchez AMJ, Borrani F, Le Fur MA, et al. Acute supra-therapeutic oral terbutaline administration has no ergogenic effect in non-asthmatic athletes. *Eur J Appl Physiol*. 2013;113(2):411-418. doi:10.1007/s00421-012-2447-0
56. Kalsen A, Hostrup M, Bangsbo J, Backer V. Combined inhalation of beta2-agonists improves swim ergometer sprint performance but not high-intensity swim performance. *Scand J Med Sci Sport*. 2014;24(5):814-822. doi:10.1111/sms.12096

57. Van Baak MA, Mayer LHJ, Kempinski RES, Hartgens F. Effect of salbutamol on muscle strength and endurance performance in nonasthmatic men. *Med Sci Sports Exerc*. 2000;32(7):1300-1306. doi:10.1097/00005768-200007000-00018

58. Collomp K, Candau R, Collomp R, et al. Effects of acute ingestion of salbutamol during submaximal exercise. *Int J Sports Med*. 2000;21(7):480-484. doi:10.1055/s-2000-7422

59. Collomp K, Candau R, Millet G, et al. Effects of salbutamol and caffeine ingestion on exercise metabolism and performance. *Int J Sports Med*. 2002;23(8):549-554. doi:10.1055/s-2002-35530

60. Hostrup M, Kalsen A, Bangsbo J, Hemmersbach P, Karlsson S, Backer V. High-dose inhaled terbutaline increases muscle strength and enhances maximal sprint performance in trained men. *Eur J Appl Physiol*. 2014;114(12):2499-2508. doi:10.1007/s00421-014-2970-2

61. Collomp K, Le Panse B, Portier H, et al. Effects of acute salbutamol intake during a Wingate test. *Int J Sports Med*. 2005;26(7):513-517. doi:10.1055/s-2004-821223

62. Pope HG, Wood RI, Rogol A, Nyberg F, Bowers L, Bhasin S. Adverse health consequences of performance-enhancing drugs: An endocrine society scientific statement. *Endocr Rev*. 2014;35(3):341-375. doi:10.1210/er.2013-1058

63. Spiller HA, James KJ, Scholzen S, Borys DJ. A descriptive study of adverse events from clenbuterol misuse and abuse for weight loss and bodybuilding. *Subst Abus*. 2013;34(3):306-312. doi:10.1080/08897077.2013.772083

64. WADA Laboratory Expert Group. *WADA Technical Document - TD2018EAAS*. 1.0. Montreal, Quebec; 2018. https://www.wada-ama.org/sites/default/files/resources/files/td2018eaas_final_eng.pdf.

65. Strahm E, Mullen JE, Gårevik N, et al. Dose-dependent testosterone sensitivity of the steroidal passport and GC-C-IRMS analysis in relation to the UGT2B17 deletion polymorphism. *Drug Test Anal*. 2015;7(11-12):1063-1070. doi:10.1002/dta.1841

66. Baume N, Saudan C, Desmarchelier A, et al. Use of isotope ratio mass spectrometry to detect doping with oral testosterone undecanoate: Inter-individual variability of 13C/12C ratio. *Steroids*. 2006;71(5):364-370. doi:10.1016/j.steroids.2005.11.004

67. Mullen J, Börjesson A, Hopcraft O, et al. Sensitivity of doping biomarkers after administration of a single dose testosterone gel. *Drug Test Anal*. 2018;10(5):839-848. doi:10.1002/dta.2341

68. Miller GD, Nair V, Morrison MS, Summers M, Willick SE, Eichner D. Intranasal delivery of Natesto® testosterone gel and its effects on doping markers. *Drug Test Anal*. 2016;8(11-12):1197-1203. doi:10.1002/dta.2106

69. Geyer H, Flenker U, Mareck U, et al. The detection of the misuse of testosterone gel. In: Schanzer W, Geyer H, Gotzmann A, Mareck U, eds. *Recent Advances in Doping Analysis 15*. 15th ed. Cologne: Sportverlag Strauss; 2007:133-142.

