*Year :* 2021

# Investigation of blood biomarkers for the improvement of the Athlete Biological Passport

Salamin Olivier

Salamin Olivier, 2021, Investigation of blood biomarkers for the improvement of the Athlete Biological Passport

Originally published at : Thesis, University of Lausanne

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Document URN : urn:nbn:ch:serval-BIB_C40B2A55DE1B0

UNIL | Université de Lausanne
Faculté de biologie et de médecine

**Institut des Sciences du Sport**
**Centre de Recherche et d'Expertise des sciences anti-Dopage (REDs)**

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**Thèse de doctorat ès sciences de la vie (PhD)**

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

**Olivier SALAMIN**

Master de l'Université de Lausanne

**Jury**

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Prof. Nelly Pitteloud, Directrice de thèse
Prof. Martial Saugy, Co-directeur
Prof. Giuseppe d'Onofrio, expert
Prof. Peter Van Eenoo, expert
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Lausanne 2021

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**Ecole Doctorale**
**Doctorat ès sciences de la vie**

# Imprimatur

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le Conseil de Faculté autorise l'impression de la thèse de

## Monsieur Olivier Salamin

Maîtrise universitaire ès Sciences en biologie médicale, Université de Lausanne

intitulée

## Investigation of blood biomarkers for the improvement of the Athlete Biological Passport

Lausanne, le 8 septembre 2021

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Lorenzo Alberio

*I have never had a single positive doping test, and I do not take performance-enhancing drugs.*

-Lance Armstrong-

## Acknowledgments

During these five incredible years, I have been fortunate to be surrounded by many people who have enabled me to accomplish the work presented in this thesis.

First of all, I would like to address my sincere thanks to Prof. Martial Saugy, my thesis director, my mentor, for giving the opportunity to carry out this thesis work and for being the first doctoral student produced from the REDs. Thank you for your precious advice and your patience, but also for giving me the opportunity to take part in annexed projects to the thesis, which brought me a lot of experience. Every time we exchanged, I could learn something new about the anti-doping world. I would also like to thank Prof. Nelly Pitteloud for accepting to be part of my thesis, for the collaboration we could establish to conduct a clinical trial involving women and testosterone and for sharing her knowledge about endocrinology. She has always shown a great optimism towards our common work, which gave me additional motivation.

Thanks also to the staff of the LAD with who I had the chance to collaborate and to be part of the daily activity. I express my sincere gratitude to Dr. Tiia Kuuranne to make me feel like home, for her constant availability and support, and for sharing her precious knowledge about the anti-doping processes. Special thanks to Dr. Raul Nicoli who introduced me to analytical chemistry and how to use properly a LC-MS instrument. Throughout the project, he helped and guided me in building up a scientific approach and in learning how to disseminate the results by oral communication or scientific publication. I would also like to thank Dr. Nicolas Leuenberger who gave me the opportunity 6 years ago to conduct my Master thesis at the LAD. Thank you for the collaboration and for giving me the opportunity to contribute to many scientific publications. I also thank all the other members of the LAD with who I spent memorable moments in and out of the lab.

A big thanks also to the colleagues from the REDs (Dr. Raphaël Faiss, Dr. Jonas Saugy, Prof. Francesco Botrè) and from the University of Geneva (Prof. Serge Rudaz and Dr. Julien Boccard) with who I had the chance to collaborate and from who I learnt a lot.

I would like to express all my gratitude to all my friends for the "off" moments and my lovely family who motivated and supported me throughout all my studies. Thank you Flavio for all the fun times we had at "La rue de la bière" which were needed after a hard day's work.

Finally, I thank Elisa from the bottom of my heart for her unfailing support throughout the years, for her presence by my side and with whom I am happy to be able to share these important moments of my life.

## List of publications

1. **Salamin O,** Jaggi L, Baume N, Robinson N, Saugy M, Leuenberger N**.** Circulating microRNA-122 as Potential Biomarker for Detection of Testosterone Abuse. PloS One. 2016 May 12;11(5):e0155248.
2. **Salamin O**, De Angelis S, Tissot JD, Saugy M, Leuenberger N. Autologous Blood Transfusion in Sports: Emerging Biomarkers. Transfu Med Rev. 2016 Jul; 30(3):109-15.
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11. **Salamin O**, Ponzetto F, Cauderay M, et al. Development and validation of an UHPLC-MS/MS method for extended serum steroid profiling in female populations. Bioanalysis. 2020; 12(11): 753-768.
12. **Salamin O**, Garcia A, Gonzalez-Ruiz V, et al. Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competition. Drug Test Anal. 2020; 12(11-12): 1649-1657.
13. **Salamin O**, Nicoli R, Langer T, et al. Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle. Drug Test Anal. 2021. Online ahead of print.

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## Summary

The implementation of the Athlete Biological Passport (ABP) represented a milestone in the fight against doping and unveiled a paradigm shift, namely the indirect detection of prohibited substances or methods through an individual, adaptive and longitudinal monitoring of biomarkers. While this tool has improved detection capability, it still suffers from sensitivity issues. To improve the discriminative performance of ABP, additional and complementary biomarkers are thus required and the blood matrix offers an immense reservoir for biomarkers discovery using emerging and sensitive –omics technologies. In the first part of the thesis, the impact of blood doping on the reticulocytes transcriptome was investigated. Three candidate genes could be highlighted as promising markers of altered erythropoiesis following an autologous blood transfusion using two different quantitative methods, with a magnitude of changes more significant than that of markers currently used in the ABP. The transcriptomic approach was applied for the detection of testosterone (T) doping using circulating microRNAs as biomarkers. Although the study enabled to identify one microRNA significantly influenced by the administration of T, it appeared that this cell-free biomarker could also be impacted by other external factors underlining a potential lack of specificity. The strategy was thus adapted toward a targeted metabolomics approach, based on recent works performed by our group, with the serum profiling of endogenous steroids using UHPLC-MS/MS. A clinical trial involving healthy cycling women and T gel administration was conducted and allowed comparing the sensitivity of the current urinary ABP biomarkers and emerging serum steroid biomarkers. While the urinary biomarkers demonstrated a moderate sensitivity mainly due to menstrual fluctuations, the longitudinal monitoring of T and DHT serum concentrations, along with the newly proposed T/androstenedione ratio, showed improved sensitivity to detect T administration. The development of an UHPLC-MS/MS method for extended serum steroid profiling enabled to expand the number of potential biomarkers with the inclusion of phase II metabolites. Because the monitoring of transcriptomic and steroidomic biomarkers requires the invasive collection of blood, dried blood spots were evaluated as alternative matrix for the measurement of these biomarkers to overcome the logistical constraints associated with blood collection. In conclusion, our work allowed expanding the range of potential biomarkers that could be integrated into the ABP for a longitudinal monitoring as complementary information to the current hematological and urinary steroidal modules. These additional doping biomarkers could ultimately be used in combination with the current ABP biomarkers as a bundle of evidence to support the scenario of the administration of a prohibited substance.

## Résumé

L'implémentation du Passeport Biologique de l'Athlète (PBA) représente une étape majeure dans la lutte contre le dopage et a dévoilé un changement de paradigme, à savoir la détection indirecte de substances ou méthodes interdites par un suivi individuel, adaptatif et longitudinal de biomarqueurs. Bien que cet outil ait amélioré la capacité de détection, il souffre néanmoins de problèmes de sensibilité. Pour améliorer la performance discriminante du PBA, des biomarqueurs supplémentaires et complémentaires sont donc nécessaires et la matrice sanguine offre un immense réservoir pour la découverte de biomarqueurs en utilisant les technologies émergentes –omics. Dans la première partie de la thèse, l'impact du dopage sanguin sur le transcriptome des réticulocytes a été étudié. Trois gènes candidats ont pu être mis en évidence comme des marqueurs prometteurs de l'érythropoïèse altérée suite à une transfusion sanguine autologue en utilisant deux méthodes quantitatives différentes, avec une ampleur de changements plus importante que celle des marqueurs actuellement utilisés dans le PBA. L'approche transcriptomique a été appliquée pour la détection du dopage à la testostérone (T) en utilisant les microARNs circulants comme biomarqueurs. Bien que l'étude ait permis d'identifier un microARN significativement influencé par l'administration de T, il est apparu que ce biomarqueur pouvait également être impacté par d'autres facteurs externes soulignant un potentiel manque de spécificité. La stratégie a ainsi été adaptée vers une approche métabolomique ciblée, basée sur des travaux récents réalisés par notre groupe, avec le profilage sérique des stéroïdes endogènes par UHPLC-MS/MS. Un essai clinique impliquant des femmes en bonne santé avec des cycles réguliers et l'administration de gel de T a été mené et a permis de comparer la sensibilité des biomarqueurs urinaires actuels du PBA et des biomarqueurs émergents de stéroïdes sériques. Alors que les biomarqueurs urinaires ont démontré une sensibilité modérée principalement due aux fluctuations menstruelles, le suivi longitudinal des concentrations sériques de T et de DHT, ainsi que du rapport T/androstènedione nouvellement proposé, ont montré une sensibilité améliorée pour détecter l'administration de T. Le développement d'une méthode UHPLC-MS/MS pour le profilage étendu des stéroïdes sériques a permis d'augmenter le nombre de biomarqueurs potentiels avec l'inclusion de métabolites de phase II. Parce que le suivi des biomarqueurs transcriptomiques et stéroïdomiques nécessite la collecte invasive de sang, les gouttes de sang séché ont été évaluées comme matrice alternative pour la mesure de ces biomarqueurs afin de surmonter les contraintes logistiques associées à la collecte de sang. En conclusion, nos travaux ont permis d'élargir la gamme de biomarqueurs potentiels qui pourraient être intégrés dans le PBA pour un suivi longitudinal en complément des modules stéroïdiens hématologiques et urinaires actuels. Ces biomarqueurs du dopage supplémentaires pourraient à terme être utilisés en combinaison avec les biomarqueurs actuels du PBA comme un ensemble de preuves pour étayer le scénario de l'administration d'une substance interdite.

# List of abreviations

- 11KT 11-ketotestosterone
- 16-en 5α-androst-16-en-3α-ol
- 17OHPROG 17α-hydroxyprogesterone
- 5α17HP 17α-hydroxyallopregnanolone
- 5αAdiol 5α-androstane-3α, 17β-diol
- 5βAdiol 5β -androstane-3α, 17β-diol
- 5α-dione 5α-androstanedione
- A Androsterone
- A4 Androstenedione
- AAF Adverse analytical finding
- AAS Anabolic-androgenic steroids
- ABP Athlete biological passport
- ABPS Abnormal profile score
- ABT Autologous blood transfusion
- ACTH Adrenocorticotropic hormone
- ADVR Anti-doping rule violation
- AKR1C3 Aldo-keto reductase family 1 member C3
- ALAS2 Delta-aminolevulinate synthase 2
- alloPreg Allopregnanolone
- APF Adverse passport finding
- APMU Athlete Passport Management Unit
- AR Androgen receptor
- AST Androsterone
- ATF Atypical finding
- ATPF Atypical passport finding
- ATPF-CPR Atypical passport finding confirmation procedure request
- BHK Baby hamster kidney
- BRB-seq Bulk RNA barcoding and sequencing
- BS Between-subject
- CA1 Carbonic anhydrase
- CAH Congenital adrenal hyperplasia
- CAS Court of arbitration for sport
- CERA Continuous erythropoietin receptor activator
- CFU-E Colony-forming units-erythroid
- CHO Chinese hamster ovary
- CIR Carbon isotope ratio
- CKD Chronic kidney disease
- CP Confirmatory procedure
- CRF Corticotropin releasing factor
- CYP Cytochrome P450
- DBS Dried blood spot

- DHEA Dehydroepiandrosterone
- DHEAS Dehydroepiandrosterone sulfate
- DHT 5α-dihydrotestosterone
- DS Dilute-and-shoot
- E Epitestosterone
- E1 Estrone
- E1S Estrone sulfate
- E2 Estradiol
- EAAS Endogenous anabolic androgenic steroid
- EPO Erythropoietin
- EPO-R Erythropoietin receptor
- ERA Erythropoietin-receptor agonist
- ERC Endogenous reference compound
- ERFE Erythroferrone
- ESA Erythropoiesis stimulating agent
- Etio Etiocholanolone
- FIFA Federation of Association Football
- FSH Follicle stimulating hormone
- GC Gas chromatography
- GC/C/IRMS Gas chromatography/combustion/isotope ratio mass spectrometry
- GH Growth hormone
- GnRH Gonadotropin-releasing hormone
- HBT Homologous blood transfusion
- HCT Hematocrit
- hCG Human chorionic gonadotropin
- HGB Hemoglobin
- HIF Hypoxia-inducible factor
- HPA Hypothalamic-pituitary adrenal
- HPG Hypothalamic-pituitary gonadal
- HRE Hypoxia response element
- HRMS High resolution mass spectrometry
- HSD Hydroxysteroid dehydrogenase
- HuEPO Human erythropoietin
- IAAF International Association of Athletics Federations
- IEF Isoelectric focusing
- INC In-competition
- IOC International Olympic Committee
- IOC-MC International Olympic Committee -Medical Commission
- IRF Immature reticulocyte fraction
- ISL International Standards for Laboratories
- ITP Initial testing procedure
- LC Liquid chromatography

- LH Luteinizing hormone
- LLE Liquid-liquid extraction
- mAR Membrane androgen receptor
- MCH Mean corpuscular hemoglobin
- MCHC Mean corpuscular hemoglobin concentration
- MCV Mean corpuscular volume
- MDMA Methylenedioxymethamphetamine
- miRNA microRNA
- MRPL Minimum required performance level
- MS Mass spectrometry
- MS/MS Tandem mass spectrometry
- NESP Novel erythropoiesis-stimulating protein
- NFID Nitrogen-flame ionization detection
- OG Olympic Games
- OOC Out-of-competition
- PAGE Polyacrylamide gel electrophoresis
- PAPS 3`-phosphoadenosine-5`-phosphosulfate
- PCOS Polycystic ovary syndrome
- PD Pregnanediol
- PEG Polyethylene glycol
- PH Prolyl hydroxylase
- pI Isoelectric point
- PLT Platelets
- PP Protein precipitation
- PREG Pregnenolone
- PROG Progesterone
- PV Plasma volume
- qPCR Quantitative polymerase chain reaction
- RBC Red blood cell
- RDW-SD Red cell distribution width
- RET Reticulocytes
- RET# Reticulocytes count
- RET% Reticulocytes percentage
- rHuEPO Recombinant human erythropoietin
- RNA-seq RNA sequencing
- RT-qPCR Reverse transcriptase quantitative polymerase chain reaction
- SAGE Serial analysis of gene expression
- SAR Sarcosyl
- SARM Selective androgen receptor modulator
- SDS Sodium dodecyl sulfate
- SERM Selective estrogen receptor modulator
- SHBG Sex hormone-binding globulin

- SLC4A1 Solute carrier family 4 member 1
- SPE Solid-phase extraction
- SRD Steroid reductase
- SSP-CPR Suspicious steroid profile confirmation procedure request
- STAT5 Signal transducer and activator of transcription 5
- SULT Sulfotransferase enzyme
- T Testosterone
- TC Target compound
- THC Tetrahydrocannabinol
- TUE Therapeutic Use Exemption
- UCI Union Cycliste Internationale
- UGT Uridine 5`-diphospho-glucuronosyltransferase
- UHPLC Ultra-high performance liquid chromatography
- WADA World Anti-Doping Agency
- WADC World Anti-Doping Code
- WBC White blood cells
- WC World Championships
- WS Within-subject

# Chapter I

Introduction

## 1.1 The anti-doping background

### 1.1.1 History of anti-doping

In the context of competition, humans have always sought to gain an advantage over their opponents in any way. The use of performance-enhancing substances has a long history both in society and in the world of sport, and the first traces of this use can be retrieved from the Ancient Olympic Games, in which athletes were reported to use special diets and herbal extracts to improve their performance. An extract from the Ephedra plant ('Mu Huang') was recommended as ergogenic aids by Chinese physicians 2700 BC and unspecified stimulants were also used by Roman gladiators to overcome fatigue and injury [1,2]. With the development of modern pharmacology and medicine, the number of potentially performance-enhancing substances has drastically increased by the end of the 19$^{th}$ century and the early 20$^{th}$ century. At that time, these substances were mainly stimulants such as cocaine, caffeine, strychnine and were principally used by cyclists and other endurance athletes in single or combined administration into specific mixtures often containing alcohol beverage [3].

The fight against doping was initiated in 1928 by the International Association of Athletics Federations (IAAF), being the first international sport body to ban the use of stimulating substances. Other federations followed suit, although these measures remained inefficient at that time in the absence of official and effective tests. No further preventive actions were undertaken although it was largely recognized that many athletes resorted to stimulating cocktails to improve their performance. It was not until 1960, following the death of a Danish cyclist during a road race at the Olympic Games (OG) in Rome and suspected of using stimulant drugs, that the real fight against doping has started and actions have been taken. The Union Cycliste Internationale (UCI) began to develop a set of rules, and in order to preserve the reputation of Olympic sports and elite sports in general, the International Olympic Committee (IOC) created a Medical Commission (IOC-MC) at the IOC Session in Athens in 1961, with the goal to design a strategy to fight drug use in Olympic sports [3,4]. Small-scale tests for stimulants were introduced at the 1964 OG with mitigate success, while in 1966 the UCI and the International Federation of Association Football (FIFA) introduced for the first time anti-doping tests in their World Championships (WC). The following year, the IOC-MC presented at the 1967 IOC Session in Tehran a motion consisting of a list of prohibited substances including principally stimulants and rules for testing for these substances at the OG. Consequently, the first tests were introduced at the Winter OG in Grenoble and Summer OG in Mexico City in 1968 [2]. At the following 1972 Munich OG, urine samples were tested for

stimulants and narcotics using gas chromatography (GC) coupled to nitrogen-selective detection (nitrogen-flame ionization detection (NFID)).

Anabolic-androgenic steroids (AAS) were first synthesized in the 1930s but their misuse by athletes was later reported in the 1960s. At the beginning of the 1970s, the use of AAS was widespread worldwide. The IAAF was the first sport organization to ban steroids in 1974 and testing based on immunoassay was set up at the European Championship in Rome the same year. In-between, the IOC included AAS to its list of prohibited substances and conducted testing for their misuse for the first time at the 1976 OG in Montreal.

In the context of the Cold War between 1970 and end of the 1980s, sport in general and doping in sport turned into massive priority not only for personal ambition of athletes and their entourage, but were also misused for nationalist and political promotion. Therefore, the development of anti-doping stagnated and progress was modest [3]. At the 1988 Summer OG in Seoul, the positive control of the world's best male sprinter Ben Johnson for stanozolol, an anabolic steroid, caused an electroshock among the sports community. Subsequent investigation revealed that at least half of the athletes competing in Seoul used AAS, which raised consciousness toward more effective doping control measures and the need for so-called 'out-of-competition' (OOC) testing in the fight against AAS doping [1,2]. This led to a 21% increase of collected samples from 1988 to 1992, and in 1991 the IAAF was the first sport organization to institute OOC testing aimed at more effective detection of AAS preferably used during training periods contrary to stimulants.

Despite increased efficiency of doping tests and testing methods, a major blow in the fight against doping, marking a turning point in anti-doping history, occurred in 1998. The soigneur of the cycling Festina team was stopped by custom officers at the Belgian-French border who discovered a large amount of prohibited substances (amphetamines, erythropoietin (EPO), growth hormone (GH), testosterone and narcotics) in his car three days before the start of the Tour de France [5]. This so-called 'Festina Affair' highlighted the necessity to harmonize anti-doping rules among anti-doping organizations, to promote and coordinate anti-doping activities both at the national and international level by a single and central body with the support of public authorities. Hence, the IOC proposed the idea of an international Anti-doping Agency and in February 1999, convened a World Conference on Doping in Lausanne in the presence of the Council of Europe and several representatives of Governments. In line with the need for an independent international agency setting unified standards for anti-doping work and coordinating the efforts of all stakeholders, the major outcome of this Conference was the agreement to create the World Anti-Doping Agency (WADA), which was officially established

in December 1999. The financial support of WADA is equally shared between public authorities (Governments) and Olympic sport. The foundation and establishment of WADA is indefinitely a major milestone of anti-doping history and has created an innovative situation with the development and implementation of a uniform set of anti-doping rules under the aegis of the World Anti-Doping Agency Code (WADC), the document harmonizing anti-doping policies in all sports and all countries [6].