70. Schulze JJ, Mullen JE, Lindgren EB, Ericsson M, Ekström L, Hirschberg AL. The impact of genetics and hormonal contraceptives on the steroid profile in female athletes. *Front Endocrinol (Lausanne)*. 2014;5(APR):1-6. doi:10.3389/fendo.2014.00050

71. Mullen J, Gadot Y, Eklund E, et al. Pregnancy greatly affects the steroidal module of the Athlete Biological Passport. *Drug Test Anal*. 2018;10(7):1070-1075. doi:10.1002/dta.2361
72. Große J, Anielski P, Sachs H, Thieme D. Ethylglucuronide as a potential marker for alcohol-induced elevation of urinary testosterone/epitestosterone ratios. *Drug Test Anal*. 2009;1(11-12):526-530. doi:10.1002/dta.110
73. Thieme D, Große J, Keller L, Graw M. Urinary concentrations of ethyl glucuronide and ethyl sulfate as thresholds to determine potential ethanol-induced alteration of steroid profiles. *Drug Test Anal*. 2011;3(11-12):851-856. doi:10.1002/dta.396
74. Albeiroti S, Ahrens BD, Sobolevskii T, Butch AW. The influence of small doses of ethanol on the urinary testosterone to epitestosterone ratio in men and women. *Drug Test Anal*. 2018;10(3):575-583. doi:10.1002/dta.2241
75. Schulze JJ, Lundmark J, Garle M, Skilving I, Ekström L, Rane A. Doping test results dependent on genotype of uridine diphospho-glucuronosyl transferase 2B17, the major enzyme for testosterone glucuronidation. *J Clin Endocrinol Metab*. 2008;93(7):2500-2506. doi:10.1210/jc.2008-0218
76. Juul A, Sørensen K, Aksglaede L, et al. A common deletion in the uridine diphosphate glucuronyltransferase (UGT) 2b17 gene is a strong determinant of androgen excretion in healthy pubertal boys. *J Clin Endocrinol Metab*. 2009;94(3):1005-1011. doi:10.1210/jc.2008-1984
77. Mareck U, Geyer H, Opfermann G, Thevis M, Schänzer W. Factors influencing the steroid profile in doping control analysis. *J Mass Spectrom*. 2008;43(7):877-891. doi:10.1002/jms.1457
78. Sottas PE, Saudan C, Schweizer C, Baume N, Mangin P, Saugy M. From population- to subject-based limits of T/E ratio to detect testosterone abuse in elite sports. *Forensic Sci Int*. 2008;174(2-3):166-172. doi:10.1016/j.forsciint.2007.04.001
79. World Anti-Doping Agency. *Athlete Biological Passport Operating Guideliens 7.0*. Montreal, Quebec; 2019. https://www.wada-ama.org/sites/default/files/resources/files/guidelines_abp_v71.pdf.
80. Sottas P-E, Robinson N, Saugy M. The athlete's biological passport and indirect markers of blood doping. In: Thieme D, Hemmersbach P, eds. *Doping in Sports: Handbook of Experimental Pharmacology*. Vol 195. Berlin, Heidelberg: Springer; 2010:305-326.
81. WADA Laboratory Expert Group. *WADA Technical Document –TD2019IRMS*. 1.0. Montreal, Quebec: World Anti-Doping Agency; 2018. https://www.wada-ama.org/sites/default/files/td2019irms_final_eng_clean.pdf.
82. Kuuranne T, Saugy M, Baume N. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. *Br J Sports Med*. 2014;48(10):848-855. doi:10.1136/bjsports-2014-093510
83. Gore CJ, Parisotto R, Ashenden MJ, et al. Second-generation blood tests to detect erythropoietin abuse by athletes. *Haematologica*. 2003;88(3):333-344. http://www.ncbi.nlm.nih.gov/pubmed/12651273.
84. Sottas P-E, Robinson N, Giraud S, et al. Statistical Classification of Abnormal Blood Profiles in Athletes. *Int J Biostat*. 2006;2(1):Article 3. doi:10.2202/1557-4679.1011
85. Bejder J, Aachmann-Andersen NJ, Bonne TC, Olsen NV, Nordsborg NB. Detection of

erythropoietin misuse by the Athlete Biological Passport combined with reticulocyte percentage. *Drug Test Anal.* 2016;8(10):1049-1055. doi:10.1002/dta.1932

86. Hullstein I, Sagredo C, Hemmersbach P. Carbon isotope ratios of nandrolone, boldenone, and testosterone preparations seized in Norway compared to those of endogenously produced steroids in a Nordic reference population. *Drug Test Anal.* 2014;6(11-12):1163-1169. doi:10.1002/dta.1745
87. Forsdahl G, Östreicher C, Koller M, Gmeiner G. Carbon isotope ratio determination and investigation of seized testosterone preparations. *Drug Test Anal.* 2011;3(11-12):814-819. doi:10.1002/dta.373
88. Cawley AT, Flenker U. The application of carbon isotope ratio mass spectrometry to doping control. *J Mass Spectrom.* 2008;43(7):854-864. doi:10.1002/jms
89. Brand WA. High precision isotope ratio monitoring techniques in mass spectrometry. *J Mass Spectrom.* 1996;31(3):225-235. doi:10.1002/(SICI)1096-9888(199603)31:3<225::AID-JMS319>3.0.CO;2-L
90. Nieschlag E, Behre HM, Bouchard P, et al. Testosterone replacement therapy: Current trends and future directions. *Hum Reprod Update.* 2004;10(5):409-419. doi:10.1093/humupd/dmh035
91. Forsdahl G, Vatne HK, Geisendorfer T, Gmeiner G. Screening of testosterone esters in human plasma. *Drug Test Anal.* 2013;5(11-12):826-833. doi:10.1002/dta.1560
92. Forsdahl G, Erceg D, Geisendorfer T, et al. Detection of testosterone esters in blood. *Drug Test Anal.* 2015;7(11-12):983-989. doi:10.1002/dta.1914
93. Fujioka M, Shinohara Y, Baba S, Irie M, Inoue K. Pharmacokinetic properties of testosterone propionate in normal men. *J Clin Endocrinol Metab.* 1986;63(6):1361-1364. doi:10.1210/jcem-63-6-1361
94. Guddat S, Fußhöller G, Geyer H, et al. Clenbuterol - regional food contamination a possible source for inadvertent doping in sports. *Drug Test Anal.* 2012;4(6):534-538. doi:10.1002/dta.1330
95. Thevis M, Geyer L, Geyer H, et al. Adverse analytical findings with clenbuterol among U-17 soccer players attributed to food contamination issues. *Drug Test Anal.* 2013;5(5):372-376. doi:10.1002/dta.1471
96. World Anti-Doping Agency. *Stakeholder Notice Regarding Meat Contamination*. Montreal, Quebec; 2019. https://www.wada-ama.org/sites/default/files/resources/files/2019-05-30-meat_contamination_notice_final.pdf.
97. Yamamoto I, Iwata K, Nakashima M. Pharmacokinetics of plasma and urine clenbuterol in man, rat, and rabbit. *J Pharmacobiodyn.* 1985;8(5):385-391. http://www.ncbi.nlm.nih.gov/pubmed/4045696.
98. Yang YG, Song LX, Jiang N, Xu XT, Di XH, Zhang M. Pharmacokinetics of ambroxol and clenbuterol tablets in healthy Chinese volunteers. *Int J Clin Exp Med.* 2015;8(10):18744-18750.
99. Nicoli R, Petrou M, Badoud F, Dvorak J, Saugy M, Baume N. Quantification of clenbuterol at trace level in human urine by ultra-high pressure liquid chromatography–tandem mass spectrometry. *J Chromatogr A.* 2013;1292:142-150. doi:10.1016/j.chroma.2012.12.008
100. Elbe AM, Overbye M. Urine doping controls: The athletes' perspective. *Int J Sport Policy.* 2014;6(2):227-240. doi:10.1080/19406940.2013.801361
101. Nørgaard-Pedersen B, Simonsen H. Biological specimen banks in neonatal screening. *Acta Paediatr Int*