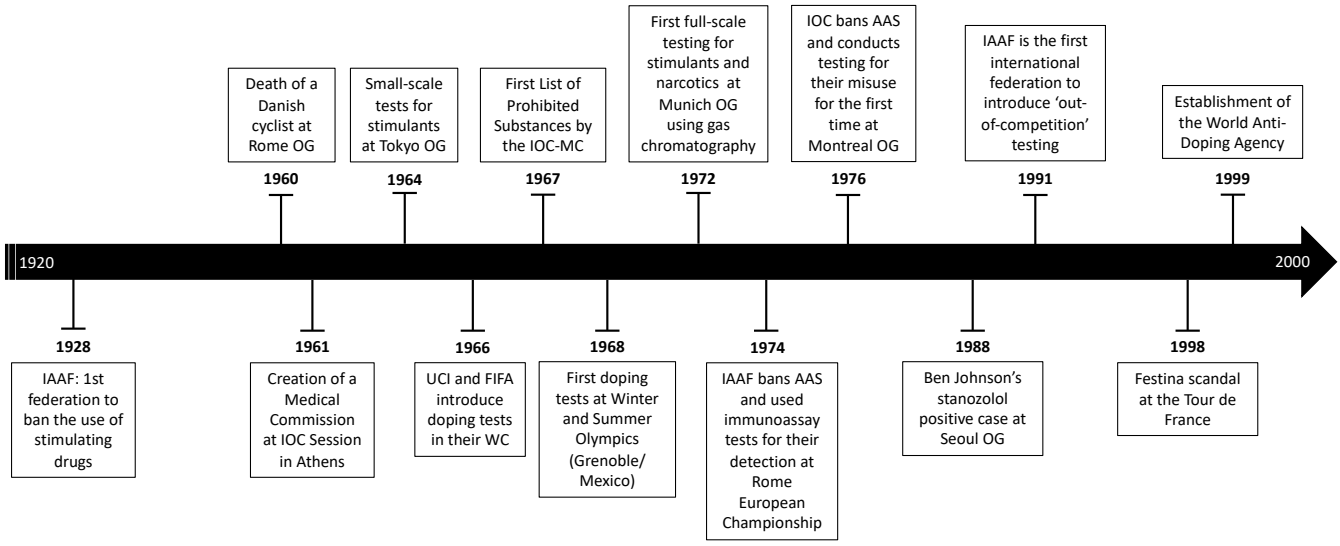


**Figure 1**. Milestones of anti-doping history until the creation of WADA.

### 1.1.2 Anti-doping regulations

As previously described, WADA is the international independent organization responsible for promoting, coordinating and monitoring the fight against doping in sport. The first major achievement of WADA was the elaboration of the WADC, which was adopted at 2nd World Conference on Doping in Sport in 2003 in Copenhagen [7]. This document was intended to replace the IOC Medical Code and the Olympic Movement Anti-Doping Code of 1999, and became the first anti-doping regulation with worldwide acceptance. It represents the base document for the fight against doping in sport and upon which the World Anti-Doping program is based. Since its adoption, with the desire to keep the document alive and evolving, the Code has been reviewed and updated twice in 2009 and 2015. In 2017, a third revision of the Code was initiated into a three-phase review process and took effect on 1 January 2021 [8]. The Code requires all signatories to have adequate anti-doping programs in place or risk being declared noncompliant with possibly serious consequences.

In the Code, doping – rather an 'anti-doping rule violation' (ADVR) - is defined as "*the occurrence of one or more of the anti-doping rule violations set forth in Article 2.1 through Article 2.11 of the Code*". In other words, it signifies that doping and subsequent suspension is not only based on (i) the presence of a prohibited substance or its metabolites or markers in an athlete's sample but also on (ii) the use or attempted use by an athlete of a prohibited substance or method, (iii) evading, refusing or failing to submit to sample collection, (iv) violation of applicable requirement regarding athlete availability for out-of-competition testing, (v) tampering or attempted tampering with any part of doping control, (vi) possession of a prohibited substance or a prohibited method, (vii) trafficking or attempted trafficking in any prohibited substances or prohibited method, (viii) administration or attempted administration of a prohibited substance or method to any athlete, (ix) complicity involving an anti-doping rule violation, (x) prohibited association and/or (xi) acts by an athlete or other person to discourage or retaliate against reporting to authorities [9].

In conjunction with the WADC (level 1 document), WADA has elaborated eight associated International Standards (level 2 documents) for the Prohibited List, for the Laboratories, for Testing and Investigations, for Therapeutic Use Exemptions (TUEs), for the Protection of Privacy and Personal Information, for Code Compliance by Signatories, for Education and for Results Managements [10]. These standards aimed at ensuring harmonization and best practice of anti-doping program among anti-doping organizations in various technical areas and are mandatory for all signatories of the Code.

The Prohibited List has been regulated by WADA since 2003 and identifies the substances and methods prohibited in- and out-of-competition, and in particular sports [11]. The List is updated and published by WADA at least annually [7]. Each annual version of the Prohibited List and all revisions are distributed promptly by WADA to each Signatory, WADA-accredited or approved laboratory, and government, and are published on WADA's website, and each Signatory shall take appropriate steps to distribute the Prohibited List to its members and constituents [9]. To be considered for inclusion in the List, a substance or a method has to fulfill at least two of the three following criteria: 1. Potential to enhance or proof of enhancing sport performance; 2. Evidence of a potential or real health risk to the athlete; 3. Violation of the spirit of sport.

The List is divided into 11 classes of prohibited substances and 3 prohibited methods (Table 1). Some substances are prohibited at all times (in- (INC) and out-of-competition (OOC)) (i.e. anabolic agents, β2-agonists, or hormone and metabolic modulators), others are only prohibited INC (i.e. stimulants, narcotics, glucucorticoids, cannabinoids), while β-blockers are prohibited

only in particular sports. Prohibited methods such as blood manipulations or gene doping are prohibited at all times. The INC period is defined as the moment starting just before midnight (at 11:59 p.m.) on the day before a competition until the end of this same competition [11]. The substances can also be distinguished between non-threshold substances, for which the simple presence in the biological sample is considered as an ADVR, and threshold substances, for which prohibition is applied above a certain threshold level of concentration and for which a quantitative determination in the matrix is mandatory.

Jointly with the 2021 WADC (Art. 4.2.3), some substances of the 2021 Prohibited List have been identified as Substances of Abuse "*because they are frequently abused in society outside of the context of sport*" [12]. Cocaine, diamorphine (heroin), methylenedioxymethampetamine (MDMA/ecstasy) and tetrahydrocannabinol (THC) are designated as Substances of Abuse. In case of an ADVR involving a Substance of Abuse, if the Athlete can establish that "*any ingestion or use occurred OOC and was unrelated to sport performance",* the period of ineligibility can be reduced to three months or less (one month) if the Athlete completes a Substance of Abuse treatment program [13].

**Table 1**. Structure of the 2021 Prohibited List

| **SUBSTANCES & METHODS PROHIBITED AT ALL TIMES** |
|---|
| S0. NON-APPROVED SUBSTANCES |
| S1. ANABOLIC AGENTS<br>1. Anabolic Androgenic Steroids (AAS)<br>2. Other anabolic agents |
| S2. PEPTIDE HORMONES, GROWTH FACTORS, RELATED SUBSTANCES AND MIMETICS<br>1. Erythropoietin (EPO) and agents affecting erythropoiesis<br>2. Peptide hormones and their releasing factors<br>3. Growth factors and growth factor modulators |
| S3. BETA-2 AGONISTS |
| S4. HORMONE AND METABOLIC MODULATORS<br>1. Aromatase inhibitors<br>2. Anti-estrogenic substances [anti-estrogens and selective estrogen receptor modulators (SERMS)]<br>3. Agents preventing activin receptor IIB activation<br>4. Metabolic modulators |
| S5. DIURETICS AND MASKING AGENTS |
| **PROHIBITED METHODS** |
| M1. MANIPULATION OF BLOOD AND BLOOD COMPONENTS |
| M2. CHEMICAL AND PHYSICAL MANIPULATION |
| M3. GENE AND CELL DOPING |
| **SUBSTANCES PROHIBITED IN-COMPETITION** |
| S6. STIMULANTS<br>A. Non-specified stimulants<br>B. Specified stimulants |
| S7. NARCOTICS |
| S8. CANNABINOIDS |
| S9. GLUCOCORTICOIDS |
| **SUBSTANCES PROHIBITED IN PARTICULAR SPORTS** |
| P1. BETA-BLOCKERS |

### 1.1.3 Anti-doping laboratory and analytical strategies

Anonymized anti-doping samples are shipped to and analyzed by dedicated anti-doping laboratories. To be authorized to proceed to anti-doping analyses, a laboratory requires a WADA accreditation based upon compliance with two international standards: the classic ISO/IEC 17025 accreditation for testing and calibration laboratories and the International Standard for Laboratories (ISL). The ISL and its related technical documents specify the criteria that must be met for accreditation and re-accreditation, as well as standards that must be met for the production of valid test results and evidentiary data [14]. In case of nonconformities with the ISL, WADA can suspend an accredited laboratory for a dedicated period of time.

Determination of the presence/absence of a prohibited substance in an anti-doping sample is performed using a two-step workflow as depicted in Fig. 2, including an Initial Testing Procedure (ITP) followed by a confirmation procedure if applicable. Collected samples for anti-doping are divided in two tamper-evident containers, namely A- and B-samples. Upon reception at the anti-doping laboratory, A-sample is opened and distributed in multiple aliquots for ITP, while B-container is directly stored (usually frozen). This bottle is only openend and analyzed in the case of an adverse analytical finding (AAF) in the A-sample and upon request of the athlete or his/her representative contesting the result of A-sample analysis.

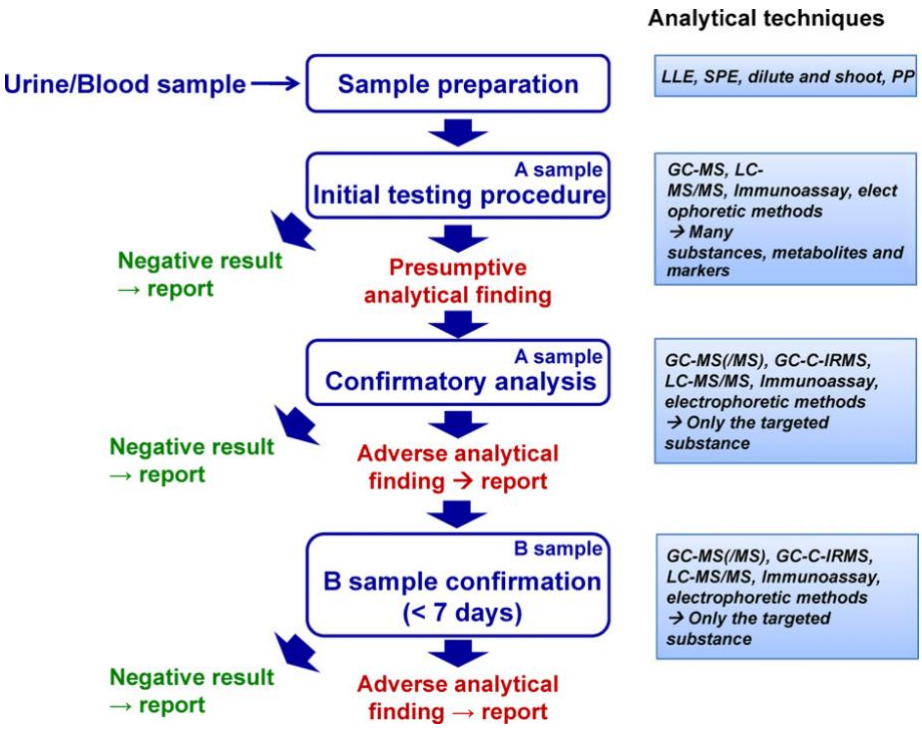


**Figure 2**. Workflow for anti-doping analyses [15]

The screening step (ITP), involving the detection of the highest number of relevant analytes, must be fast to handle the flux of samples, large, selective and sensitive to avoid false-positive and false-negative results [15]. In situation of a suspect result, a second and confirmatory analysis is performed on A-sample. This procedure is specifically dedicated for the detection of the suspected substance and may require a quantitative estimation with sufficient precision and accuracy. The same confirmatory procedure will be performed in case of B-sample analysis.

As illustrated in Table 1, a wide range of chemical structures is covered by the Prohibited List, which necessitates the use of analytical strategies with fast turnaround, high specificity and sensitivity [16]. These compounds have very different physico-chemical properties, hence several methods are required in parallel to cover all the different categories and ensure the quality of the analytical results [16]. Furthermore, the inclusion of characteristic drug metabolites may extend the window of detection and provide an additional evidence for unambiguous identification of the prohibited substance. These analytical techniques mainly include chemical (separation techniques) and biological (immunological tests) analyses. Proteins and peptides are principally detected using biochemical and immunological assays, while small molecules are usually separated and analyzed using chromatography-mass spectrometry (MS) based methods (GC-MS or liquid chromatography (LC)-MS). For the latter, specific identification criteria, including chromatographic and mass spectrometric identification criteria, shall be followed by Laboratories for confirmation procedure [17]. Furthermore, reference material (positive control) is required for a parallel analysis, which should match for both the chromatographic and MS elements of the analysis. Along with the various physicochemical properties of the doping agents, multiple sample preparations are employed to achieve sufficient level of sensitivity and selectivity, such as liquid-liquid extraction (LLE), solid phase extraction (SPE) or protein precipitation (PP). Owing to the high sensitivity and selectivity of the latest generation of MS detectors, generic sample preparation using non-selective *dilute-and-shoot* (DS) allows screening for a high number of prohibited substances for which expected urinary concentrations are relatively high [16]. Finally, to ensure homogenous results among WADA-accredited laboratories, minimal requirement performance levels (MRPL) have been established by WADA for non-threshold substances [18]. Initially, these capacities have been mainly defined for substances analyzed with chromatography-MS based methods, but MRPL have recently been constituted for the immunological detection of Erythropoietin-Receptor Agonists (ERAs) [19]. Threshold compounds are covered by another technical document dedicated to decision limits of threshold substances [20].

### 1.1.4 Biological matrices

Historically, urine was the preferred biological specimen used for anti-doping analysis. This matrix acts as a waste bin of the organism in which exogenous substances are accumulated before excretion. At that time, the anti-doping strategy principally relied on the ability to test this fluid as urine collection had no associated legal constraints and was not considered as a medical act [21]. Urine collection presents the advantage of being non-invasive with a large volume available for analysis. To facilitate its excretion, xenobiotic biotransformation through phase I and II reactions is necessary to convert the drug into more hydrophilic products. This phenomenon is an asset for anti-doping analysis since drug metabolites, which can be detected in urine for a longer time than the parent compound after the last administration, may provide long-term information of the use of a prohibited substance (long-term metabolites) [22–25].

Despite these benefits, the use of urine does not allow for an accurate estimation of the dose-response relationship. This may indeed lead to difficulties of interpretation for substances that are prohibited only INC such as stimulants and recreational drugs for determining the time of initial exposure [21]. Moreover, the risk of manipulation of this matrix is relatively elevated and consequent time of collection imposes organizational constraints to athletes especially directly after certain competitions [26]. To circumvent the large detection windows generated with long-term metabolites, doping practices evolved towards the administration of low doses of pseudo-endogenous substances such as testosterone.

In opposition, blood matrix is a much better option to obtain the dose-effect response of a substance and to get a better biological signature of doping [27]. This biological specimen would definitely be the most useful for INC-testing to evaluate the period during which the substance was most likely to have been administered [21]. Furthermore, the collection of this biological fluid would be much faster at the end of a competition compared to urine sampling. Besides, large scale blood testing allows evaluating the prevalence of doping similarly to epidemiological studies [28,29]. The blood compartment is also a more suitable matrix for the investigation of the biological signature of a doping substance through holistic approaches such as metabolomics in order to find new doping biomarkers. Initially, blood samples were used for health tests associated with so-called ‘no-start rule’ by few sport federations before being used as an accepted matrix for anti-doping [30]. Nowadays, blood specimen is used for detection of some peptide hormones in serum such as growth hormone (GH) or continuous erythropoietin receptor activator (CERA) as well as indirect detection through the monitoring of hematological parameters using whole blood. However, blood matrix is not free from some constraints. In addition to its invasiveness, the collection of blood sample is associated with strict and

cumbersome pre-analytical conditions that need to be followed implying important expensive logistical organization to ensure the validity of the sample.

Dried blood spots (DBS) have been proposed as alternative and complementary method for blood sampling. This principle is quite simple and requires the collection of a few drops of capillary blood by pricking the finger or the heel and its deposit on an adapted filter paper. DBS have been used for neonatal screening for decades [31], but it is only in recent years that they have been considered for anti-doping purposes. This process benefits from minimal invasiveness, simplicity of sample collection, chemical stability and facilitated transport with reduced costs. Moreover, DBS requires minimal sample preparation and the resulting extracts can be directly analyzed using LC-MS. It could constitute a definitive asset for INC-testing as described above, and it could allow for more frequent sampling with limited constraints. The collection of multiple spots could also enable various analyses for either direct detection of prohibited substances or indirect detection through the monitoring of doping biomarkers.

While this methodology has already been used in cyclist competitions for the detection of tramadol under the aegis of a medical rule [32], WADA recently launched an extensive collaboration between the different stakeholders for the development and implementation of DBS testing in a near future [33].

## 1.2 Blood manipulation

### 1.2.1 Physiology of erythropoiesis

Erythropoiesis, the process corresponding to the production of red blood cells (RBCs), is a well-controlled system involving several pathways and proteins. Among them, human EPO (HuEPO) is the main actor. This glycoprotein is mainly produced by peritubular fibroblasts in the kidney and to a much lesser extent in perisinusoidal cells in the liver. EPO synthesis is regulated at the transcriptional level and *EPO* expression is sensitive to tissue $O_2$ pressure [34]. Under normoxia condition, the *EPO* promoter is inhibited by GATA-2 and nuclear factor κB [35]. In addition, the hypoxia-inducible factor (HIF) prolyl hydroxylases (PH) hydroxylates the prolyl residues in the C-terminus of $O_2$-labile HIF-α subunits, which consequently undergoes proteasomal degradation (Fig. 3) [36]. When the level of $O_2$ drops (hypoxia), the oxygen-dependent activity of HIF-PH decreases, stabilizing HIF-α subunits which can translocate into the nucleus and dimerize with HIF-1β. This HIF heterodimers activates the *EPO* enhancer by binding to its hypoxia response element (HRE) in cooperation with co-activator p300 subsequently leading to *EPO* transcription. The resulting EPO molecule is a 165 amino acids proteins containing three N-linked and one O-linked glycans [36,37]. The N-glycans are

particularly important for the biological activity because it mediates the EPO molecular stability, the affinity to the receptor, and thus its serum half-life [38].

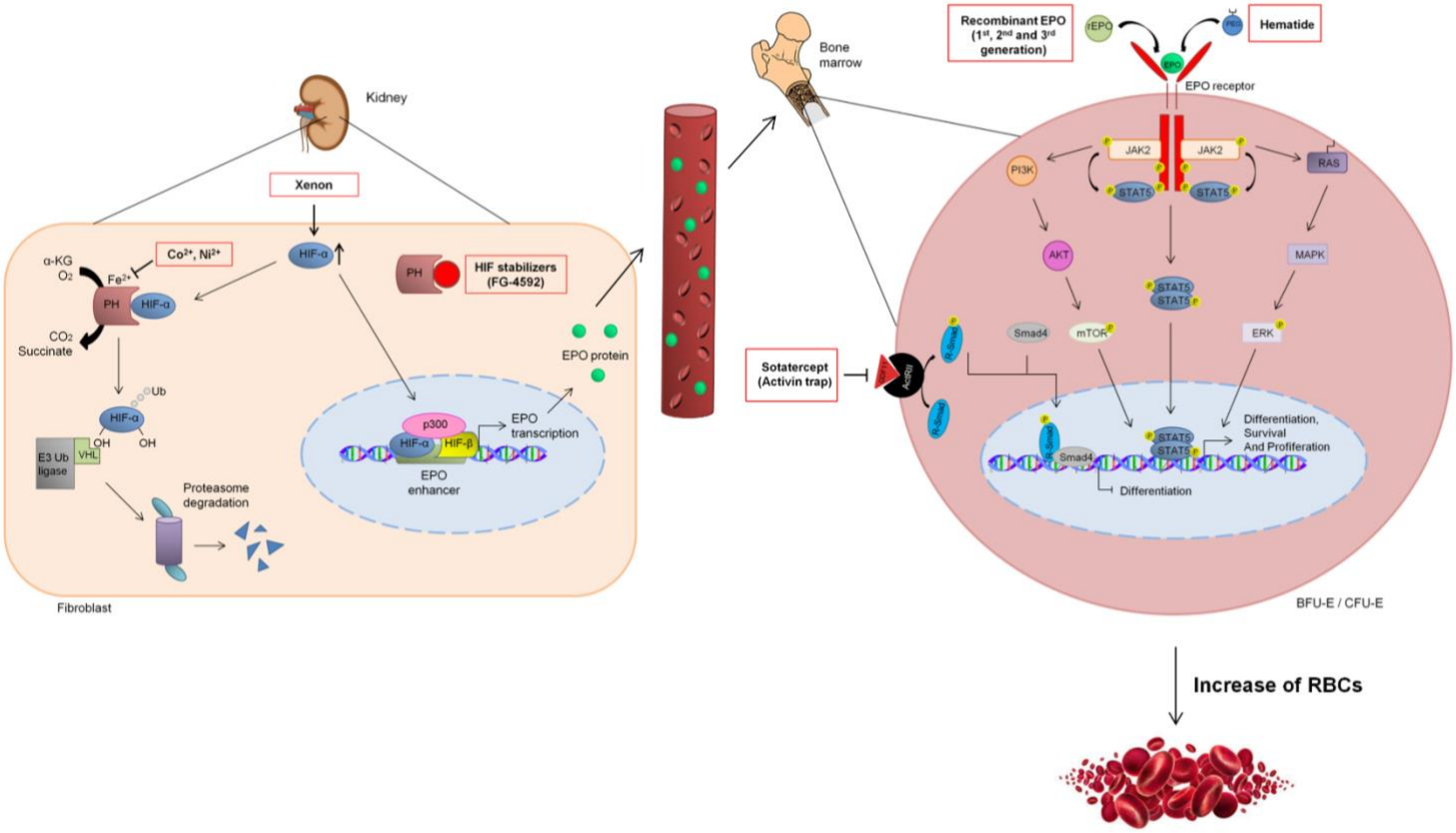


**Figure 3.** Schematic model of the control of EPO synthesis and consequent red blood cell production. The sites of action of erythropoiesis-stimulating agents are also highlighted [37]

After circulating through the blood system, EPO enters the bone marrow and binds to its receptor (EPO-R) abundantly expressed at the surface of colony-forming units-erythroid (CFU-Es) (Fig. 3). EPO binding induces the activation of EPO-R associated Janus kinase 2 (JAK2), which further induces intracellular signaling pathways involving different kinases and the signal transducer and activator of transcription 5 (STAT5) [34]. This signaling ultimately leads to the survival, proliferation and differentiation of erythroblasts into reticulocytes (RET) that enter circulation. This pathway is a slow acting process and generally takes 3-4 days before the increase of RET. These immature cells, still containing functional residual nucleic acid material [39], mature within 1-2 days to erythrocytes, whose lifespan is approximately 120 days, and are therefore a good indicator of bone marrow activity. Consecutive to RET increase, the concentration of the main blood oxygen carrier, hemoglobin (HGB), will also increase to restore level of circulating $O_2$.