J Paediatr Suppl. 1999;88(432):106-109. doi:10.1111/j.1651-2227.1999.tb01172.x

102. Sadones N, Capiau S, De Kesel P, Lambert WE, Stove CP. Spot them in the spot : analysis of abused substances using dried blood spots. *Bioanalysis*. 2014;6(17):2211-2227. doi:10.4155/bio.14.156
103. Ignjatovic V, Pitt J, Monagle P, Craig JM. The utility of dried blood spots for proteomic studies: Looking forward to looking back. *Proteomics - Clin Appl*. 2014;8(11-12):896-900. doi:10.1002/prca.201400042
104. Déglon J, Thomas A, Daali Y, et al. Automated system for on-line desorption of dried blood spots applied to LC/MS/MS pharmacokinetic study of flurbiprofen and its metabolite. *J Pharm Biomed Anal*. 2011;54(2):359-367. doi:10.1016/j.jpba.2010.08.032
105. Tretzel L, Thomas A, Piper T, et al. Fully automated determination of nicotine and its major metabolites in whole blood by means of a DBS online-SPE LC-HR-MS/MS approach for sports drug testing. *J Pharm Biomed Anal*. 2016;123:132-140. doi:10.1016/j.jpba.2016.02.009
106. Tretzel L, Thomas A, Geyer H, Delahaut P, Schänzer W, Thevis M. Determination of Synacthen® in dried blood spots for doping control analysis using liquid chromatography tandem mass spectrometry. *Anal Bioanal Chem*. 2015;407(16):4709-4720. doi:10.1007/s00216-015-8674-6
107. Rosting C, Sæ CØ, Gjelstad A, Halvorsen TG. Evaluation of water-soluble DBS for small proteins: a conceptual study using insulin as a model analyte. *Bioanalysis*. 2016;8(10):1051-1065. doi:10.4155/bio-2016-0002
108. Tretzel L, Thomas A, Geyer H, et al. Use of dried blood spots in doping control analysis of anabolic steroid esters. *J Pharm Biomed Anal*. 2014;96:21-30. doi:10.1016/j.jpba.2014.03.013
109. Alfazil AA, Anderson RA. Stability of benzodiazepines and cocaine in blood spots stored on filter paper. *J Anal Toxicol*. 2008;32(7):511-515. doi:10.1093/jat/32.7.511
110. Thomas A, Kohler M, Schänzer W, Kamber M, Delahaut P, Thevis M. Determination of Synacthen in urine for sports drug testing by means of nano-ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2009;23(17):2669-2674. doi:10.1002/rcm.4176
111. Chaabo A, De Ceaurriz J, Buisson C, Tabet JC, Lasne F. Simultaneous quantification and qualification of synacthen in plasma. *Anal Bioanal Chem*. 2011;399(5):1835-1843. doi:10.1007/s00216-010-4565-z
112. Holub M, Tuschl K, Ratschmann R, et al. Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clin Chim Acta*. 2006;373(1-2):27-31. doi:10.1016/j.cca.2006.04.013
113. Wilhelm AJ, den Burger JCG, Swart EL. Therapeutic drug monitoring by dried blood spot: Progress to date and future directions. *Clin Pharmacokinet*. 2014;53(11):961-973. doi:10.1007/s40262-014-0177-7
114. Kim JH, Woenker T, Adamec J, Regnier FE. Simple, miniaturized blood plasma extraction method. *Anal Chem*. 2013;85(23):11501-11508. doi:10.1021/ac402735y
115. Emmons G, Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis*. 2010. doi:10.4155/bio.10.159
116. Protti M, Catapano MC, Samolsky Dekel BG, et al. Determination of oxycodone and its major metabolites in haematic and urinary matrices: Comparison of traditional and miniaturised sampling

approaches. *J Pharm Biomed Anal*. 2018;152:204-214. doi:10.1016/j.jpba.2018.01.043