Erythropoiesis is regulated by a feedback mechanism measuring blood oxygenation and iron availability to ensure that the production of RBCs is equal to their destruction and to ensure homeostasis in oxygen delivery to body tissues [40]. In addition, *HIF-PH* are also HIF-target genes which can explain the decline of EPO during prolonged exposure to hypoxia [34].

Erythropoiesis and iron metabolism are two processes tightly linked. Indeed, iron is essential for the synthesis of hemoglobin which is vital for the delivery and storage of oxygen [41]. Approximately 20 mg of iron is daily required by the bone marrow to maintain an efficient erythropoiesis [42]. The basal systemic iron level is mainly maintained by the recycling of iron from senescent erythrocytes with a low contribution from dietary absorption (1-2 mg/day), mainly to compensate for the slight iron losses caused by cell desquamation and minor blood loss [42].

Hepcidin is the main regulator of iron homeostasis and availability for erythropoiesis. This hormone is a 25-amino acid peptide produced by hepatocytes [43]. Hepcidin acts by binding to the iron exporter (ferroportin) expressed on duodenal enterocytes, hepatocytes and macrophages causing its internalization and degradation, which consequently decreases the iron availability for HGB synthesis [44]. The synthesis and secretion of hepcidin by the liver is regulated by many factors such as iron stores, inflammation, hypoxia and erythropoiesis [45]. The erythroid hormone erythroferrone (ERFE), which is produced by erythroblasts in response to EPO, is the erythroid regulator and mediates the suppression of hepcidin expression resulting in an increased iron availability for HGB synthesis (Fig. 4) [46]. This factor is thus the direct connection between iron metabolism and erythropoiesis.

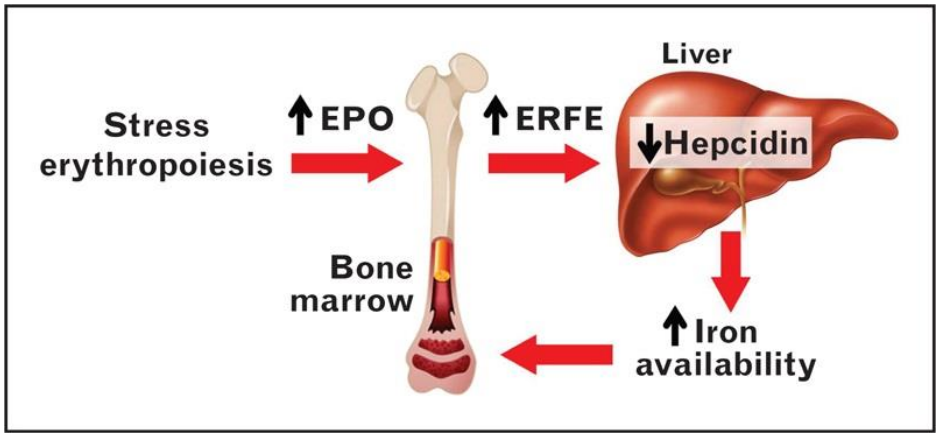


**Figure 4. Erythropoietic regulation of iron homeostasis**. In case of stress erythropoiesis (e.g. blood loss), increased EPO levels induce an increase of ERFE production by erythroblasts in the bone marrow. ERFE then suppresses hepcidin expression in hepatocytes, resulting in an increased iron availability for HGB synthesis [41].

### 1.2.2 Blood doping

The concept that the oxygen carrying capacity of exercising muscles is a limiting factor for aerobic performance has been well recognized for decades. For many years, the improvement of the oxygen-carrying capacity of the blood was one of the prime targets for some athletes [47]. Therefore, besides altitude training, they started to experiment blood transfusions already in the 1970s to enhance their endurance.

A blood transfusion process consists of three distinct phases: first the withdrawal of approximately 450 mL of blood (one bag), storage as RBC concentrates, and finally the reinfusion or transfusion of the stored blood. A blood transfusion is labelled *homologous* (HBT) if the reinfused blood originates from another individual with a matched blood group, while it is denoted *autologous* blood transfusion (ABT) if the reinfused RBCs originate from a previous blood donation from the same individual. Withdrawal of some units of blood unsurprisingly decreases RBC mass and HGB concentration within few days. The attenuated HGB level, and thus oxygen transport capacity, consequently stimulates the erythropoietic activity with increase of EPO concentration and RET percentage (RET%) within one day and one week respectively [47]. When the blood is reinfused, it causes an increase of RBC mass and HGB concentration [48]. Following a sufficient recovery period for regeneration to pre-withdrawal levels, the reinfusion generates an increase of HGB above baseline values inducing a negative feedback on EPO production and RET [49]. The increase of RBC mass consecutive to blood transfusion ultimately leads to an improvement of physical performance even with a low-volume transfusion [50,51]. Blood withdrawal is usually performed during off- or preparation-phase to limit the impairment on performance, while blood reinfusion generally occurs few days before a competition or during a multiple-days event (e.g. stage races).

The misuse of blood transfusion requires a sophisticated methodology and carries the risk of adverse effects. When recombinant human erythropoietin (rHuEPO) was commercially marketed between 1987 and 1989 for the treatment of anemic patients with chronic kidney disease (CKD), athletes quickly started to resort to this product due to its easy access and significant impact on performance. Furthermore, although rHuEPO was readily placed on the prohibited list, no validated detection was available until 2000, which caused its widespread abuse among athletes. In 1985, the first production of rHuEPO by transfection of mammalian cell lines (Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells) was possible following the isolation and cloning of human *EPO* gene [52]. The use of different cell lines for the production of rHuEPO results in differences in glycosylation and sulfation pattern of the protein, producing variations in the biochemical and biological properties of the different

epoetins [53]. Commercially produced epoetins are followed by a Greek letter depending on the composition and nature of the glycosylation pattern (α, β, δ, ω, κ). First-generation rHuEPOs are characterized by short half-life, thus requiring multiple injections to increase erythropoiesis. To improve the limited half-life and reduce the frequency of troublesome administrations, a second-generation rHuEPO was pharmaceutically developed under the name of novel erythropoiesis-stimulating protein (NESP) or darbepoetin alfa by mutating five amino acids in the protein backbone of EPO [54]. It resulted in additional sialic acid residues, increasing the half-life by 3-fold and thus lowering the dosing intervals [55]. The first collaboration between a pharmaceutical industry (Amgen) and anti-doping laboratories arose from the marketing of NESP. Few years later, the third-generation rHuEPO continuous erythropoietin activator (CERA) was marketed. This formulation consists in an epoetin β coupled to methoxy polyethylene glycol (PEG) moiety resulting in an increased molecular size that hinders its glomerular filtration and increasing its serum half-life [56]. Before the introduction of the drug into the market, Roche developed a CERA-specific ELISA and provided it to WADA [57].
The benefic effect of rHuEPO on performance was rapidly recognized after its introduction in the market explaining its almost generalized used in the 1990s [58]. Similarly to endogenous EPO, administration of rHuEPO has a rapid impact on RET increasing their production within few days. The effect on HGB concentration takes more time and depends on iron stores available for HGB synthesis. Indeed, iron supplementation is often necessary to potentiate rHuEPO effect. The cessation of a long-term use of rHuEPO generates a suppression of RET production by the negative feedback mentioned previously. This doping substance is usually misused in the preparation phase to increase the RBC mass and consequently $VO_{2max}$ just before competition [59,60]. The administration of rHuEPO low doses can also be used to stabilize RET fluctuations caused by ABT as highlighted by "*Operation Aderlass*" [61]. Besides rHuEPO, other substances may serve as erythropoiesis-stimulating agents (ESAs) such as EPO-mimetic peptides, HIF stabilizers, xenon, cobalt or activin traps [37]. Their mechanisms of action are described in Fig. 3.

### 1.2.3 Direct detection

Homologous blood transfusion is directly and easily detected using flow cytometry which identifies variations in blood group antigens [62,63]. On the contrary, there is currently no approved direct detection method for the autologous variant of transfusion.
The first method to detect rHuEPO in biological samples was developed based on isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) in the early 2000s [64]. This

technique exploits the dissimilar isoelectric point (pI) between HuEPO and rHuEPO due to differences in carbohydrate composition. The detection capacity was then improved with the development of an approach based on sodium dodecyl sulfate (SDS)-PAGE using distinction by the molecular weight [65]. Both methods allowed detecting first- and second-generation rHuEPO in urine but this approach was limited for CERA due to its non-filtration by the kidneys and non-excretion in urine. Therefore, CERA detection needed to be performed with serum samples. To optimize protein separation using electrophoresis, SDS was replaced with sarcosyl (SAR) which is only binding to the protein-part of CERA and not to its PEG-chain like SDS [66]. The screening and confirmation for ERAs in urine and blood can be performed either by IEF-PAGE and/or SAR-PAGE or SDS-PAGE in accordance with the technical document for the analysis of ERAs [67]. The only exception is that the confirmation procedure of first-generation rHuEPO can only be carried out with SAR- or SDS-PAGE. The HBT and rHuEPO tests are not included into the ITP of anti-doping laboratories and are conducted upon specific requests from the testing authority and/or WADA. It is to be noted that HBT tests are less and less conducted with a decrease of 80% in the number of tests conducted between 2015 (771) and 2019 (163), and with only few laboratories accredited for this specific analysis. Between 2013 and 2017, no AAFs was reported for HBT, while in 2018 and 2019, three and two AAFs were reported respectively. For the detection of other ESAs such as HIF stabilizers, LC-MS based methods included in ITP are performed [68].

## 1.3 Endogenous anabolic androgenic steroids

### 1.3.1 Androgen biosynthesis and action

Androgens, which may also be defined as endogenous anabolic androgenic steroids (EAAS), are naturally occurring steroids with a core structure of 19 carbon atoms that are synthesized from cholesterol. They play a major role in metabolic homeostasis and in reproductive health in men and women, and their signaling through the activation of androgen receptor is mainly mediated by the active androgens testosterone (T) and 5α-dihydrotestosterone (DHT).

*De novo* androgen biosynthesis only occurs in the cortex of the adrenal gland and in the gonads, which contain specific steroidogenic enzymes such as the cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) [69]. Adrenal androgen biosynthesis occurs in the *zona reticularis* via the $\Delta^5$ classical pathway from pregnenolone (PREG) to dehydroepiandrosterone (DHEA) (Fig. 5). The majority of resulting DHEA is sulfated by the major DHEA sulfotransferase (SULT2A1) abundantly expressed in the *zona reticularis*, resulting in high level of circulating DHEAS (low micromolar concentration) [70,71]. Due to the low expression

of *HSD3B2* and aldo-keto reductase family 1 member C3 (*AKR1C3)* in the adrenal, only small amounts of DHEA are further converted to androstenedione (A4) and T [72]. Therefore, the adrenal gland secretes mainly androgen precursors and only very low amounts of active androgens.

In the Leydig cells of the testes, androgen biosynthesis also follows the $\Delta^5$ classical pathway. Nevertheless, due to the large presence of HSD3B2 and 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) enzymes, the subsequent biosynthesis continues from DHEA to A4 and T [71,72]. The $\Delta^4$ pathway also contributes in a minor way to the production of T by the testes [73]. The steroid output from the testes is thus predominantly the active androgen T with low levels of precursors A4 and DHEA [71].

In the ovaries, the steroid biosynthesis is distinct between two cell types, namely granulosa and theca cells, due to cell-specific enzyme expression. In granulosa cells, the *de novo* biosynthesis stops at the level of progesterone (PROG) and PREG due to absence of *CYP17A1* expression. These precursors diffuse to theca cells, which express *CYP17A1* and *HSD3B2*, and serve as substrates for the synthesis of A4 that can be secreted or converted into T by AKR1C3 [72]. Nonetheless, the majority of A4 diffuses back to granulosa cells in which it is converted to estrone (E1), estradiol (E2) and E1-sulfate (E1S) [74]. In addition to this *de novo* biosynthesis, ovaries are also able to use DHEA of adrenal origin for the production of androgens and estrogens [72,75]. With the exception of testicular T production, the majority of $C_{19}$ steroids produced by the adrenal and the ovaries are inactive androgen precursors.

The peripheral tissue activation of precursors, also known as “steroid intracrinology” [76,77], represents a significant contribution to the pool of effective androgens, especially in women. The activated androgens are subsequently inactivated prior to being released from the cell for excretion [72]. In peripheral tissues, circulating T can be further activated to a more potent androgen DHT by 5α-reductase type 2 (SRD5A2). DHT biosynthesis can also occur via the alternate 5α-androstanedione (5α-dione) pathway that bypasses T as intermediate (Fig. 5) [72]. This pathway plays a major role for peripheral DHT generation from circulating precursors other than T [72]. DHT can also be produced by a so-called “backdoor” pathway that by-passes T, A4 and DHEA [78]. In this pathway, 17α-hydroxyprogesterone (17OHPROG) and PROG are subsequently 5α-reduced by SRD5A1 and 3α-reduced by AKR1C. Allopregnanolone (alloPreg) and subsequent 17α-hydroxyallopregnanolone (5α17HP) yields to androsterone (AST) through the 17,20-lyase activity of CYP17A1. Androsterone, which is an inactive metabolite of DHT under normal circumstances, can be reactivated by the sequential 17β-reduction and 3α-oxidase reactions [71]. This pathway plays an important role in human

hyperandrogenic disorders, such as congenital adrenal hyperplasia (CAH) in which 17OHPROG accumulates [79]. Finally, 11-oxygenated androgens such as 11-ketotestosterone (11KT) contribute to the pool of active androgens. They are the results of the peripheral activation of 11-oxygenated $C_{19}$ precursors originating from adrenal, and 11KT binds and activates the human androgen receptor with a similar affinity and potency than T [80,81]. It was demonstrated for example that 11-oxygenated androgens were the predominant $C_{19}$ steroids in women with polycystic ovary syndrome (PCOS) [82].

The daily production of T is of approximately 7 mg per day in men and around 0.1-0.4 mg/day in females. The serum T concentration demonstrates a bimodal distribution among sex and ranges typically between 8.8-30.9 nmol/L in men and 0.4-2.0 nmol/L in women [83,84]. In men, 95% of T originates from the Leydig cells in the testes with a small contribution from the adrenal cortex [85]. In women, T is produced in small amounts by the ovaries and adrenal glands, while 50% is synthesized by intracrine conversion in peripheral tissues [72]. Circulating T is loosely bound mostly to albumin and to a lesser extent bound tightly to sex hormone-binding globulin (SHBG), with a very low fraction circulating in a free and biologically active form (1 to 2%). Fluctuations in SHBG level (rise or decrease) can therefore influence the androgen activity and have clinical consequences.

T is responsible for the development of male seconday sex characteristic (androgenic effect) as well as the increase of muscular mass (lean body mass) and physical strength (anabolic effect). Precursor and active androgens reach the target tissue cell by passive diffusion thanks to their lipophilic characteristics. In the cytoplasm, active androgens (T, DHT or 11KT) exert their effects by binding to the androgen receptor (AR). AR is a nuclear receptor that upon activation translocates into the nucleus and binds to DNA acting as a transcription factor regulating multiple genes expression [86]. Androgens may also have biological effects as antiestrogens by competing for the estrogen receptor [86]. Alternative non-genomic actions which are distinct from the classical nuclear AR have also been reported for androgen receptors [87]. For instance, AR can interact with some signal transduction proteins in the cytoplasm, rapidly altering cell signaling or ion transport [88]. These reactions are rapid, in seconds or minutes, while genomic actions take several hours to days to occur.

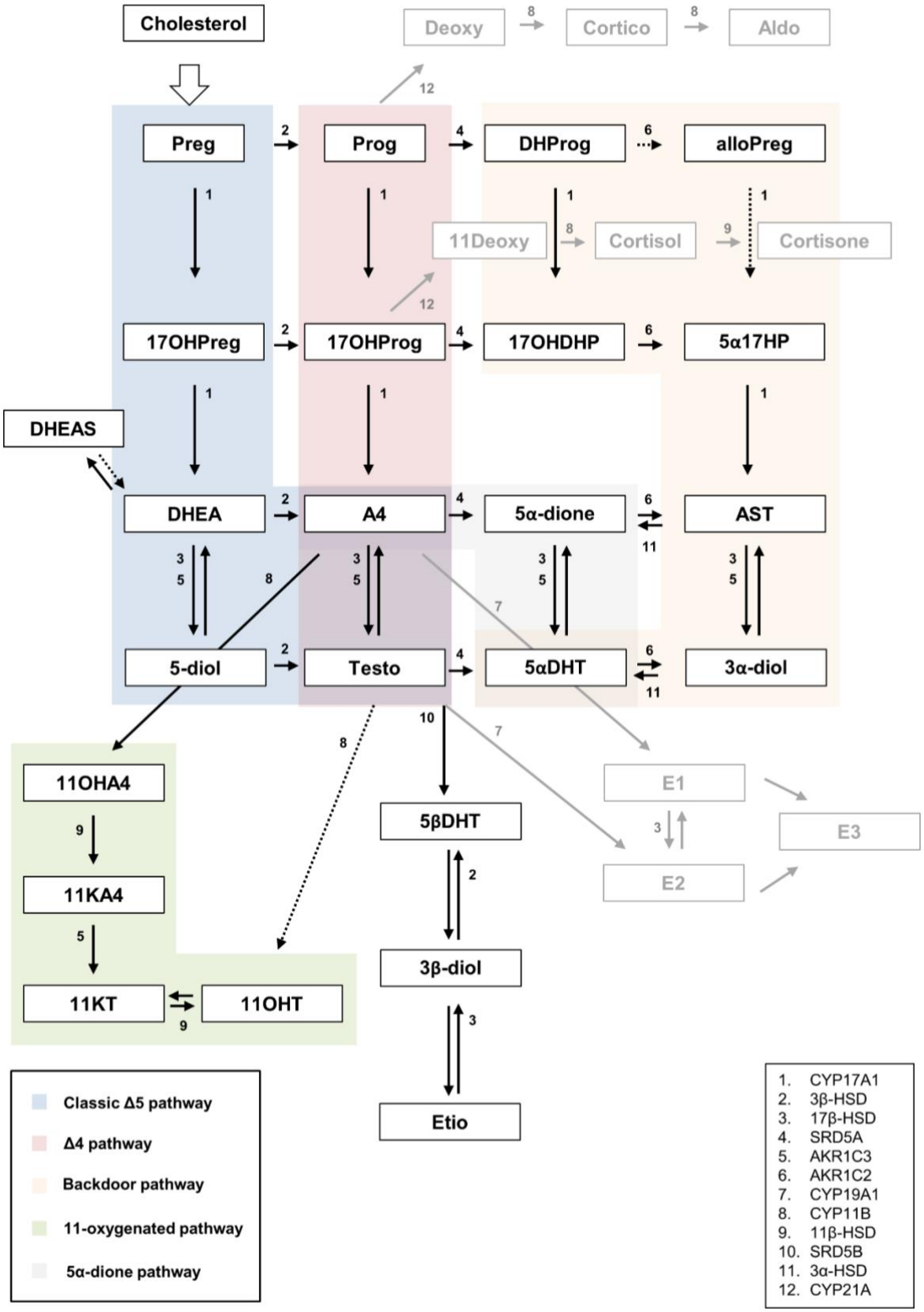


**Figure 5.** Schematic overview of androgen biosynthesis.

### 1.3.2 Androgen regulation and excretion

The gonadal steroidogenesis is regulated by the hypothalamic-pituitary-gonadal (HPG) axis initiated at puberty. The normal functioning of the HPG is essential for the maintenance of reproductive function [89]. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), a 10-amino acid neuropeptide, in a pulsatile manner to reach the pituitary which secretes gonadotropins, namely the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). These gonadotropins in turn stimulates the gonads (testes in males and ovaries in females) to produce androgens (testes and theca cells by LH) and estrogens (granulosa cells by FSH). The sex hormones (androgens in men and estrogens and progesterone in women) provide a negative feedback at the hypothalamus and pituitary level to decrease the release of GnRH and/or LH. The production of adrenal androgens is on the other side regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Briefly, acute stress exposure simulates the paraventricular nucleus of hypothalamus to secrete corticotropin releasing factor (CRF) which in turn causes the release of pituitary adrenocorticotropic hormone (ACTH) [90]. ACTH subsequently stimulates the biosynthesis and release of glucocorticoids and androgen precursors. Glucocorticoids (cortisol) successively reduces the secretion of CRF and ACTH by a negative feedback. On the contrary, adrenal androgen precursors do not contribute to the feedback inhibition of the HPA axis [71].

As indicated above, steroids are inherently lipophilic compounds which need to be transformed into more polar compounds to be excreted in urine. In addition to phase I reactions that transform precursors into active and inactive compounds with the introduction of new functional groups to the steroid substrate, phase II metabolism is required to transform steroids into inactive and polar compounds through conjugation reactions. Phase I and phase II reactions are usually sequential but some steroids can also undergo phase II metabolism without being phase I transformed during androgen inactivation [71].

The biotransformation of androgens into conjugated metabolites is of high importance, as the excretion of unconjugated steroids is low, accounting for less than 3% of the total EAAS urine pool [91]. Glucuronidation corresponding to the addition of a glucuronic acid catalyzed by Uridine 5`-diphospho-glucuronosyltransferases (UGTs) is the main conjugation reaction of androgens in human (Fig.6) [92]. The second main phase II reaction is the sulphoconjugation mediated by sulfotransferase enzymes (SULTs) with the transfer of a sulpho-moiety from the co-substrate 3`-phosphoadenosine-5`-phosphosulfate (PAPS) to the substrate. While glucuronidation is irreversible and ultimately inactivates the androgen, sulfated steroid hormones can actively be imported into specific target cells via uptake carriers and be

interconverted by steroid sulfatase to contribute to the overall regulation of steroid responsive organs [93]. Polymorphism altering the expression of UGT and/or SULT enzymes can contribute to large inter-individual variation observed in the concentration of steroids in urine [94].

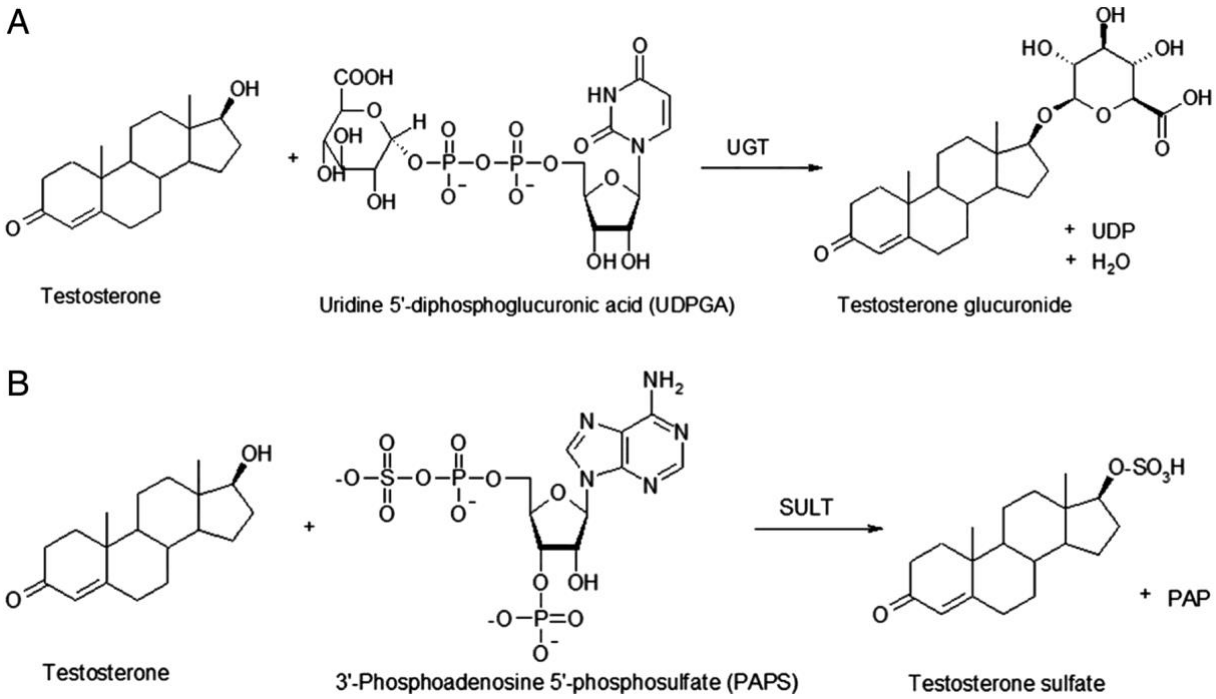


**Figure 6.** (A) Glucuronidation and (B) sulfoconjugation of testosterone [92].

### 1.3.3 Testosterone doping

T and its synthetic derivatives (AAS) are available in various formulations and dosages on the pharmaceutical market as well as on the black market. Exogenous T formulation was initially intended for the testosterone replacement therapy of classical hypogonadism and was further extended to other non-endocrine diseases such as anemia, muscular dystrophy or dermatological diseases [95]. Right from its isolation and synthesis, T was quickly used for performance enhancement purposes due to its anabolic effects [96]. Exogenous T can be administered via various available preparations: oral, parenteral (intra-muscular injections), transdermal (gel/cream or patch), subdermal (implants), buccal (tablets) or nasal (Table 2). Oral and parenteral administration of natural T does not exert significant effects in the human body due to rapid metabolism by the liver (first-pass effect) [97]. Therefore, the addition of esters at the 17β-position (esterification) increased the half-life and the action of both oral use and intra-muscular injections [96]. In the anti-doping context, synthetic AAS are readily detected in urine since their metabolites are not normally present while testosterone formulations are much more

difficult to detect due to the necessity to distinguish between the exogenous and endogenous origin. There is inevitably only sparse information on doses and modes of administration of EAAS used by athletes [98,99]. While exogenous T is particularly effective in strength sports promoting muscle mass and with effects on lean body mass in a dose-dependent manner [100], its use in endurance sports may also improve recovery. Indeed, low T doses can compensate the lower amount of endogenous T induced by overtraining [101], and can increase glycogen synthase activity to refill the glycogen store in muscles [102]. The administration of EAAS has also the potential to rapidly increase aggressiveness and motivation, which can be beneficial for increased competitive drive. While this effect is subsequent to recent administration, athletes rather use EAAS in pre-competition phases to exploit the persistence of EAAS action [103]. Oral and transdermal T administrations have a short detection window and are therefore preferred by athletes over parenteral administration, which can be detected for a longer time.

| T preparation | T formulation type | Dosing Scheme |
|---|---|---|
| Testosterone undecanoate | Oral | 2-4 capsules at 40 mg/day |
| Testosterone tablets | Buccal | 30 mg twice a day |
| Testosterone enanthate | Intramuscular injection | 200-250 mg every 2-3 weeks |
| Testosterone cypionate | Intramuscular injection | 200 mg every 2 weeks |
| Testosterone undecanoate | Intramuscular injection | 1000 mg every 10-14 weeks |
| Testosterone implants | Implantation under the abdominal skin | 4 implants at 200 mg every 5-6 months |
| Transdermal testosterone patch | Non-scrotal skin | 2 systems per day or every second day depending on preparation |
| Transdermal testosterone gel (1-2.5%) | Non-scrotal skin | Starting dose 40-62.5 mg/day depending on preparation |
| Testosterone solution (2%) | Axillary | Starting dose 60 mg/day |

**Table 2**. Mode of application and dosage of various testosterone preparations for clinical use. Adapted from [104].

In healthy men, the exogenous administration of T induces a negative feedback for the endogenous production of androgens through the HPG axis as described in the previous chapter. It is therefore common for T misuse cure to be followed by the administration of human chorionic gonadotropin (hCG) hormone, also prohibited in sport, to stimulate endogenous

production of T. Furthermore, T and AAS administration is often combined with aromatase inhibitor (also included in the Prohibited list) in men to avoid side effects such as gynecomastia caused by increased conversion of testosterone to estradiol. Females are particularly more sensitive to testosterone than males [105] and hence, due to the existing low basal concentration, an increase in the concentration of T is proposed to lead to a significant benefit in performance. Notably, a study has recently demonstrated that a moderate increase of testosterone levels in young women, following a 10-weeks treatment with 10 mg transdermal testosterone, improved significantly physical performance, and increased total lean mass, type II fibre size and capillarization [106,107].

### 1.3.4 Detection of testosterone

As previously highlighted, to detect the misuse of EAAS, doping control laboratories require methods that allow differentiation between endogenous steroids and their synthetic copies. Historically, a marker of its misuse composed by the ratio of urinary glucuroconjugated testosterone to epitestosterone (T/E) measured with GC-MS was proposed in the early 1980s [108]. Epitestosterone (E) is an isomer of T whose metabolism is believed to be independent although the exact paths of biosynthesis are unknown [109]. E is mostly formed in the testes while another part of the production probably occurs in the adrenal gland [110]. The T/E marker is based on the fact that exogenous T administration increases this ratio due to intrinsic T increase but also by E decrease due to negative feedback, at least in men. Following its implementation, an initial threshold of 6 was set based on population studies and in 1992 a ratio exceeding this value was considered to reflect the administration of testosterone "*unless there is an evidence that this ratio is due to a physiological or pathological condition"* [98,111]. In 2004, this threshold was lowered to 4 by WADA. However, the use of population-based threshold had some limitation and the case of a top-level sprinter at the end of the 1990s, who, with the help of his expert endocrinologist, convinced his national federation that the increased T/E value of his positive urine sample was probably due to alleged physiological conditions, urged the anti-doping community for the need of a complementary tool offering a direct detection [111]. Consequently, the development of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) provided a confirmatory method to assist a more timely decision [112].

This method allows discriminating an exogenous T supply from its endogenous production by precisely measuring the variations of the isotopic abundance of $^{13}C$ and $^{12}C$. It relies on the evidence that pharmaceutically produced T, obtained from one plant material, demonstrates a

depleted carbon isotope ratio (CIR) in comparison to endogenous T synthesized from multiple sources of carbon [112]. Therefore, the diet change can have an impact of the CIR depending on the geographic situations [113,114] and the use of exogenous T with isotopic compositions similar to or even within the normal range may remain unnoticed [115]. In accordance with the WADA technical document (TD2019IRMS) [116], the isotopic composition of the target compounds (TCs) androsterone (A), etiocholanolone (Etio), 5α-androstane-3α, 17β-diol (5αAdiol), 5β-androstane-3α, 17β-diol (5βAdiol), T and E is compared with those of endogenous reference compounds (ERCs) known to be unaffected by EAAS administration. The most used ERCs are pregnanediol (PD), 5α-androst-16-en-3α-ol (16-en), 11β-hydroxyandrosterone or 11-keto-etiocholanolone. The natural abundance CIR of each analyte is expressed as $\delta^{13}C$ values in per mil (‰) against a virtual carbon isotopic international standard (Vienna Pee Dee Belemnite) [117]. The calculated $\delta^{13}C$ value of each TC is then subtracted from the $\delta^{13}C$ value of ERC and the $\Delta\delta^{13}C$ values of the different TCs are used as criteria to establish the positivity of a sample. The following criteria need to be fulfilled for positivity (Table 3) [116]:

| | |
|---|---|
| i. | The $\Delta\delta^{13}C$ value of the ERC-T pair and of one of the ERC-5αAdiol or ERC-5βAdiol pairs are both > 3 ‰ |
| ii. | The $\Delta\delta^{13}C$ values of the ERC-5αAdiol and ERC-5βAdiol pairs are both > 3 ‰ |
| iii. | The concentration of E is > 50 ng/mL in females or > 200 ng/mL in males (SG-adjusted, if needed) and the $\Delta\delta^{13}C$ value of the ERC-E pair is > 4 ‰ |
| iv. | The $\Delta\delta^{13}C$ value of the ERC-A pair is > 3 ‰ or the $\Delta\delta^{13}C$ value of the ERC-Etio pair is > 4 ‰ |
| v. | The $\Delta\delta^{13}C$ value of the ERC-A pair is between 2 ‰ - 3 ‰ or the $\Delta\delta^{13}C$ value of the ERC-Etio pair is between 3 ‰ - 4 ‰, and the $\Delta\delta^{13}C$ value of one of the ERC-5αAdiol or ERC-5βAdiol pairs is > 3 ‰ |
| vi. | The $\Delta\delta^{13}C$ value of the ERC-5αAdiol pair is > 4 ‰ and the $\delta^{13}C$ value of 5αAdiol is ≤ -27 ‰ (e.g. DHT administration); |
| vii. | The $\Delta\delta^{13}C$ value of either the ERC-formestane, ERC-boldenone or ERC-boldenone Metabolite(s) pairs is greater than 4 ‰ |

**Table 3**. GC/C/IRMS positivity criteria according to the WADA technical document.

A GC/C/IRMS result can also be reported as inconclusive (atypical finding; ATF) when: only one of the combined criteria specified in point i, ii, v or vi is met; due to technical limitations

(insufficient sample volume or very low concentrations of TCs or ERC(s) or presence of interfering compounds); if the laboratory estimates that the results are not consistent with the endogenous origin of the urinary metabolites although the criteria for positivity are not met. In addition to T, GC/C/IRMS can also be used for the detection of other EAAS such as E, DHEA, A4 or DHT [118–120].

Because the GC/C/IRMS analysis is a confirmatory procedure, it is not included in the ITP procedure in the same way as EPO and HBT methods, and therefore is not performed on all urine samples. Indeed, this analysis is time-consuming and expensive, requiring numerous purification steps to obtain clean extracts, and therefore cannot be applied to all samples. Originally, GC/C/IRMS was used as confirmatory analysis in case of T/E value above fixed thresholds based on population studies. However, athletes with naturally low T/E values never exceeded those thresholds even after EAAS administration. Furthermore, the intermittent and low-dose use of T gel did not generate significant T/E alterations. These limitations led the anti-doping community to shift from a population-based approach to individualized steroid profiling [121–123].

## 1.4 Athlete Biological Passport

As described previously, the classical principle of an anti-doping analysis relies on the direct detection of an exogenous substance in urine or blood sample of athletes. While this approach is efficient for synthetic substances (not produced by the organism), it is much more struggling when an endogenous molecule (naturally produced by the human organism) such as EPO or T is administered. Therefore, the implementation of the Athlete Biological Passport (ABP) introduced a paradigm shift in the fight against doping, namely the indirect detection based on individual monitoring of biomarkers indicative of doping [124]. The response of individual metabolism to performance-enhancing drug intake is investigated rather than the substance itself. The presumed advantage of this new approach is that the drug's effect on metabolism lasts longer than its detection in the urine or blood, and is independent from the commercialization of new pharmaceutical substances that may be diverted as performance-enhancer, which would necessitate adjustments of the analytical workflow for their direct detection.

While biomarkers have been used for decades for screening, diagnosis or monitoring of many disorders or for T detection (T/E ratio), the innovation of ABP relies on a longitudinal and personalized follow-up of specific markers using individual limits generated by a Bayesian adaptive model. In the model, individual limits are initially based on population reference

values and then adapted taking into account athlete's previous measurements for each selected parameter (the athlete becoming his/her own reference) and eliminating the inter-individual variability of the biomarker under consideration. Before the introduction of any observation into the model, the mean of the predictive distribution of expected values for the athlete is the population mean and its variance corresponds to the sum of the between-subject (BS) and within-subject variance (WS) [125]. The predictive distribution of expected values is then computed iteratively as a function of the athlete's test history assuming a normally distributed marker and a universal WS variance at a 99% specificity level (corresponding to 0.5 and 99.5 percentiles) using the iteration presented in Fig. 7 [125,126]. Assuming a universal WS, for each new points, the BS variance reduces and becomes individualized [126]. Therefore, the model requires a prior assessment of intra- and inter-individual variability of the marker under consideration.

$$
\begin{aligned}
&n = 0: \\
&X_1 = \mathrm{BS}_{\mathrm{variance}} \\
&X_2 = \mathrm{POP}_{\mathrm{mean}} \\
&\text{Predictive mean} = X_2 \\
&\text{Predictive variance} = X_1 + \mathrm{WS}_{\mathrm{variance}} \\
&N \rightarrow n+1: \\
&\quad A = X_1 \\
&\quad B = X_2 \\
&X_1 = \frac{1}{\frac{1}{A} + \frac{1}{WS_{\mathrm{var}}}} \\
&X_2 = \frac{X_1 \times B}{A} + \frac{X_1 \times RES\_N}{WS_{\mathrm{var}}} \\
&\text{Predictive mean} = X_2 \\
&\text{Predictive variance} = X_1 + \mathrm{WS}_{\mathrm{variance}}
\end{aligned}
$$

**Figure 7**. Model used for Bayesian inference iteration [125].

This approach is of particular interest when the intra-individual variability of the biological marker is lower than the corresponding inter-individual variability [126]. Abnormal deviation of a biomarker from athlete's own reference values (normality), resulting in atypical passport finding (ATPF), may therefore indicate either doping or a potential medical/pathological condition which would necessitate further investigation. Thus, the ABP can be used either to pursue an ADRV in accordance with Article 2.2 of the WADC or to identify athletes for specific target testing such as GC/C/IRMS or rHuEPO analysis through intelligent and timely interpretation of passport data [127]. Similar to a forensic approach, the ABP corresponds to a platform for the evaluation of multiple pieces of scientific evidence.

The ABP is organized in three distinct modules: the haematological module for the detection of any form of blood doping, the steroidal module for the detection of direct and indirect forms of doping with anabolic agents and the endocrinological module for the detection of doping with growth factors [124]. The concept of ABP was discussed and further elaborated for

antidoping application by WADA already in 2002 [124]. The hematological and steroidal modules were implemented in the network of WADA-accredited laboratories in 2008 and 2014 respectively, while the endocrinological module still requires further development.

For the harmonization of the ABP program between the different stakeholders, WADA published in 2009 the Athlete Biological Passport Operating Guidelines that are regularly updated [127]. These guidelines are associated with technical documents dedicated to the analysis of the markers used in each module.

### 1.4.1 The hematological module

The hematological module of the ABP was first implemented in 2008 by UCI followed by WADA one year later. The module aims to identify any form of blood doping, including ESAs use and blood transfusion (homologous and autologous) or manipulation either via target testing or directly based on atypical passport data. The following blood variables are considered within the hematological module: hematocrit (HCT), HGB concentration, RBC count, RET%, RET count (RET#), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW-SD), immature reticulocyte fraction (IRF), platelets (PLT) and white blood cells (WBC). In addition, two multiparametric markers are included into the module, namely the OFF-Score combining HGB and RET% (HGB (g/L)-60*RET%$^{½}$) and the Abnormal Profile Score (ABPS) combining seven markers (HCT, HGB, RBC, RET%, MCV, MCH and MCHC) [128]. The OFF-Score is particularly valuable for the identification of an off-phase, namely the period following multiple rHuEPO injections, or following a blood transfusion, characterized by suppressed RET% and an elevated HGB. Among these variables, the Bayesian model is applied on the longitudinal monitoring of HGB, RET%, OFF-Score and ABPS calculating upper and lower limits (red lines) at each additional sample (blue line) as presented in Fig. 8.

Among the four markers plotted longitudinally, ATPFs are generated only in case of abnormal values in the two primary markers, namely HGB and OFF-Score. Nevertheless, RET%, a secondary marker, is a key marker for the evaluation of hematological profile and is always taken into consideration in combination with HGB when a doping scenario is assessed [129,130]. On the contrary, the ABPS marker is not subject to thorough evaluation and is rather used as marker of sample degradation. To assist in the interpretation of the profile, additional information associated with each sample added into the ABP software such as hemodilution state, possible altitude exposure or use of altitude simulation and blood loss are available.

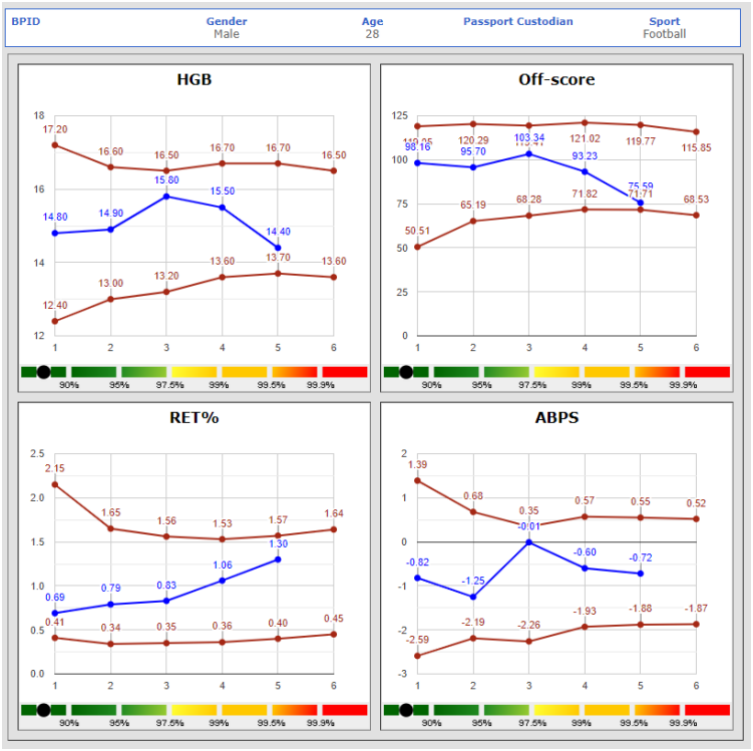


**Figure 8**. Hematological passport of a male athlete tested on 5 occasions.

The application of the hematological module relies on the analysis of whole blood samples. Consequently, the collection of this matrix is associated with standardized protocols [131] and stringent pre-analytical conditions that need to be respected to ensure the validity of the sample [132,133]. Furthermore, to prevent data dispersion of hematological results, all anti-doping laboratories use the same automated hematology analyzer for whole blood analysis in accordance with a technical document for analytical requirement (TD2021BAR), implying for example the use of external and internal controls, analysis in duplicate or homogenization of the sample for 15 minutes prior to analysis. [134,135].

### 1.4.2 The steroidal module

The steroidal module of ABP, which was first implemented on 1 January 2014, aims to identify EAAS when administered exogenously, in particular T, its precursors [such as androstenediol, androstenedione and DHEA)] or its active metabolite DHT, as well as its inactive epimer E, and other anabolic agents such as selective androgen receptor modulators (SARMs). It is composed by the urinary concentrations, measured by GC-MS(/MS), of free and glucuro-conjugated fractions of T, E, A, Etio, 5αAdiol and 5βAdiol. These concentrations are adjusted with the specific gravity of urine sample to eliminate the dilution effect and they are combined into five specific ratios, namely T/E, A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E, that are

longitudinally monitored (Fig. 9). Each ratio owns its proper characteristics and may provide crucial information for the evaluation of the complete steroid profile [136]. Moreover, these ratios are robust and do not change due to circadian rhythm, or physiological conditions [111,137]. The T/E ratio is the primary marker defined as the most specific to doping and the other four ratios are secondary markers that provide supporting evidence of doping in isolation or in combination with other markers [127].