117. Ryona I, Henion J. A book-type dried plasma spot card for automated flow-through elution coupled with online SPE-LC-MS/MS bioanalysis of opioids and stimulants in blood. *Anal Chem*. 2016;88(22):11229-11237. doi:10.1021/acs.analchem.6b03691
118. Antelo-Domínguez Á, Ángel Cocho J, Jesús Tabernero M, María Bermejo A, Bermejo-Barrera P, Moreda-Piñeiro A. Simultaneous determination of cocaine and opiates in dried blood spots by electrospray ionization tandem mass spectrometry. *Talanta*. 2013;117:235-241. doi:10.1016/j.talanta.2013.09.010
119. Antunes MV, Raymundo S, De Oliveira V, et al. Ultra-high performance liquid chromatography tandem mass spectrometric method for the determination of tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen in dried blood spots - Development, validation and clinical application during breast . *Talanta*. 2015;132:775-784. doi:10.1016/j.talanta.2014.10.040
120. Cox HD, Rampton J, Eichner D. Quantification of insulin-like growth factor-1 in dried blood spots for detection of growth hormone abuse in sport. *Anal Bioanal Chem*. 2013;405(6):1949-1958. doi:10.1007/s00216-012-6626-y
121. Cox HD, Hughes CM, Eichner D. Sensitive quantification of IGF-1 and its synthetic analogs in dried blood spots. *Bioanalysis*. 2014;6(19):2651-2662. doi:10.4155/bio.14.109
122. Cox HD, Eichner D. Mass spectrometry method to measure membrane proteins in dried blood spots for the detection of blood doping practices in sport. *Anal Chem*. 2017;89(18):10029-10036. doi:10.1021/acs.analchem.7b02492
123. Cox HD, Miller GD, Lai A, Cushman D, Eichner D. Detection of autologous blood transfusions using a novel dried blood spot method. *Drug Test Anal*. 2017;9(11-12):1713-1720. doi:10.1002/dta.2323
124. Ferro P, Ventura R, Pérez-Mañá C, Farré M, Segura J. Evaluation of fibronectin 1 in one dried blood spot and in urine after rhGH treatment. *Drug Test Anal*. 2017;9(7):1011-1016. doi:10.1002/dta.2108
125. Höppner S, Delahaut P, Schänzer W, Thevis M. Mass spectrometric studies on the in vivo metabolism and excretion of SIRT1 activating drugs in rat urine, dried blood spots, and plasma samples for doping control purposes. *J Pharm Biomed Anal*. 2014;88:649-659. doi:10.1016/j.jpba.2013.10.022
126. Lange T, Walpurgis K, Thomas A, Geyer H, Thevis M. Development of two complementary LC-HRMS methods for analyzing sotatercept in dried blood spots for doping controls. *Bioanalysis*. 2019;11(10):923-940. doi:10.4155/bio-2018-0313
127. Lawson G, Cocks E, Tanna S. Quantitative determination of atenolol in dried blood spot samples by LC-HRMS: A potential method for assessing medication adherence. *J Chromatogr B Anal Technol Biomed Life Sci*. 2012;897:72-79. doi:10.1016/j.jchromb.2012.04.013
128. Kojima A, Nishitani Y, Sato M, Kageyama S, Dohi M, Okano M. Comparison of urine analysis and dried blood spot analysis for the detection of ephedrine and methylephedrine in doping control. *Drug Test Anal*. 2016;8(2):189-198. doi:10.1002/dta.1803

129. Mommers J, Mengerink Y, Ritzen E, Weusten J, van der Heijden J, van der Wal S. Quantitative analysis of morphine in dried blood spots by using morphine-d3 pre-impregnated dried blood spot cards. *Anal Chim Acta*. 2013;774:26-32. doi:10.1016/j.aca.2013.03.001

130. Möller I, Thomas A, Geyer H, Schänzer W, Thevis M. Development and validation of a mass spectrometric detection method of peginesatide in dried blood spots for sports drug testing. *Anal Bioanal Chem*. 2012;403(9):2715-2724. doi:10.1007/s00216-012-6043-2

131. Peng SH, Segura J, Farré M, De La Torre X. Oral testosterone administration detected by testosterone glucuronidation measured in blood spots dried on filter paper. *Clin Chem*. 2000;46(4):515-522.

132. Protti M, Catapano MC, Samolsky Dekel BG, et al. Determination of oxycodone and its major metabolites in haematic and urinary matrices: Comparison of traditional and miniaturised sampling approaches. *J Pharm Biomed Anal*. 2018;152:204-214. doi:10.1016/j.jpba.2018.01.043

133. Reverter-Branchat G, Bosch J, Vall J, et al. Determination of recent growth hormone abuse using a single dried blood spot. *Clin Chem*. 2016;62(10):1353-1360. doi:10.1373/clinchem.2016.257592

134. Reverter-Branchat G, Ventura R, Ezzel Din M, Mateus J, Pedro C, Segura J. Detection of erythropoiesis-stimulating agents in a single dried blood spot. *Drug Test Anal*. 2018;10(10):1496-1507. doi:10.1002/dta.2418

135. Rosting C, Gjelstad A, Halvorsen TG. Water-soluble dried blood spot in protein analysis: A proof-of-concept study. *Anal Chem*. 2015;87(15):7918-7924. doi:10.1021/acs.analchem.5b01735

136. Salamin O, Gottardo E, Schobinger C, et al. Detection of stimulated erythropoiesis by the RNA-based 5'-aminolevulinate synthase 2 biomarker in dried blood spot samples. *Clin Chem*. 2019;65(12):1563-1571. doi:10.1373/clinchem.2019.306829

137. Saussereau E, Lacroix C, Gaulier JM, Goulle JP. On-line liquid chromatography/tandem mass spectrometry simultaneous determination of opiates, cocainics and amphetamines in dried blood spots. *J Chromatogr B Anal Technol Biomed Life Sci*. 2012;885-886:1-7. doi:10.1016/j.jchromb.2011.11.035