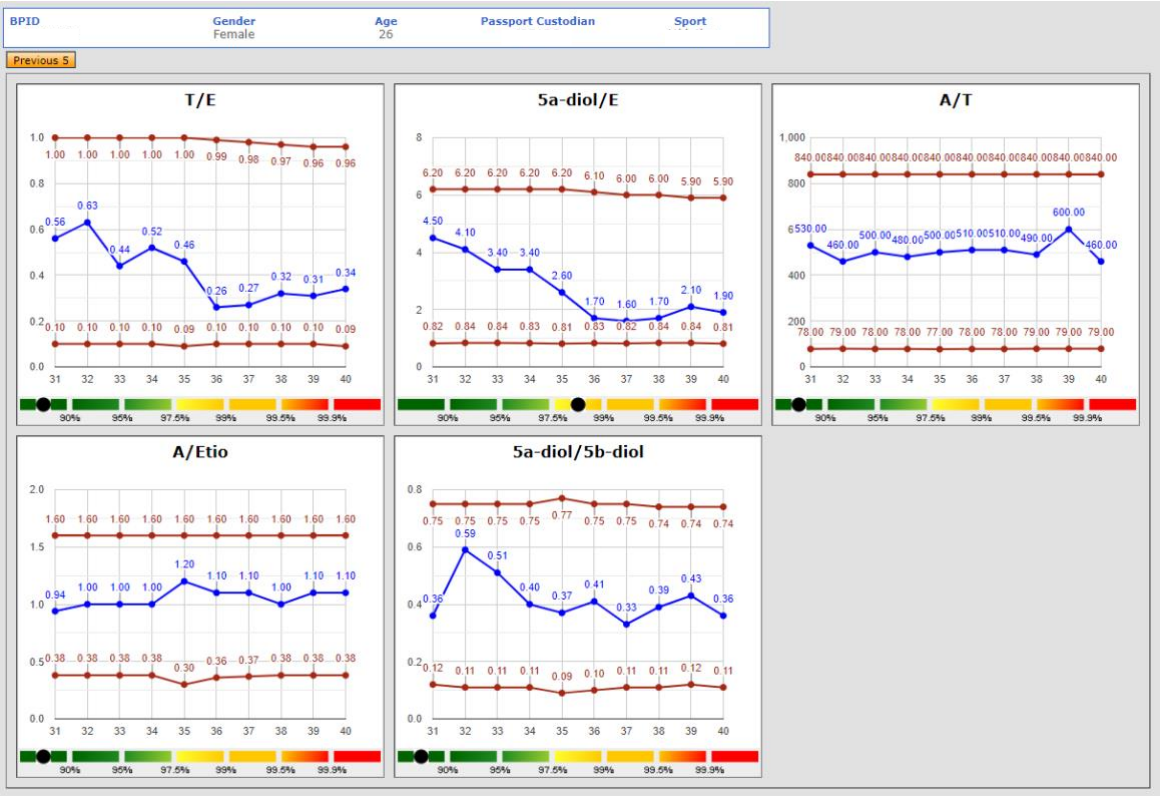


**Figure 9**. Urinary steroidal passport of a female athlete tested on 40 occasions.

Similar to whole blood analysis, to ensure data harmonization between the different anti-doping laboratories and avoid any bias in the ABP, the analysis of urine samples for the steroidal profile is performed according to a technical document for the analysis of EAAS (TD2021EAAS) [138]. Along with the analysis of the steroid profile, the presence of factors impacting the steroid profile such as microbial contamination, alcohol consumption, 5α-reductase inhibitors and ketoconazole has to be evaluated and taken into consideration for profile interpretation. However, contrary to whole blood analysis, a confirmatory procedure (CP) can be requested in case of abnormal value. An ATPF confirmation procedure request (ATPF-CPR) is automatically generated by an atypically high T/E value, while in case of abnormality in any of the other ratios, it is upon responsibility of the passport management unit to advise a CP. When

there is no existing steroidal passport for an athlete (i.e. first sample), a suspicious steroid profile confirmation procedure request (SSP-CPR) can be generated in case of T/E > 4, [T] or [E] (adjusted for SG) > 200 ng/mL in males or > 50 ng/mL in females, [A] or [Etio] > 10'000 ng/mL, [5αAdiol] > 250 ng/mL in males or > 150 ng/mL in females. If the abnormal T/E value is confirmed, a GC/C/IRMS analysis is automatically requested while for the other ratios, it depends on the recommendation given by the passport management unit. This module offers the asset that each time a urine sample is collected (much more often than blood), it is analyzed for the steroidal profile as the method is included in the ITP. In addition to the detection of EAAS, this tool also allows identifying possible urine sample tampering or swapping.

### 1.4.2 Management of individual profiles

The management and evaluation of the biological data in the ABP is under the responsibility of the Athlete Passport Management Unit (APMU). Since 2019, the WADA accredited anti-doping laboratories are defined as the host organizations for the APMUs [139]. The administrative and functional framework of the APMU is described in the WADA technical document TD2021APMU [140]. It is the duty of the APMU to perform an initial review of the passport to provide target testing recommendations, to assess the sample validity or to assure support to the Passport Custodian (e.g. national anti-doping organization, regional or international sport federation) to define specific analyses, test distribution plan and target testing. These tasks thus necessitate having expertise in anti-doping administration and management, being familiar with pre-analytical and analytical procedures, and possessing knowledge in physiology to ensure valuable recommendation for the Passport Custodian. [47].

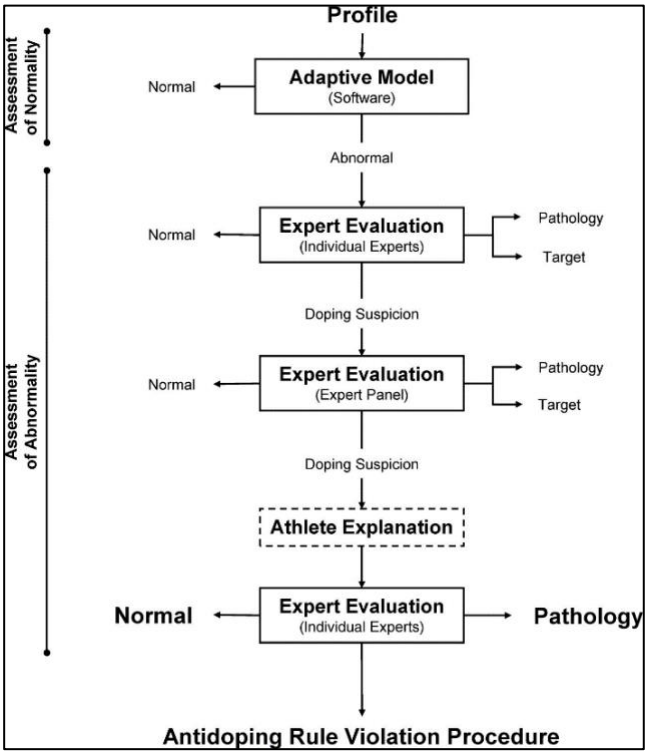


**Figure 10**. Evaluation process for data of ABP [141].

In case of an ATPF, abnormal value of a secondary marker, or atypical profile, the APMU is responsible to send the profile to an external expert for review and suggestions of further actions [137]. To assure anonymity and confidentiality during the whole procedure, the APMU is the link between the Passport Custodian and the external experts [137,139]. The expert evaluates whether the findings are indicative of doping or might originate from other causes and associates a specific recommendation along its profile interpretation (Fig. 10) [47,141]. Depending on the recommendation of the initial review, the APMU may undertake different actions (Table 4). In the situation of a "likely doping" evaluation, the passport is sent to two additional other experts for independent review (Fig. 10). A unanimous opinion among the expert panel is required to proceed further towards declaring an Adverse Passport Finding (APF) [127].

| **Expert evaluation** | **APMU action** |
|---|---|
| Normal | Continue normal Testing plan |
| Suspicious | Recommend target testing, sample analysis and/or request further information |
| Likely doping | Send to a panel of three experts including the initial expert |
| Likely medical condition | Inform the athlete as soon as possible via the Passport Custodian (or send to other experts) |

**Table 4**. Expert review and APMU actions.

### 1.4.3 Confounding factors

Since its implementation, the ABP has demonstrated a strong deterrent effect. Indeed, the number of atypical passports and extreme values have undoubtedly reduced [142] and the introduction of ABP was associated with a decrease of performance in certain disciplines [143,144]. It suggests that athletes resort to less efficient doping scheme with lower doses that are also less dangerous for their health and at less effective times. Nevertheless, it also suggests that athletes accustomed to this indirect detection method and adapted their doping regimen accordingly. Indeed, they returned to archaic undetectable methods such as autologous blood transfusions or resort to microdoses of rHuEPO or T gel that are hardly detectable using the modules of ABP [125,145]. The evaluation of an individual profile is a complex task demanding to assess that the fluctuations observed in the markers cannot be explained by other causes than doping. This complexity is further reinforced by the impact of various confounding

factors that have the potential to influence the values of the measured markers and consequently to lead to misinterpretation of ABP biomarkers variation.

In the context of the hematological module, concentration-based variables such as HGB are particularly prone to be affected by plasma volume (PV) variations. These changes may complicate the interpretation of a hematological profile and may hinder distinction between natural physiological variations and doping-induced changes in cell mass. In addition to variations caused by body position prior to venipuncture [146], HGB concentration can be influenced by the tourniquet time [147], diurnal or seasonal variations [148,149], saline injection [147], water intake or dehydration [150,151] and acute or chronic exercise [152–156] among others. Furthermore, hypoxia is another environmental condition widely used by athletes in their preparation phase which has the potential to confound the fingerprint of blood doping [157]. Indeed, altitude exposure induces an initial (within hours to 2 days) HGB increase mainly caused by hemoconcentration. HGB concentration then continues to increase until reaching a plateau depending on the altitude dose [157]. Similar to HGB, RET% increases in response to the hypoxic stress within the first days before returning to baseline after 10 days. Upon return from altitude, HGB normalizes within two weeks while RET% declines from baseline five to nine-days post-altitude due to negative feedback in a similar manner than after rHuEPO administration. While the response to altitude demonstrates high inter-individual variability, the baseline levels for the response of ABP parameters are of certain importance and the iron status is also a major criterion for an appropriate response to hypoxia [158,159]. Nevertheless, a recent study demonstrated that the combination of rHuEPO treatment and hypoxic exposure provided an additive erythropoietic response with an increased sensitivity of ABP at altitude [160]. As iron metabolism is deeply related to the erythropoietic process, iron supplementation (oral or injection), which is non-prohibited in sports, has also the potential to alter the ABP markers notably by increasing RET% with a larger increase in iron-deficient subjects (e.g. females) [161,162]. Pregnancy also induces changes in erythropoiesis with an increase of RET% typically paired with a decrease of HGB concentration [163]. Other prohibited substances although not considered as blood doping have also the potential to alter the erythropoietic markers such as diuretics (increase of HGB) or testosterone injections (increase of RET%) [164].

On the other hand, the hematological module includes the collection of whole blood sample, a “living” matrix, which requires stringent collection, transport and analysis conditions to be respected to reduce pre-analytical and analytical uncertainties and to ensure proper interpretation of the profiles [132,133]. Those strict criteria therefore imply important logistical

requirements (specialized phlebotomist, temperature-controlled transport, immediate sample processing) associated with extensive costs. Depending on the geographic situation of the sample collection, the collection time to analysis can be relatively long, leading to the invalidity of sample and the impossibility to use it for the consideration of a doping scenario.

Regarding the steroidal module, while the longitudinal monitoring of urinary EEAS concentrations certainly ameliorated the detectability of steroid doping in comparison to the T/E > 4 cut-off previously used, the approach still suffers from the influence of some confounding factors altering the steroid excretion and thus urinary steroid concentrations [92]. These factors can be of endogenous origin such as age, gender or ethnicity. The T/E ratio values may indeed vary in function of the age notably during puberty, which is associated with the development of sexual organs and characteristics [165–167]. In women, the menstrual cycle can influence the concentration of urinary EAAS. In particular, E concentration was demonstrated to vary in function of the menstrual phases with higher values in the mid-end of the cycle, principally impacting the T/E and 5αAdiol/E ratios and complexing the interpretation of the profile [168,169]. Pregnancy is also associated with an increased E excretion and a subsequent decrease of T/E and 5αAdiol/E ratios [170].

As explained previously, androgens are submitted to phase II reactions such as glucuronidation to be excreted in urine. Differences in the activity of the enzymes involved in this pathway due to polymorphism can therefore generate variability in the urinary steroid concentrations. The most documented genetic polymorphism in relation with the ABP urinary steroid profile is the one relative to UGT2B17 enzyme, responsible for the glucuro-conjugation of T. The *del/del* genotype results in low urinary T levels and therefore lower T/E ratio values that are nearly unaltered by T administration [171]. It was demonstrated that the *del/del* genotype was associated with ethnicity and was particularly prevalent in the Asian population [172]. This polymorphism can explain in part the bimodal distribution of T/E values among athletes population [173]. In addition to those endogenous factors, external factors such as drug administration, diet and environmental conditions contribute to alteration of steroids metabolism and urinary excretion. Various medications have been identified as possible sources of alterations such as oral contraceptive pills, hCG, glucocorticoids or nonsteroidal anti-inflammatory drugs [174–177]. Ketoconazole and 5α-reductase inhibitors (e.g. finasteride) are of particular importance for the interpretation of urinary steroid profile due to their suppressing effect on the synthesis and/or excretion of T and some metabolites [178,179]. Consequently, these compounds shall be monitored and reported by anti-doping laboratories to facilitate profile evaluation. Aside its recreational consumption, ethanol can influence the metabolic

pathway linked to steroids biotransformation via competitive inhibition of enzymes involved in alcohol and steroids metabolism [180,181]. The effect of ethanol usually results in an increase of T excretion associated with a decrease of A and Etio excretion, leading to higher T/E and lower A/T values [180]. In women, the urinary steroid concentrations are particularly sensitive to ethanol consumption [182]. Although its effects were reported to be negligible, green tea consumption has recently been suggested as potential confounding factor due to its suppressing effect on the activity of UGT2B17 enzyme [183].

Finally, the intrinsic nature of the urinary matrix makes it prone to bacterial contamination and the enzyme activity related to microorganisms may lead to an increase or a drop of steroid concentrations or even to the hydrolysis of conjugated T metabolites [92]. Thus, markers of degradation, namely 5α-androstanedione and 5β-androstanedione which originate from bacterial transformation of A and Etio glucuronide respectively, are screened by anti-doping laboratories to reveal adulteration of the biological samples with microorganisms and assess the validity of the sample [184]. For CP, to ensure that the elevated T/E ratio is not caused by bacterial contamination, the ratio between free T and total T shall also be monitored.

Despite the recent addition of 5αAdiol/E ratio to the steroidal module, the same basic and classical biomarkers have been monitored in both modules of the ABP since its implementation. As highlighted in this chapter, these markers can on certain occasions lack sensitivity due to the use of low doping doses but also specificity caused by confounding factors. These limitations underlines the need of additional biomarkers to improve these two aspects and to contribute to gathering a bundle of evidence to support the scenario of the administration of a prohibited substance.

## 1.5 Doping biomarkers

A biomarker can be described as "*a molecule that indicates an alteration of the physiological state of an individual in relation to health or disease state, drug treatment, toxins and other challenges of the environment"* (which can be defined as the 'exposome') [185]. They have been used as surrogate indicators of the status of the body in disease screening, diagnosis, characterization, and monitoring; for predicting and treating adverse drug reactions; for identifying cell types; or for pharmacodynamics and dose-response studies [186]. In the anti-doping context, a biomarker of doping is defined as a biological variable indicating the response, with a high degree of certainty, to the use of a prohibited substance or method [187]. The variations in doping biomarkers can directly be used as a whole to sanction an athlete but also to target specific analyses (such as rHuEPO tests or GC/C/IRMS analysis), to collect

additional samples from the athlete or to investigate an athlete or group of athletes [187]. These same biomarkers can also be exploited to estimate the prevalence of doping in an athletic population [28,29] but also to pinpoint changes in athletes behavior [30,142].

The ideal characteristics of a doping biomarker are similar to those used in diagnostics, clinical or pharmaceutical chemistry and are summarized in Table 5 [188,189]. While in some clinical situations the false positive rate may be disregarded in favor of high true positive rate (sensitivity), the prevention of false positive cases is primordial in the anti-doping context due to the heavy consequences of a potential anti-doping sanction [187]. Doping biomarkers therefore need to be highly discriminatory due to the variability inherent in biological systems [190].

| Characteristic | Definition |
|---|---|
| **Specificity** | Informative of the status of a specific organ or pathology and able to differentiate perturbed state without false positive |
| **Sensitivity** | Rapid and significant release upon the administration of prohibited substance, ability to identify true positive |
| **Predictive** | Long half-life in sample and response proportional to the dose administered |
| **Robust** | Rapid, simple, accurate and inexpensive detection and unconfounded by environment and unrelated conditions |
| **Translatable** | Data can be used to bridge pre-clinical and clinical results |
| **Non-invasive** | Present in accessible fluid sample |

**Table 5.** Characteristics of an ideal biomarker. Adapted from [188].

Concerning the discovery of novel doping biomarkers, the main difference with disease related field is that the response of selected biomarkers is monitored at the individual level in opposition to the classical approach comparing normal and diseased states in two distinct populations. Consequently, the clinical trial design and data analysis should be designed and performed at the individual and not population level [187]. The design should also allow robust baseline sampling and measurement of within-subject changes in the different biomarkers evaluated over time using longitudinal approach, which could further enable to discriminate between natural within-subject variability and treatment-induced variability of the evaluated biomarkers. Regarding biomarkers discovery, the emergence of large-scale -omics technologies has provided some new insights for the investigation of novel doping biomarkers using targeted

(*a priori* information) or more holistic (untargeted) approaches. The development of these methods enabled the exploration of multi-layers of data (transcriptome, proteome, metabolome) for the study of the biological and human systems which can be influenced by various factors such as doping and which can supply multiple putative biomarkers. The inherent nature of blood makes it a suitable matrix for biomarkers discovery because it is more informative of the disturbed state than urine, which rather represents the waste bin constituted by the metabolites of the perturbed homeostasis.

Search for novel biomarkers using approaches from systems biology may result in the discovery of a large panel of promising biomarkers candidates, which need to be selected for further validation [190]. In addition to the critical sensitivity and specificity, a low ratio within/between-subject variance will be favored. For the validation process, the sources of biomarker variation mainly consisting of the variations between individual (between-subject) and variations within individuals (within-subject), including biological, sample collection/transportation and analytical variations, need to be assessed [187]. This process shall be carried out on a larger population of elite athletes. Finally, considering a possible implementation, similar to whole blood analysis or urine analysis for EAAS, the analytical procedure needs to be highly harmonized among laboratories with a low measurement uncertainty to avoid any significant bias when a new sample is added to the passport.

The process from discovery to implementation of a doping biomarker is therefore a complex procedure with an important bottleneck in-between.

## 1.6 Aim of the thesis

Despite its indisputable deterrent effect demonstrated since its implementation, the ABP suffers from some limitations mainly linked to its sensitivity, to the influence of various confounding factors and the lack of integration of novel potential biomarkers for both the hematological and the urinary steroid modules. Thus, complementary and additional strategies need to be investigated to overcome these limitations and further improve the ABP strategy by increasing the relative likelihoods of biomarkers variations are being due to doping than other causes. Blood offers an ideal matrix for novel biomarkers discovery using -omics technologies but also benefits from the expertise of clinical medicine for the interpretation of certain data from endogenous compounds. Moreover, in comparison to urine matrix, blood is less prone to manipulation or bacterial contamination and is much more informative of the metabolism perturbations with an instant snapshot of athlete's physiological condition at the moment of sample collection. Consequently, the general aim of the thesis was to investigate blood biomarkers originating from multi-layers of data for the improvement of the ABP. As the use of this matrix implies the invasive collection of venous blood, alternative matrix such as dried blood spots was also evaluated.

First, transcriptomic biomarkers originating from immature red blood cells were evaluated as a complementary approach to the current hematological module for the detection of blood doping. In the second part, the blood-born research for biomarkers was applied to testosterone detection. A transcriptomic approach was applied for the detection of testosterone doping using circulating microRNAs as biomarkers. As the outcome was not satisfactory in term of sensitivity and specificity, the strategy was adapted toward a targeted metabolomic approach benefiting from the previous knowledge developed by our research group to highlight putative biomarkers of T administration in healthy women with regular menstrual cycle. Accordingly, a UHPLC-MS/MS method was further developed for the quantification of endogenous free steroids as well as their phase I and phase II metabolites in serum and in DBS. The analysis included newly discovered markers of doping use with T, associated with classic clinical markers.

# Chapter II

## Transcriptomic biomarkers of doping

## 2.1 Transcriptomic biomarkers of altered erythropoiesis

### 2.1.1 Introduction

The genome corresponds to the genetic material of an organism consisting of DNA including genes and non-coding DNA. The genes (the coding regions) are a sequence of nucleotides in DNA that encode for the synthesis of proteins and are first transcribed into RNA. Accordingly, the transcriptome represents the set of all coding and non-coding RNA transcripts and the quantitative analysis of the RNA transcripts produced by the genotype at a given time is defined as transcriptomics [191]. While the genome is nearly invariable, the transcriptome is in a constant state of flux reflecting the responsive changes of the cell milieu and metabolic activity [192].

The emergence of high-throughput technologies such as microarray, RNA sequencing (RNA-seq) or quantitative polymerase chain reaction (qPCR) allowed for an accessible application of transcriptomics and a promising alternative in the research of doping biomarkers, especially in the context of blood doping. Indeed, while they expelled their nucleus, the most immature RET fraction (IRF) in circulation still contains functional residual nucleic acid material that is essential for reticulocyte maturation into RBC [39]. Consequently, the remnant mRNA contained in RET is hypothesized to encode a reservoir of information regarding erythropoiesis [193]. As blood manipulations alter the erythropoietic processes and thus the production of immature RBCs, their gene expression should also be affected, reflecting the bone marrow activity.

Already in 2004 and 2009, Varlet-Marie *et al.* identified and confirmed several genes that were differentially expressed in whole blood after administration of rHuEPO using serial analysis of gene expression (SAGE) and reverse-transcriptase qPCR (RT-qPCR) [194,195]. More recently, Durussel *et al.* investigated the blood transcriptional signature of rHuEPO in two distinct groups receiving rHuEPO injections for 4 weeks, composed of endurance-trained Caucasian males at sea level and Kenyan runners at moderate altitude [196]. The functions of the identified altered genes were mainly related to the functional and structural properties of the RBCs. Despite a pilot study evaluating the expression profile of T lymphocytes following blood reinfusion [197], no study investigated the alteration of reticulocytes gene expression following autologous blood transfusion.

### 2.1.2 Article 1: Impact of blood transfusion on gene expression in human reticulocytes

In this first study, on the basis of the blood transcriptional signature of rHuEPO [196], a subset of genes was selected for monitoring their expression following ABT in seven healthy male volunteers. The transcriptomic response of the pre-determined subset of genes was assessed via digital multiplex mRNA profiling using Nanostring® nCounter Analysis system. This method uses color-coded molecular barcodes able to directly hybridize to many different types target molecules with the advantage of being quantitative and thus amplification-free. Total RNA was extracted and quantified from blood stabilized in Tempus Blood RNA Tubes.

Three candidate gene markers, namely delta-aminolevulinate synthase 2 (*ALAS2*), carbonic anydrase (*CA1*) and solute carrier family 4 member 1 (*SLC4A1*), were the most affected by ABT with a downregulation 6 to 9 days after reinfusion. Interestingly, proteins coded by those genes were tightly implicated into biological processes of reticulocytes such as heme synthesis or gas exchange. The variations observed in genes expression were more marked than for conventional blood markers such as RET% after ABT suggesting an early reflection erythropoietic alterations caused by blood re-infusion.

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## Impact of blood transfusion on gene expression in human reticulocytes

*To the Editor*: Blood transfusion is a frequently performed therapeutic procedure that requires regular evaluation, particularly for its indications, effectiveness, and risks. There are indeed an increasing percentage of blood transfusions considered to be inappropriate and their efficiency has raised questions. The identification of new specific biomarkers of blood transfusion would be of particular relevance for the monitoring of this method. These markers would also be applicable for anti-doping purposes.

Infusion of blood results in a rapid increase of circulating red blood cells (RBCs), which impairs endogenous production and release of immature RBCs. This impact of transfusion is mediated by the suppression of erythropoietin (EPO) [1,2]. We hypothesized that blood re-infusion may cause a decrease in the expression of genes related to structural and functional components of reticulocytes and red blood cells (RBCs). The present study aimed to investigate the transcriptional response of a subset of genes, whose functions are related to reticulocyte metabolism, after autologous blood transfusion (ABT) using the digital multiplex mRNA profiling.

Seven healthy male volunteers (age range, 20–35 years; body mass index, 18–30), that were eligible for blood donation according to national regulations were included in the study. Details regarding the clinical trial (NCT02423135) were previously described [2]. Briefly, during the control phase, all volunteers were infused with saline solution. Fourteen days later, all volunteers donated one full bag of blood (approximately 500 mL). The concentrated RBCs were stored at ~4°C until re-infusion 36 days later. RNA expression was measured using Nanostring® nCounter Analysis System (Nanostring Technologies, Seattle, WA). Gene expression analysis was performed at baseline (D-4 and D-1) and after the infusion of 0.5 L of saline solution (0.9% NaCl, BBraun, Cressier, Switzerland) on day 3, 6, and 9 and after re-infusion of one's own blood (0.28 L) on day 6, 9, and 15. Blood samples were drawn into Tempus Blood RNA tubes (Life Technologies, Carlsbad, CA) to stabilize genomic material and were stored at −20°C until further extraction. A one-way ANOVA followed by post hoc pairwise comparisons (*t*-tests adjusted by Bonferroni corrections and Tukey's Honestly Significant Difference) were used to test differences between samples taken during saline or transfusion phase.

Based on the blood transcriptional signature of recombinant human erythropoietin [3], a subset of 45 genes (following removal of the ten housekeeping genes) was selected for evaluation of their expression following ABT. Agglomerative clustering (heat maps) resulted in the identification 27 genes that were commonly down-regulated 6, 9, and 15 days after blood re-infusion in seven volunteers (Supporting Information S1 Table and Supporting Information S1 Fig.). Delta-aminolevulinate synthase 2 (*ALAS2),* carbonic anhydrase *(CA1)*, and solute carrier family 4 member 1 (*SLC4A1)* were observed to have the greatest fold-change following blood re-infusion than at baseline. A marked decrease in gene expression was observed 6 days after ABT, although the number of transcripts for each gene was significantly decreased 9 days, after re-infusion of blood (Fig. 1A). At day 15, gene expression remained low, and was not significant. During the control phase, the number of transcripts of the candidate genes did not vary significantly 3, 6, and 9 days and stayed steady after saline infusion (Fig. 1B). Finally, the expression of the housekeeping genes (*ACTB, ACTR10, MRFAP1, TBP, TRAP1*) remained constant throughout the phases, indicating that the changes observed in gene expression are not cell count-based (Supporting Information S2 Fig.). It suggests that the variations of gene expression observed during the transfusion phase are specific to the re-infusion of blood.

Although they have shed their nucleus, circulating blood reticulocytes still retain quantities of functional residual acid material which is essential for their maturation into erythrocyte [4]. These remaining copies contained in circulating reticulocytes are hypothesized to reflect gene expression activity of erythroblast into bone marrow [5]. Erythroid precursors express at their surface a receptor specific to EPO. When secreted upon hypoxia, EPO targets developing erythroblasts and controls their differentiation and proliferation. It also contributes to the release of reticulocytes through a diminution of the normal marrow-peripheral blood barrier.

Previously, Durussel et al. demonstrated that EPO injection influenced the gene expression in human reticuloctytes [3]. As blood transfusion suppresses erythropoiesis through the decrease of EPO concentration [1,2], similar changes in mRNA expression specific to reticulocytes were expected.

Our study demonstrates that the transfusion of autologous blood triggers a down-regulation of genes that are involved in biological processes related to reticulocytes and RBCs.

ALAS2 is involved in the heme synthesis, whereas CA1 and SLC4A1 are responsible for the transport of oxygen and carbon dioxide. Thus, these candidate genes are specific to erythropoiesis. The magnitude of the changes of the genes transcripts was more

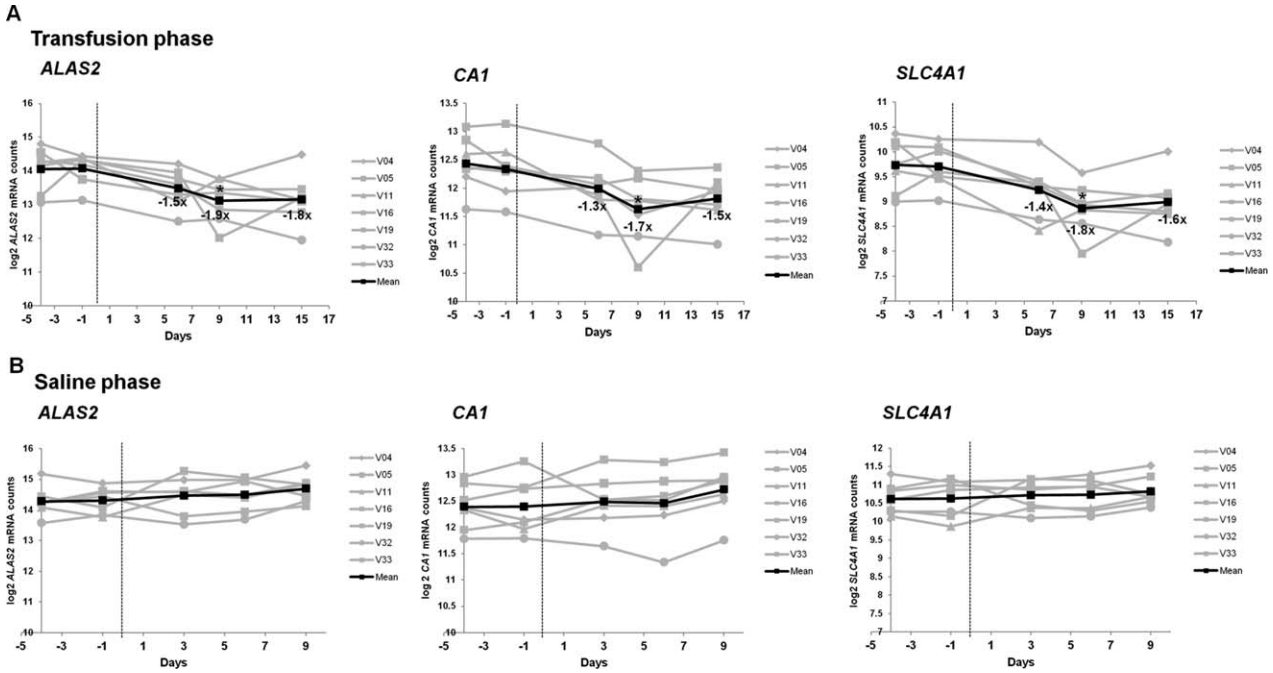


**Figure 1**. A: Transfusion phase. Expression of *ALAS2*, *CA1*, and *SLC4A1* before (D-4 and D-1) and 6, 9, and 15 days ($n = 7$) after autologous blood transfusion. The dashed line indicates transfusion. Gene expression fold-changes were calculated on original/normalized mean data and are annotated below the time points 6, 9, and 15 days post-transfusion relative to baseline (mean of the data from 1 and 4 days pre-transfusion). Raw counts were normalized to internal levels of five reference genes, *ACTR10, ACTB, MRFAP1, TBP,* and *TRAP1*. The *Y* axis represents log2-transformed normalized counts of the genes. $^{\#}P \leq 0.05$, statistically significant difference compared with baseline values. B: Saline phase. Expression of *ALAS2*, *CA1*, and *SLC4A1* before (D-4 and D-1) and 3, 6, and 9 days ($n = 7$) after saline infusion. The dashed line indicates saline infusion. Raw counts were normalized to internal levels of five reference genes, *ACTR10, ACTB, MRFAP1, TBP,* and *TRAP1*. The *Y* axis represents log2-transformed normalized counts of the genes. No statistically significant difference was observed between time points.

 doi:10.1002/ajh.24458

important compared to the small physiological effect of ABT on peripheral blood markers [2].

In anti-doping field, autologous blood transfusion is assessed by measuring hematological parameters via the Athlete Biological Passport (ABP) which involves brittle biological materials. It requires costly investments in the pre-analytical steps to ensure the validity of the analyses [6]. To overcome actual challenges, our study proposed the inclusion of transcriptomic biomarkers, whose sensitivity is greater than that of classical variables, into the adaptive model of the ABP, coupled with easy-to-use collection blood tubes that stabilize genomic material for up to 5 days at room temperature and for years when kept frozen. However, before a potential integration of these three innovative biomarkers into the adaptive model of the ABP, intrinsic and extrinsic factors that may affect the expression of these genes must also be fully characterized.

In summary, our results demonstrate that autologous blood transfusion triggered a down-regulation of genes whose function is linked to the metabolism of immature RBCs. Following autologous infusion of stored RBCs, expression of the genes *ALAS2*, *CA1*, and *SLC4A1* was markedly decreased. The profiling of reticulocytes transcriptome offers a new clinical way for the study of erythroid biology in response to blood transfusion. Futhermore, these transcriptomic biomarkers may also serve for the detection of autologous blood transfusion in an anti-doping context as they appeared to be more sensitive than classic hematological biomarkers.

## Acknowledgment

The authors would like to thank the Genomic Technologies Facility at the University of Lausanne for Nanostring analyses and their assistance for the understanding of nSolver software.

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*Additional Supporting Information may be found in the online version of this article.*

*Contract grant sponsor: World Anti-Doping Agency (WADA); Contract grant number: 12C14NL; Contract grant sponsor: Département Universitaire de Médecine et Santé Communautaire (DUMSC); Contract grant number: 06/2015.*

*Conflict of interests: The authors declare that they have no conflicts of interest relevant to the manuscript submitted to American Journal of Hematology.*

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*Received for publication: 7 June 2016; Revised: 30 June 2016; Accepted: 5 July 2016*

*Published online: 7 July 2016 in Wiley Online Library (wileyonlinelibrary.com)*

*DOI: 10.1002/ajh.24470*

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## Barriers and facilitators to research participation among adults, and parents of children with sickle cell disease: A trans-regional survey

*To the Editor*: Patient recruitment into sickle cell disease (SCD) clinical trials has not always been robust, leading to delayed results or even failure to trial completion [1]. Disparities impacting participation in research include race, lower socioeconomic status and lower health literacy [2]. We sought to extend previous research by surveying adults with SCD and parents of children with SCD across geographically diverse regions in the U.S. We hypothesized that demographic variables such as lower income and education would present barriers to interest in research participation, as would psychosocial variables (depression symptoms and lower health literacy). We also evaluated patient/family preferences in use of technology for being contacted about research.

We surveyed a convenience sample of adults, and parents of children with SCD, all genotypes. We used the Mid-South Clinical Data Research Network [3] survey to gather socio-demographic information, depression symptoms (Patient Health Questionnaire—PHQ-2) and health literacy (Brief Health Literacy Screening). We assessed participant interest in varying research types and asked if they would want to be contacted for research using electronic communication—e-mail, text messaging, and social media—as well as traditional face-to-face conversations in clinic or phone calls. We examined differences in demographic and psychosocial variables between participants who responded they were interested in each research type versus those that were not (study outcome) using Fisher's exact and Wilcoxon tests, and created logistic regression models for each research type, analyses performed in R version 3.2.2.

Sample characteristics included: 211 adults and 331 parents of children with SCD; ages 1–70 years for individuals with SCD; over 50% female; 97% Black/African American race, 2% Hispanic ethnicity. Median education for adults with SCD and parents was some college education, and 75% reported "good" health literacy. However, 45% reported it was "somewhat" to "very difficult" to pay monthly bills. Twenty-two percent of adults obtained scores of $\geq$3 on the PHQ-2 (vs. 14% for pediatrics). Most had participated in research previously, with adults (63%) participating more frequently than pediatrics (56.5%). Most participants (95%) expressed interest in research, preferring text messaging (52.1% of adults) and e-mail (58.9% of parents) as modes of contact. Participants were most interested in completing research surveys (83%), followed by studies involving phone/internet contact or community meetings (76%); and giving blood samples (74%). Adults and parents were least interested in participating in studies where they would have to take medication (58%) or where they/their child would have to stay in the hospital (52%).

Adults, and parents of children with SCD were more likely to express interest in several types of research if they had previously participated in research (Table I in Supporting Information). Higher health literacy increased the odds of interest in giving a blood sample for parents. Adults from the Mid-South were less likely to express interest in studies involving medications, compared with the Midwest and West. Adults with depressive symptoms were more likely to express interest in medication studies, while parents of children with more symptoms of depression were less interested in medication studies. Parents were more likely to express interest in studies requiring hospital admission if they had difficulty paying bills and higher scores on the PHQ-2.

In regression analyses, when other variables were in the model, female gender predicted interest in completing surveys, and depressive symptoms predicted interest in medication studies and studies requiring hospitalization for adults. For parents, lower health literacy predicted interest in research involving hospitalization; depression symptoms and higher health literacy predicted interest in giving a blood sample, and male gender, younger age of child and depression symptoms predicted interest in phone/internet studies.

Our hypotheses were partially supported, as we found previous research participation was associated with interest in subsequent research participation, for adults and parents [4], and lower health literacy was associated with less interest in some research types, for parents. Contrary to expectations, parents expressed greater interest in studies requiring hospital admissions if they had difficulty paying bills, and in studies involving giving blood samples if they had more depressive symptoms; and adults with more depression symptoms expressed greater interest in studies requiring hospitalization or taking medication. The only regional variation we found was that adults from the Mid-South expressed less interest in studies involving medications, possibly related to lower prior participation compared with the other two regions.

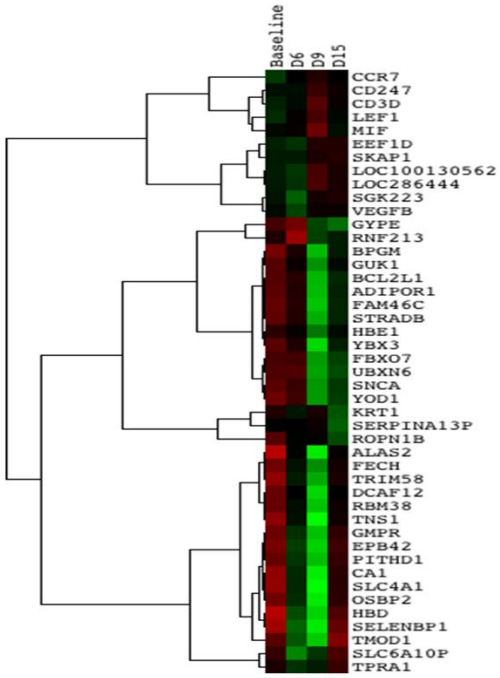
Our findings are critical to consider, as exploration of new pharmacotherapeutic approaches is growing rapidly, yet more than half of adults, and parents of children with SCD, were not interested in participating in studies involving taking medication or hospital admission. Our results may reflect poor communication between providers/researchers and patients or historic mistrust [5]. Our findings that depressive symptoms in both adults and parents of children with SCD, and difficulty paying bills for parents were associated with greater interest in more invasive research may highlight some of the cognitive challenges [6] and vulnerability of the SCD population.

We recognize the limitations with our survey that relied on self-report about future events and that did not directly evaluate the influence of trust, concerns about harm, issues in relation to time commitments and cognitive impairment. Our convenience sample may not be representative of the broader population of patients with SCD although we demonstrated that adults with SCD and parents in several regions across the U.S. are interested in research participation. Our findings support the need for clear, detailed information about research, at appropriate literacy levels, delivered through channels that are attractive to potential research participants (i.e., email and text messaging). When designing educational materials or approaching patients, researchers must be vigilant those individuals with SCD and cognitive impairment, and/or those with lower income and depressive symptoms are not being subtly coerced into research participation, and that negative perceptions are addressed, given past research misuses. By understanding

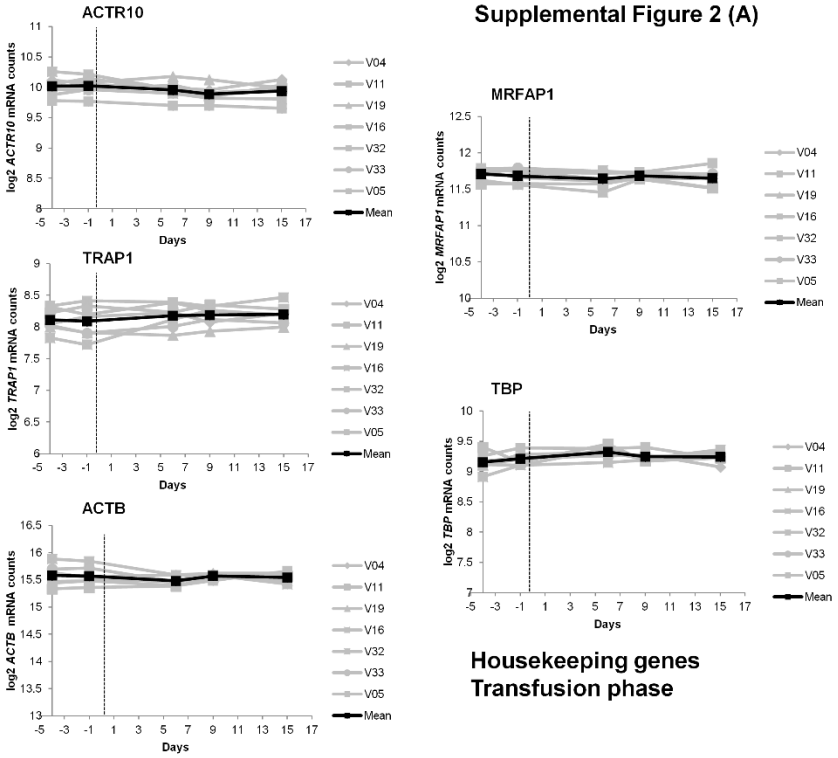
# Supplemental Material

## Supplementary figures

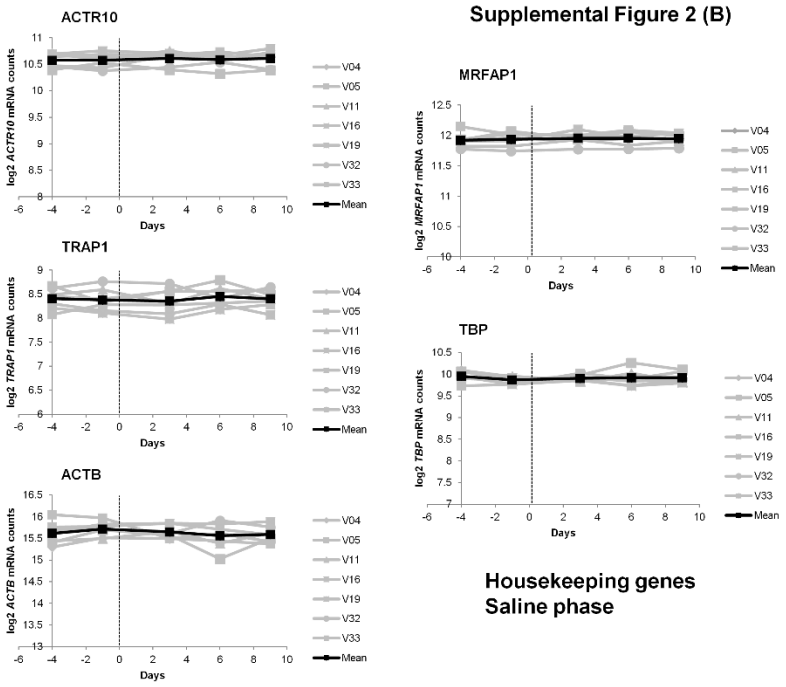
**Supplemental Figure 1**




**Supplemental Figure 2 (A)**




**Housekeeping genes**
**Transfusion phase**




## Supplementary table

### Supplemental Table 1

| Gene Symbol | NCBI | Gene Name |
|---|---|---|
| ADIPOR1 | NR_046083.1 | adiponectin receptor 1 |
| ALAS2 | NM_001037968.3 | aminolevulinate, delta-, synthase 2 |
| BCL2L1 | NM_001191.2 | BCL2-like 1 |
| BPGM | NM_199186.1 | 2,3-biphosphoglycerate mutase |
| CA1 | NM_001738.2 | carbonic anhydrase 1 |
| DCAF12 | NM_015397.2 | DDB1 And CUL4 Associated Factor 12 |
| EPB42 | NM_000119.2 | erythrocyte membrane protein band 4.2 |
| FAM46C | NM_017709.3 | family with sequence similarity 46, member C |
| FBXO7 | NM_001033024.1 | f-box protein 7 |
| FECH | NM_000140.3 | ferrochelatase |
| GMPR | NM_006877.3 | guanosine monophosphate reductase |
| GUK1 | NM_000858.5 | guanylate kinase 1 |
| HBE1 | NM_005330 | hemoglobin, epsilon 1 |
| HBD | NM_000519.3 | hemoglobin, delta |
| OSBP2 | NM_030758.3 | oxysterol binding protein 2 |
| PITHD1 | NM_020362.4 | PITH domain containing 1 |
| RBM38 | NM_183425.2 | RNA binding motif protein 38 |
| SELENBP1 | NM_003944.2 | selenium binding protein 1 |
| SLC4A1 | NM_000342.3 | solute carrier family 4, anion exchanger, member 1 |
| SNCA | NM_000345.2 | synuclein alpha |
| STRADB | NM_018571.4 | STE20-related kinase adaptor beta |
| TMOD1 | NM_003275 | tropomodulin 1 |
| TNS1 | NM_022648 | tensin 1 |
| TRIM58 | NM_015431.3 | tripartite motif-containing 58 |
| UBXN6 | NM_025241.2 | UBX domain protein 6 |
| YBX3 | NM_003651.3 | Y box binding protein 3 |
| YOD1 | NM_018566.3 | YOD1 OTU deubiquinating enzyme 1 homolog |