138. Thomas A, Geyer H, Guddat S, Schänzer W, Thevis M. Dried blood spots (DBS) for doping control analysis. *Drug Test Anal*. 2011;3(11-12):806-813. doi:10.1002/dta.342

139. Thomas A, Geyer H, Schänzer W, et al. Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem*. 2012;403(5):1279-1289. doi:10.1007/s00216-011-5655-2

140. Thomas A, Thevis M. Analysis of insulin and insulin analogs from dried blood spots by means of liquid chromatography–high resolution mass spectrometry. *Drug Test Anal*. 2018;10(11-12):1761-1768. doi:10.1002/dta.2518

141. Tretzel L, Thomas A, Geyer H, Pop V, Schänzer W, Thevis M. Dried blood spots (DBS) in doping controls: a complementary matrix for improved in- and out-of-competition sports drug testing strategies. *Anal Methods*. 2015;7(18):7596-7605. doi:10.1039/c5ay01514f

142. Tretzel L, Görgens C, Geyer H, et al. Analyses of meldonium (Mildronate) from blood, dried blood spots (DBS), and urine suggest drug incorporation into erythrocytes. *Int J Sports Med*. 2016;37(06):500-

502. doi:10.1055/s-0036-1582317

143. Verplaetse R, Henion J. Quantitative determination of opioids in whole blood using fully automated dried blood spot desorption coupled to on-line SPE-LC-MS/MS. *Drug Test Anal*. 2016;8(1):30-38. doi:10.1002/dta.1927

144. Ask KS, Pedersen-Bjergaard S, Gjelstad A. Dried blood spots on carboxymethyl cellulose sheets: Rapid sample preparation based on dissolution and precipitation. *Chromatographia*. 2016;79(7-8):509-514. doi:10.1007/s10337-016-3039-7

145. Täuber U, Schröder K, Düsterberg B, Matthes H. Absolute bioavailability of testosterone after oral administration of testosterone-undecanoate and testosterone. *Eur J Drug Metab Pharmacokinet*. 1986;11(2):145-149. doi:10.1007/BF03189840

146. Farah CS, Reinach FC. The troponin complex and regulation of muscle contraction. *FASEB J*. 1995;9(9):755-767. doi:10.1096/fasebj.9.9.7601340

147. Szczesna D, Zhao J, Jones M, Zhi G, Stull J, Potter JD. Phosphorylation of the regulatory light chains of myosin affects Ca 2+ sensitivity of skeletal muscle contraction. *J Appl Physiol*. 2002;92(4):1661-1670. doi:10.1152/japplphysiol.00858.2001

148. Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW. Tissue distribution and ontogeny of steroid 5 alpha-reductase isozyme expression. *J Clin Invest*. 1993;92(2):903-910. doi:10.1172/JCI116665

149. Fraysse B, Vignaud A, Fane B, et al. Acute effect of androgens on maximal force-generating capacity and electrically evoked calcium transient in mouse skeletal muscles. *Steroids*. 2014;87:6-11. doi:10.1016/j.steroids.2014.05.005

150. Calbet JAL, Chavarren J, Dorado C. Fractional use of anaerobic capacity during a 30- and a 45-s Wingate test. *Eur J Appl Physiol Occup Physiol*. 1997;76(4):308-313. doi:10.1007/s004210050253

151. Hostrup M, Bangsbo J. Limitations in intense exercise performance of athletes – effect of speed endurance training on ion handling and fatigue development. *J Physiol*. 2017;595(9):2897-2913. doi:10.1113/JP273218

152. Seyrek M, Yildiz O, Ulusoy HB, Yildirim V. Testosterone relaxes isolated human radial artery by potassium channel opening action. *J Pharmacol Sci*. 2007;103(3):309-316. doi:10.1254/jphs.FP0060883

153. McErlain-Naylor S, King M, Pain MT homa. G. Determinants of countermovement jump performance: a kinetic and kinematic analysis. *J Sports Sci*. 2014;32(19):1805-1812. doi:10.1080/02640414.2014.924055

154. McLellan CP, Lovell DI, Gass GC. The role of rate of force development on vertical jump performance. *J Strength Cond Res*. 2011;25(2):379-385. doi:10.1519/JSC.0b013e3181be305c

155. Crewther BT, Cook CJ, Gaviglio CM, Kilduff LP, Drawer S. Baseline strength can influence the ability of salivary free testosterone to predict squat and sprinting performance. *J Strength Cond Res*. 2012;26(1):261-268. doi:10.1519/JSC.0b013e3182185158

156. Crewther B, Carruthers J, Kilduff L, Sanctuary C, Cook C. Temporal associations between individual changes in hormones, training motivation and physical performance in elite and non-elite trained men.