### 2.1.3 Article 2: Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion

In the second article, the results from Article 1 were confirmed integrating additional volunteers (7) and time points (8) of the ABT clinical trial and using RT-qPCR as alternative quantitative method. This study supported the finding that the decrease of expression of the three candidate genes actually begun 6 days after re-infusion of one's own blood, with the number of transcripts significantly downregulated 9 days after the intervention and remaining low until 15 days. The results demonstrated a strong correlation between RT-qPCR and Nanostring® nCounter technology, suggesting that both methods are suitable for the quantification of RET mRNA.
Conventional blood markers of the hematological module of ABP such as RET% and IRF demonstrated a similar response due to the inhibitory effect of ABT on erythropoiesis but to a lesser extent than the transcriptomic biomarkers. The expression of the three genes exhibited a stronger correlation with IRF than with RET%, suggesting that those biomarkers reflect more substantially early erythropoietic changes and are therefore reliable predictors of bone marrow's response to ABT.
This study confirms and consolidates that ABT triggers a down-regulation of genes encoding proteins implicated in functional and structural processes of immature RBCs. It reinforces the idea that transcriptomic biomarkers might be promising complements to conventional hematological parameters of the ABP in a longitudinal monitoring to improve the detection of blood doping.

Received: 12 April 2017 | Revised: 16 June 2017 | Accepted: 22 June 2017

DOI: 10.1002/dta.2240

WILEY

SHORT COMMUNICATION

# Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion

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**Funding information**
World Anti-Doping Agency, Grant/Award Number: Grant No. 12C14NL

Autologous blood transfusion is a powerful means of improving performance and remains one of the most challenging methods to detect. Recent investigations have identified 3 candidate reticulocytes genes whose expression was significantly influenced by blood transfusion. Using quantitative reverse transcription polymerase chain reaction as an alternative quantitative method, the present study supports that delta-aminolevulinate synthase 2 (*ALAS2*), carbonic anhydrase (*CA1*), and solute carrier family 4 member 1 (*SLC4A1*) genes are down-regulated post-transfusion. The expression of these genes exhibited stronger correlation with immature reticulocyte fraction than with reticulocytes percentage. Moreover, the repression of reticulocytes' gene expression was more pronounced than the diminution of immature reticulocyte fraction and reticulocyte percentage following blood transfusion. It suggests that the 3 candidate genes are reliable predictors of bone marrow's response to blood transfusion and that they represent potential biomarkers for the detection of this method prohibited in sports.

**KEYWORDS**

IRF, transcriptomics, transfusion

## 1 | INTRODUCTION

Blood manipulations are strictly prohibited by the World Anti-Doping Agency (WADA) although they remain a significant problem in sports. Autologous blood transfusion (ABT) stands as a particular challenge for sports authorities, due to the lack of direct detection method. ABT is currently indirectly detected with the hematological module of the Athlete Biological Passport (ABP).[1] It consists in a longitudinal monitoring of various hematological parameters (hemoglobin level (Hb) and reticulocytes percentage (Ret%)) to identify suspicious patterns of altered erythropoiesis.

Indeed, blood manipulations leave a characteristic fingerprint on the athlete's physiology.[2,3] For instance, blood reinfusion suppresses erythropoiesis with a subsequent decrease of erythropoietin (EPO) concentration through a negative feedback system.[4,5] It leads to a diminution of the release of immature red blood cells (RBCs) from the bone marrow, and thus a decline of Ret% and immature reticulocytes fraction (IRF).

Based on that assumption, Salamin et al. demonstrated by a pilot study that the expression of genes involved in biological processes related to reticulocytes decreases following ABT using digital multiplex mRNA profiling.[6] These genes seemed particularly more sensitive to ABT than Ret%.

The primary aim of our study was to confirm the results of this pilot study using additional volunteers (7) and time points (8), and quantitative reverse transcription polymerase chain reaction (RT-qPCR) as alternative quantitation method. Secondly, it was demonstrated that the expression of those genes was better correlated with IRF than with Ret%.

## 2 | MATERIAL AND METHODS

### 2.1 | Study design

Fifteen healthy male volunteers (age range, 20–35 years; body mass index, 18–30), that were eligible for blood donation according to national regulations, were included in the study approved by the human research ethics committee of the Canton de Vaud (Switzerland; Protocol 06/14). Details regarding the clinical trial were previously described (NCT02423135).[4,9] Briefly, during the control phase, all volunteers were infused with 500 mL of saline solution (NaCl 0.9%,

  

B.Braun, Crissier, Switzerland). Fourteen days later, all volunteers donated one full bag of blood (approximately 500 mL) using international standard methods of transfusion. Blood was drawn into Anticoagulant Citrate Phosphate Dextrose Solution, conditioned and concentrated in RBC for an approximate volume of 280 mL. The procedure to perform RBC concentration was described in Leuenberger et al.[9] The concentrated RBCs were stored at ~4°C in a solution containing saline, adenine, dextrose, and mannitol (ADSOL preservation solution, Fenwal Laboratories, Lake Zurich, IL, USA) until re-infusion 36 days later. Blood samples were obtained using standard procedures from the antecubital vein. Blood was drawn into EDTA tubes (K2EDTA, 7.2 mg, 4 mL, BD Vacutainer, Plymouth, UK) and Tempus Blood RNA tubes (Life Technologies, Carlsbad, CA, USA) at 1 and 4 days before (baseline) and 1, 2, 3, 6, 9, and 15 days after saline infusion or re-infusion.

## 2.2 | Hematological and gene expression analyses

The whole blood from the EDTA samples was analyzed for blood variables using a fully automated hematology analyzer (Sysmex XN 2000, Sysmex, Norderstedt, Germany).

Total RNA was extracted from blood stabilized in Tempus Blood RNA Tubes using Tempus Spin RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was assessed using a Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). RNA was aliquoted and stored at −20°C until further analysis.

The amount of 200 ng of extracted RNA was first reverse-transcribed into cDNA using Roche Transcriptor Universal cDNA Master kit (Roche Diagnostics). The resulting cDNA was diluted 10-fold and 4 µL of the diluted cDNA was used in a 10 µL-PCR amplification reactions using specific primers of target genes (*ALAS2*, *CA1*, *SLC4A1*). *TBP*, *MRFAP1*, *ACTR10* genes were selected as housekeeping genes for expression normalization from publicly available database. These genes were selected due to their well expression and stability in whole blood (mean 27 Ct). PCR amplification was performed using a Roche LightCycler 480 real-time PCR system. Raw data and normalization were performed with LightCycler 480 software (release 1.5.0). All primers sequences are available on the Qiagen website (https://www.qiagen.com/ch/shop/pcr/rt2-qpcr-primer-assays).

## 2.3 | Statistical analyses

Normalized data were first log2-transformed and a one-way ANOVA followed by *post hoc* pairwise comparisons (Tukey's Honestly Significant Difference) were used to test differences between time points during each phase. Correlations were calculated with Pearson's correlation method. Statistical analyses were performed using R software.

# 3 | RESULTS AND DISCUSSION

Based on the blood transcriptional signature of recombinant human EPO (rHuEPO),[8] Salamin et al. demonstrated that delta-

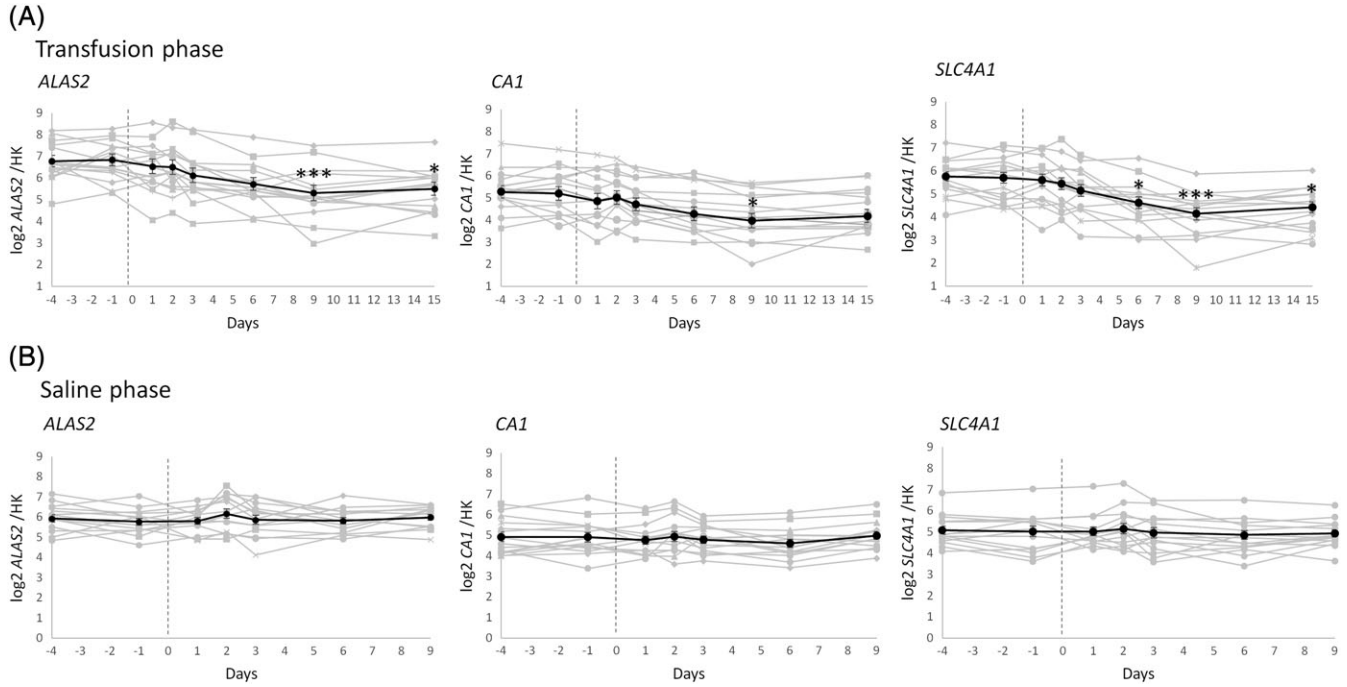


**FIGURE 1** (A) transfusion phase. Relative expression of *ALAS2*, *CA1*, and *SLC4A1* before (D-4 and D-1) and 1, 2, 3, 6, 9, 15 days after autologous blood transfusion ($n$ = 15). Dashed line indicates blood transfusion. Data were normalized to the corresponding levels of housekeeping genes (HK: *TBP*, *MRFAP1*, *ACTR10*). The *Y* axis represents log2-transformed relative expression of the genes. Grey lines indicate individual monitoring of gene expression and black lines indicate mean (±SE) of the 15 independent subjects. *$P \leq 0.05$; *** $P \leq 0.001$, statistically significant difference compared with baseline values (mean of the data from 1 and 4 days pre-transfusion). (B) saline phase. Relative expression of *ALAS2*, *CA1*, and *SLC4A1* before (D-4 and D-1) and 1, 2, 3, 6, and 9 days after saline infusion ($n$ = 14). Dashed line indicates saline infusion. Data were normalized to the corresponding levels of housekeeping genes (HK: *TBP*, *MRFAP1*, *ACTR10*). The *Y* axis represents log2-transformed relative expression of the genes. Grey lines indicate individual monitoring of gene expression and black lines indicate mean (±SE) of the 14 independent subjects. No statistically significant difference was observed between time points

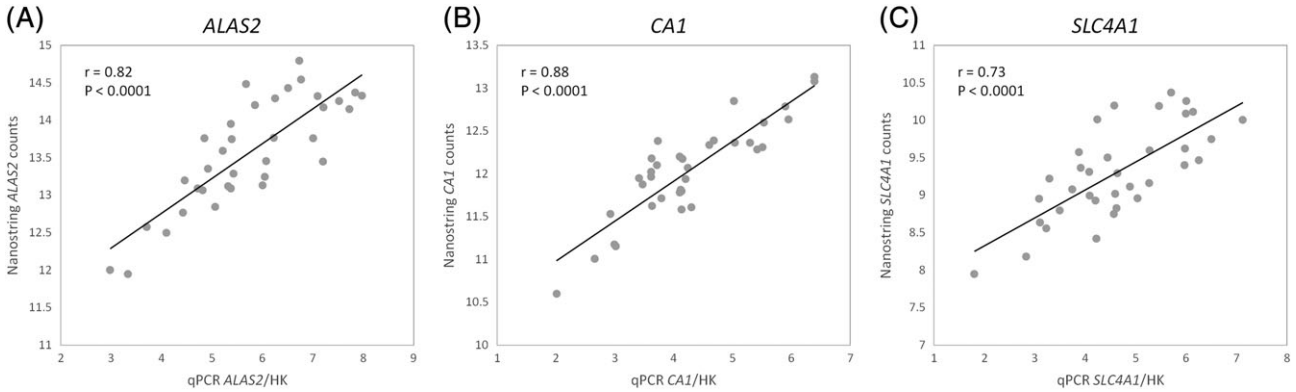


**FIGURE 2** Correlation between Nanostring nCounter system and RT-qPCR results after autologous blood transfusion. (A) *ALAS2*. Pearson correlation of Nanostring and RT-qPCR results (Pearson $r = 0.81$; $P < 0.0001$; $n = 35$). *X* axis represents the log2-relative expression of *ALAS2* (RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring). (B) *CA1*. Pearson correlation of Nanostring and RT-qPCR results (Pearson $r = 0.88$; $P < 0.0001$; $n = 35$). *X* axis represents the log2-relative expression of *CA1*(RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring). (C) *SLC4A1*. Pearson correlation analysis of Nanostring and RT-qPCR results (Pearson $r = 0.7$; $P < 0.0001$; $n = 35$). *X* axis represents the log2-relative expression of *SLC4A1*(RT-qPCR) and *Y* axis represents the log2-normalized mRNA counts of the gene (Nanostring)

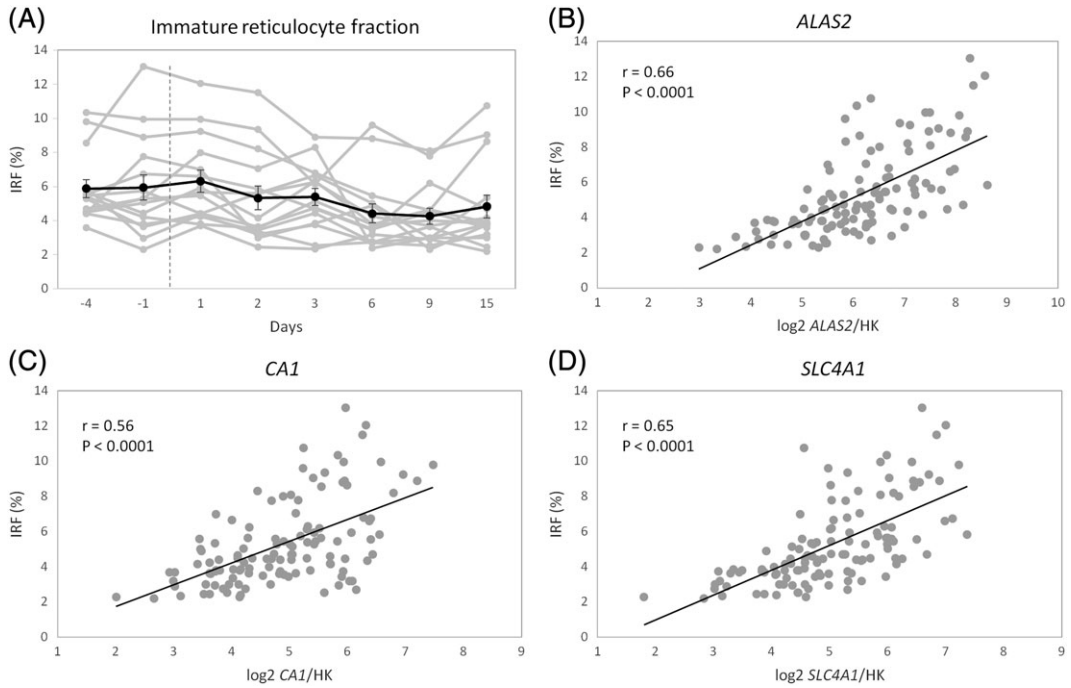


**FIGURE 3** (A) impact of transfusion on IRF. Longitudinal monitoring of IRF before (D-4 and D-1) and 1, 2, 3, 6, 9, 15 days after autologous blood transfusion ($n = 15$). Dashed line indicates blood transfusion. Grey lines indicate individual monitoring of IRF and black line indicates mean (±SE) of the 15 independent subjects. (B) relationship of IRF and *ALAS2* after ABT. (C) relationship of IRF and *CA1* after ABT. (D): Relationship of IRF and *SLC4A1* after ABT. Line represents linear regression of data. *X* axis represents relative expression of the gene and *Y* axis represents IRF (%)

aminolevulinate synthase 2 (*ALAS2*), carbonic anhydrase (*CA1*), and solute carrier family 4 member 1 (*SLC4A1*) were down-regulated after reinfusion of donated blood.[6] Using a different method of quantitation (RT-qPCR), we confirmed the results of this pilot study with additional subjects ($n = 15$ vs 7) and time points tested ($t = 8$ vs 5) (Figure 1A). The expression of the three target genes exhibited a significant maximum decrease 9 days ($P < 0.05$) after blood transfusion. The diminution of the number of copies was also significant at 15 days for *ALAS2* and *SLC4A1*, and at 6 days for *SLC4A1*. These results corroborate the pilot study and certify that the decrease of gene expression actually begins 6 days after the reinfusion of one's own blood. Because ABT could be performed few days (1–2 days) before an anti-doping control, other biomarkers such as circulating miRNAs could complement this detection strategy.[3] After saline infusion, the number of transcripts of the three genes did not vary substantially and remained constant throughout the time points (Figure 1B). Finally, the expression of the housekeeping genes (*TBP*, *MRFAP1*, *ACTR10*) used for normalization demonstrated no variation and was uniform among the subjects (data not shown).

Interestingly, the results obtained with the RT-qPCR correlated with those of Nanostring nCounter technology (*ALAS2*, $r = 0.81$; *CA1*, $r = 0.88$; *SLC4A1*, $r = 0.7$) (Figure 2) suggesting that both

medium-throughput mRNA abundance measurement technologies are robust and sensitive for quantitation of reticulocytes' mRNA. While RT-qPCR analysis is less expensive, can profile smaller subset of specific genes such as candidate gene validation and is considered as the gold standard for nucleic acid quantification, Nanostring technology does not necessitate reverse transcription and has the ability to profile directly Tempus tubes lysed whole blood without any purification steps.[10]

The reinfusion of one's own blood triggered a decline of the erythropoietic activity with a diminution of Ret% being maximal at 9 days.[4] ABT also produced a similar, although non-significant, decrease in IRF with a comparable maximal decrease at 9 days (Figure 3). The measure of this parameter exhibited some variability for several volunteers which could be rationalized by the absence of normalization in comparison with gene expression. However, the variability of these individuals did not influence the overall trend after autologous transfusion. Both hematological parameters fluctuate in a similar manner to that of the three candidate genes investigated.