Biol Sport. 2016;33(3):215-221. doi:10.5604/20831862.1201810

157. Healy ML, Gibney J, Pentecost C, Wheeler MJ, Sonksen PH. Endocrine profiles in 693 elite athletes in the postcompetition setting. *Clin Endocrinol (Oxf)*. 2014;81(2):294-305. doi:10.1111/cen.12445
158. Chung T, Kelleher S, Liu PY, Conway AJ, Kritharides L, Handelsman DJ. Effects of testosterone and nandrolone on cardiac function: a randomized, placebo-controlled study. *Clin Endocrinol (Oxf)*. 2007;66(2):235-245. doi:10.1111/j.1365-2265.2006.02715.x
159. Yu J-G, Bonnerud P, Eriksson A, Stål PS, Tegner Y, Malm C. Effects of long term supplementation of anabolic androgen steroids on human skeletal muscle. Alway SE, ed. *PLoS One*. 2014;9(9):e105330. doi:10.1371/journal.pone.0105330
160. Parkinson AB, Evans NA. Anabolic androgenic steroids. *Med Sci Sport Exerc*. 2006;38(4):644-651. doi:10.1249/01.mss.0000210194.56834.5d
161. Sottas P-E, Robinson N, Saugy M, Niggli O. A forensic approach to the interpretation of blood doping markers. *Law, Probab Risk*. 2008;7(3):191-210. doi:10.1093/lpr/mgm042
162. Sottas PE, Robinson N, Fischetto G, Dollé G, Alonso JM, Saugy M. Prevalence of blood doping in samples collected from elite track and field athletes. *Clin Chem*. 2011;57(5):762-769. doi:10.1373/clinchem.2010.156067
163. Voss SC, Flenker U, Majer B, Schänzer W. Stability tests for hematological parameters in antidoping analyses. *Lab Hematol*. 2008;14(3):24-29. doi:10.1532/LH96.08005
164. Gray BP, Teale P, Pearce CM. Analysis of methyloxime derivatives of intact esters of testosterone and boldenone in equine plasma using ultra high performance liquid chromatography tandem mass spectrometry. *Drug Test Anal*. 2011;3(4):206-213. doi:10.1002/dta.237
165. Nadarajah N, Skadberg Ø, Adaway J, Brede C. Multiplexed analysis of steroid hormones in saliva by LC-MS/MS with 2-hydrazinopyridine derivatization. *Clin Mass Spectrom*. 2017;4-5:1-10. doi:10.1016/j.clinms.2017.08.001
166. Nicoli R, Petrou M, Badoud F, Dvorak J, Saugy M, Baume N. Quantification of clenbuterol at trace level in human urine by ultra-high pressure liquid chromatography-tandem mass spectrometry. *J Chromatogr A*. 2013;1292:142-150. doi:10.1016/j.chroma.2012.12.008
167. Mei J. Dried Blood Spot sample collection, storage, and transportation. In: Li W, Lee MS, eds. *Dried Blood Spots: Applications and Techniques*. ; 2014:21-31. doi:10.1002/9781118890837.ch3
168. Blicharz TM, Gong P, Bunner BM, et al. Microneedle-based device for the one-step painless collection of capillary blood samples. *Nat Biomed Eng*. 2018. doi:10.1038/s41551-018-0194-1
169. Salamin O, Mignot J, Kuuranne T, Saugy M, Leuenberger N. Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion. *Drug Test Anal*. 2018;10(3):604-608. doi:10.1002/dta.2240
170. Durussel J, Haile DW, Mooses K, et al. Blood transcriptional signature of recombinant human erythropoietin administration and implications for antidoping strategies. *Physiol Genomics*. 2016;48(3):202-209. doi:10.1152/physiolgenomics.00108.2015

PAPER I

PAPER II

PAPER III

PAPER IV

ISBN 978-87-7209-334-5

UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

ADD
ANTI DOPING DANMARK