Depending on the maturation stage, reticulocytes contains variable amounts of RNA. They are thus divided into low-fluorescence reticulocytes (LFR), middle-fluorescence reticulocytes (MFR), and high-fluorescence reticulocytes (HFR) populations, of which the HFR fraction includes the most immature reticulocytes.[11] Although not fully exploited in anti-doping context, IRF appeared as a particular sensitive indicator of bone marrow function and of erythropoietic status in clinic as it includes a count of the most immature reticulocytes.[12,13] Pearson's correlation between the genes, Ret%, and IRF was calculated, and the level of significance determined. A stronger correlation between IRF and *ALAS2*, *CA1*, and *SLC4A1* (Figure 3) was discovered than for Ret% and these three genes (Figure S1). This can be easily explained by the more abundant RNA content in IRF compared to Ret%, which influences greatly the gene expression analysis.

Although the gene expression significantly correlates with IRF, the magnitude of the changes in the number of transcripts was more significant than that of IRF or Ret%. It suggests that in response to ABT, the copies remaining in immature RBCs reflect more substantially the bone marrow activity than variations of IRF or Ret%. Indeed, *ALAS2*, *CA1*, and *SLC4A1* genes all code for proteins implicated in reticulocyte and erythrocyte metabolism and are thus specific to these sorts of cells and to erythropoiesis.[7,14,15] It suggests that transcriptomic biomarkers might be promising complements to hematological parameters of the ABP in a longitudinal monitoring for the detection of transfusion. Moreover, as demonstrated by Durussel et al., these genes might also serve as biomarkers of rHuEPO administration.[8]

However, to ensure the specificity of the genes' response to ABT and a possible integration into the adaptive model of the ABP, the impact of intrinsic and extrinsic factors such as altitude exposure or physical exercise must be fully characterized. Because the genes' functions are closely related to reticulocytes' metabolism, the influence of those confounding factors on IRF measurement should also be valuable for the transcriptomic biomarkers.

In conclusion, our results confirm and consolidate that autologous blood transfusion triggers a down-regulation of genes implicated in reticulocytes life cycle. The decrease of *ALAS2*, *CA1*, and *SLC4A1* expression correlated better with IRF than with Ret%. This stronger correlation could be explained by the higher amount of RNA contained in IRF compared to Ret%. The fluctuations of the number of transcripts seemed also more important than that of IRF or Ret%, suggesting that combined with those markers, they can improve the sensitivity of ABT detection.

## CONFLICT OF INTERESTS

The authors declare that they have no competing of interest relevant to the manuscript submitted to *Drug Testing and Analysis*.

## ACKNOWLEDGEMENT

This work was financially supported by a grant from the World Anti-Doping Agency (WADA) (Grant No. 12C14NL).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Salamin O, Mignot J, Kuuranne T, Saugy M, Leuenberger N. Transcriptomic biomarkers of altered erythropoiesis to detect autologous blood transfusion. *Drug Test Anal*. 2018;10:604–608. https://doi.org/10.1002/dta.2240

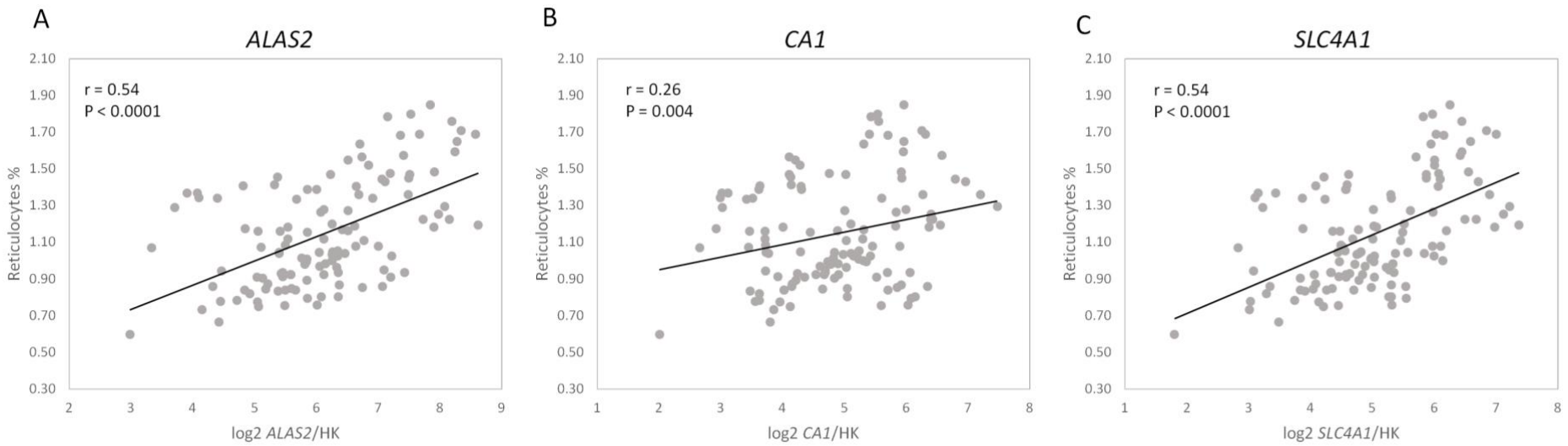


**Figure S1. (A) Correlation between Ret% and *ALAS2* after ABT**. Pearson correlation analysis shows a significant positive correlation of Ret% and *ALAS2* expression (Pearson $r = 0.54$; $P < 0.0001$; $n = 120$). *X* axis represents relative expression of *ALAS2* and *Y* axis represents Ret%. **(B) Correlation between Ret% and *CA1* after ABT**. Pearson correlation analysis shows a significant positive correlation of Ret% and *CA1* expression (Pearson $r = 0.25$; $P < 0.009$; $n = 120$). *X*-axis represents relative expression of *CA1* and *Y*-axis represents Ret%. **(C) Correlation between Ret% and *SLC4A1* after ABT.** Pearson correlation analysis shows a significant positive correlation of Ret% and *SLC4A1* expression (Pearson $r = 0.54$; $P < 0.0001$; $n = 120$). *X* axis represents relative expression of *SLC4A1* and *Y* axis represents Ret%.

### 2.1.4 Article 3: Detection of Stimulated Erythropoiesis by the RNA-Based 5'-Aminolevulinate Synthase 2 Biomarker in Dried Blood Spot Samples

The monitoring of transcriptomic biomarkers involves the invasive collection of venous blood sample from antecubital vein. DBS offer a viable alternative strategy to blood collection, especially in the anti-doping context with a simplified collection procedure and reduced transport costs. Hence, this article describes the development of a method for the simultaneous quantification of linear and circular forms of *ALAS2* RNA, one of the candidate transcriptomic biomarkers of blood doping, in DBS using RT-qPCR. For this purpose, an extraction method was developed and optimized for a trustworthy analysis of gene expression in DBS. The method was then applied to monitor stimulated erythropoiesis following a blood withdrawal in male subjects. The intervention triggered the stimulation of erythropoiesis and thus an increase of *ALAS2* expression peaking 6 days later. A strong correlation between results obtained for capillary finger-prick DBS and venous blood samples collected in Tempus tubes was observed suggesting that DBS represent a suitable matrix for monitoring *ALAS2* gene expression.

Moreover, the methodology was applied for the monitoring of exogenous stimulation of erythropoiesis through rHuEPO injections in a proof-of-concept study with two male subjects. *ALAS2* expression demonstrated a significant increase in DBS following three consecutive injections of therapeutic rHuEPO doses.

The results obtained in this study highlighted the potential of DBS as alternative matrix for the monitoring of transcriptomic biomarkers, which represent a promising complement to the hematological parameters of the ABP for the detection of blood manipulation.

**Clinical Chemistry** 65:12
1563–1571 (2019)



# Detection of Stimulated Erythropoiesis by the RNA-Based 5′-Aminolevulinate Synthase 2 Biomarker in Dried Blood Spot Samples

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**BACKGROUND:** Despite implementation of the Athlete Biological Passport 10 years ago, blood doping remains difficult to detect. Thus, there is a need for new biomarkers to increase the sensitivity of the adaptive model. Transcriptomic biomarkers originating from immature reticulocytes may be reliable indicators of blood manipulations. Furthermore, the use of dried blood spots (DBSs) for antidoping purposes constitutes a complementary approach to venous blood collection. Here, we developed a method of quantifying the RNA-based 5′-aminolevulinate synthase 2 (*ALAS2*) biomarker in DBS.

**MATERIALS:** The technical, interindividual, and intraindividual variabilities of the method, and the effects of storage conditions on the production levels of *ALAS2* RNA were assessed. The method was used to monitor erythropoiesis stimulated endogenously (blood withdrawal) or exogenously (injection of recombinant human erythropoietin).

**RESULTS:** When measured over a 7-week period, the intra- and interindividual variabilities of *ALAS2* expression in DBS were 12.5%–42.4% and 49%, respectively. Following withdrawal of 1 unit of blood, the *ALAS2* RNA in DBS increased significantly for up to 15 days. Variations in the expression level of this biomarker in DBS samples were more marked than those of the conventional hematological parameters, reticulocyte percentage and immature reticulocyte fraction. After exogenous stimulation of erythropoiesis via recombinant human erythropoietin injection, *ALAS2* expression in DBS increased by a mean 8-fold.

**CONCLUSIONS:** Monitoring of transcriptomic biomarkers in DBS could complement the measurement of hematological parameters in the Athlete Biological Passport and aid the detection of blood manipulations.



The hematological module of the Athlete Biological Passport (ABP)[7], which is based on the longitudinal monitoring of hematological parameters, was implemented by the World Anti-Doping Agency almost 10 years ago *(1)*. This tool introduced a new paradigm in antidoping, namely, the indirect detection of blood manipulations via individual follow-up of specific biomarkers. Although this method of detection has been an important deterrent to doping, as demonstrated by a reduction in extreme blood values *(2)*, the same hematological parameters have been monitored for a decade. Moreover, athletes are accustomed to the indirect detection method and adapted their blood doping regimen using microdoses of recombinant human erythropoietin (rhEPO) or microtransfusions, which are barely detectable using classical hematological parameters *(3, 4)*. Hence, antidoping research has focused on the discovery of additional biomarkers capable of increasing the sensitivity of the ABP through different "omics" technologies *(5)*.

In addition to other biomarkers *(4, 6)*, longitudinal monitoring of transcriptomic biomarkers from immature reticulocytes has been used to detect rhEPO injections and autologous blood transfusion *(7–9)*. The expression levels of several genes are markedly affected by blood doping, in-

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Received May 3, 2019; accepted August 26, 2019.
Previously published online at DOI: 10.1373/clinchem.2019.306829


[7] Nonstandard abbreviations: ABP, Athlete Biological Passport; rhEPO, recombinant human erythropoietin; ALAS2, delta-aminolevulinate synthase 2; DBS, dried blood spot; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; L, linear form; L+C, linear + circular forms; Ret%, reticulocyte percentage; IRF, immature reticulocyte fraction.

cluding that encoding 5′-aminolevulinate synthase 2 (ALAS2), an enzyme involved in the heme biosynthesis pathway that is particularly active during the biosynthesis of hemoglobin and thereby specific to erythropoietic processes.

Circular RNAs are abundantly produced in human cells, are essential in enucleated blood cells, and regulate different cellular processes by acting as microRNA sponges or transcriptional activators *(10)*. Furthermore, circular RNAs derive from back-spliced transcripts and are joined head to tail at splice sites, which renders them more stable and resistant to exonuclease-mediated digestion than linear RNAs *(10)*. Hence, quantification of these forms might be useful for doping detection. In a forensic context, analysis of the *ALAS2*[8] circular RNA form facilitates the detection of messenger RNA markers during body fluid identification *(11)*, suggesting that it could also be applied to blood manipulation monitoring.

Application of the ABP in antidoping requires whole-blood collection, which requires specialized personnel, refrigerated transport in a short time window, and sample analysis in <12 h. The use of dried blood spots (DBSs) as a complementary matrix could overcome these limitations. Collection of DBS involves the transfer of a limited volume of blood originating from a finger prick onto filter paper. This process benefits from minimal invasiveness, simplicity of sample collection, facilitated transport and storage, and reduced cost. Although DBSs have been used to screen for metabolic diseases in the pediatric environment for decades *(12)*, only recently have studies investigated their use for the direct detection of blood doping *(13)* and human growth hormone abuse *(14)* and their indirect detection through potential biomarkers *(15–18)*.

In this study, we developed a method of measuring the linear and circular forms of *ALAS2* RNA in DBS. The method was used to monitor erythropoiesis that was stimulated naturally through blood withdrawal or exogenously through rhEPO injection.

## Materials and Methods

### DBS SPOTTING METHOD

DBS were collected from 3 healthy male volunteers with different expression levels of *ALAS2*. A blood drop was generated on the finger with a dedicated lancet (Glucolet®2, Bayer), and a 20-μL blood sample was spotted onto Whatman 903™ protein saver cards (GE Healthcare). The cards were dried for a minimum of 1 h at room temperature and stored at 4 °C, unless used for stability analysis.

### DBS EXTRACTION

The whole DBS was excised from the card and transferred into a clean 2-mL conical polypropylene microcentrifuge tube. Total RNA was extracted with the miRNeasy® Mini Kit (Qiagen), according to the manufacturer's protocol with minor modifications. QIAzol lysis reagent (1 mL; Qiagen) was added to the tube, which was then incubated at 37 °C for 15 min with shaking, sonicated for 15 min, and then incubated for another 15 min at 37 °C with shaking. Chloroform (250 μL) was then added, and the sample was vortex-mixed twice and then centrifuged for 15 min at 13 400*g* after a 5-min incubation. The aqueous phase (525 μL) was transferred to a clean 2-mL conical polypropylene microcentrifuge tube, mixed with ethanol (800 μL), and transferred into an RNeasy Mini spin column. After washing, the RNA was eluted with 50 μL of RNase-free water.

### REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

The RNA was reverse-transcribed into complementary DNA in a final volume of 20 μL with the Transcriptor First Strand Complementary DNA Synthesis Kit (Roche Life Science), according to the manufacturer's instructions. The resulting complementary DNA was diluted 10-fold with nuclease-free water, and a 4 μL aliquot was used as a template for PCR amplification (final volume 10 μL) of the linear (*ALAS2L*) and linear + circular (*ALASL+C*) forms of *ALAS2*. Details regarding the primers' design for amplification of ALAS2L and ALASL+C were previously described by Zhang et al. *(11)*. Combination of TATA-box binding protein (*TBP*), Morf4 family-associated protein 1 (*MRFAP1*), actin-related protein 10 (*ACTR10*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) combined with the linear and circular forms of regulator of cell cycle (*RGCC*) were tested and used as reference genes for expression normalization, according to publicly available data sets and previous research *(10)*. PCRs were performed with a Roche LightCycler® 480 System [with the SYBR Green I/HRM Dye (465–510) program]. Raw and normalized data were analyzed with LightCycler 480 software (release 1.5.0). The *TBP*, *MRFAP1*, and *ACTR10* primer sets were purchased from Qiagen (primer sequences are available at *(19)*. The *ALAS2*, *GAPDH*, and *RGCC* primer sets were purchased from MicroSynth, and the sequences are provided in Table 1 in the online Data Supplement (see Table 1 in the online Data Supplement).

[8] Human Genes: *ALAS2,* 5′-aminolevulinate synthase 2; *TBP,* TATA-box binding protein; *MRFAP1,* Morf4 family-associated protein 1; *ACTR10,* actin-related protein 10; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *RGCC,* regulator of cell cycle.

#### INTRA- AND INTERASSAY VARIABILITY

Intra- and interassay variability was assessed with DBSs from 3 healthy male volunteers with different levels of *ALAS2* expression (see above). For intraassay variability, DBS from each volunteer were analyzed in quintuplicate within the same experiment. For the interassay variability analyses, quintuplicates were analyzed on 5 consecutive days.

#### STABILITY

The effect of different storage conditions on quantification of the *ALAS2* RNAs was assessed. In the first experiment, DBS cards from the 3 volunteers (see above) were divided into 2 groups and stored at room temperature or 4 °C for 1, 2, or 3 weeks. In the second experiment, the DBS cards from 2 volunteers were divided into 2 groups and stored at 37 °C or 4 °C for 4, 8, or 24 h. For each condition, DBS were extracted in duplicate.

## Clinical Study

#### LONGITUDINAL STUDY AND TAP™ PILOT STUDY

For the longitudinal study, DBS samples were obtained from 6 healthy volunteers (5 men and one woman) over a 7-week period. Informed written consent was obtained from each participant. Once a week, each volunteer provided at least 2 finger prick DBS (on Whatman 903 protein saver cards). DBS analyses of *ALAS2* expression levels were performed in duplicate.

To assess the efficiency of the TAP™ push-button blood collection device (SeventhSense Biosystems) for the measurement of *ALAS2* expression in DBS, capillary blood was collected from 10 healthy volunteers (8 men and 2 women) with both finger pricks and TAP devices positioned on the arm. Blood samples (20 μL) were deposited onto filter paper (Whatman 903 protein saver cards), left to dry for at least 1 h at room temperature, and then stored at 4 °C.

#### BLOOD WITHDRAWAL STUDY

A randomized, single-blind, placebo-controlled trial was approved by the Human Research Ethics Committee of the Canton of Vaud in Switzerland (Protocol: 2016–00324). Sixteen white men aged 20–35 years with a serum ferritin concentration ≤50 μg/L ± 10% and a body mass index of 18–30 who were eligible for blood donation according to national regulations were randomized to receive an intravenous injection of iron or placebo (NCT03014921). Written informed consent was obtained from each participant before participation. The iron supplement was a 250-mL perfusion of 0.9% NaCl (B. Braun Medical) combined with 10 mL of Ferinject®, a 500-mg complex of ferric carboxymaltose (Vifor Pharma). The placebo was a 250-mL perfusion of 0.9% NaCl (B. Braun Medical). Two weeks after iron or placebo injection, 1 unit of blood (approximately 450 mL) was withdrawn from each volunteer.

Whole-blood samples were collected in anticoagulated EDTA tubes (K2EDTA, 7.2 mg, 4 mL; BD Vacutainer, ref. 368861) and Tempus® Blood RNA tubes (Life Technologies) 1 day before and up to 30 days after blood withdrawal. DBS samples (Whatman 903 protein saver cards) were generated by depositing 20 μL of finger prick blood onto cards, which were left to dry for a minimum of 1 h at room temperature and stored at 4 °C. Hematological variables were measured in whole-blood samples in anticoagulated EDTA tubes with a fully automated hematology analyzer (Sysmex XN 2000, Sysmex). Total RNA was extracted from blood in Tempus tubes using the MagJET Whole Blood RNA Kit (Thermo Scientific), according to the manufacturer's instructions. RT-qPCR was performed as described earlier.

#### PILOT STUDY: ADMINISTRATION OF rhEPO

Two healthy volunteers were recruited at the Clinical Trial Unit at IMIM with the approval of the local Ethics Committee and the Spanish Medicine Agency. Details regarding the pilot clinical trial have been described previously (Code IMIMFTCL/EPO) *(13)*. Briefly, the 2 volunteers received 3 consecutive subcutaneous therapeutic doses (3500 IU, approximately 50 UI/kg) of first-generation rhEPO (Eprex®, Janssen-Cilag) at 48-h intervals. Finger prick DBSs were collected 3 days and immediately before the first injection and 1, 2 (just before the second administration), 3, 4 (just before third administration), and 7 days afterward. Blood drops were directly deposited onto Whatman FTA® DMPK-C cards (GE Healthcare), which were left to dry for a minimum of 4 h and stored at 4 °C.

#### STATISTICAL ANALYSES

Unless otherwise specified, data are expressed as the mean (±SE). Normality of data was determined with the Shapiro test. In cases of nonnormality, data were log2-transformed for statistical analyses. One-way repeated ANOVAs followed by post hoc pairwise comparisons (Tukey's honestly significant difference) were used to test differences between samples collected before and after blood withdrawal. Correlations were calculated with Pearson's correlation method. $P < 0.05$ was considered statistically significant. Statistical analyses were performed with R software.

## Results

#### INTRAASSAY AND INTERASSAY VARIABILITY

DBSs from 3 individuals with different *ALAS2* expression levels were analyzed in quintuplicate on the same day. The intraday coefficients of variation for *ALAS2*L were 3.6%, 12.5%, and 7.3% for volunteers 1, 2, and 3,

**Table 1. The effects of storage conditions on the expression levels of *ALAS2*L and *ALAS2*L+C in DBSs.[a]**

| 4 °C vs room temperature | 1 week | 2 weeks | 3 weeks |
|---|---|---|---|
| Linear *ALAS2* | −3.24% | −17.15% | −14.10% |
| Linear + circular *ALAS2* | −3.04% | −2.73% | −1.66% |
| **4 °C vs 37 °C** | **4 h** | **8 h** | **24 h** |
| Linear *ALAS2* | −6.30% | −11.00% | −22.9% |
| Linear + circular *ALAS2* | −5.70% | −3.50% | −13.20% |

[a] Values are expressed as the percentage reduction in *ALAS2*L and *ALAS2*L+C in samples stored at room temperature or 37 °C, relative to those in the refrigerated samples (4 °C). Data were normalized to the corresponding expression levels of reference genes.

respectively. Co-analysis of *ALAS2*L+C increased these intraday variabilities to 8.8%, 23.6%, and 9.03%, respectively. To assess interday variability, DBSs from the 3 volunteers were analyzed in quintuplicate over 5 days. The coefficients of variation for volunteer 1 were 14.14% for *ALAS2*L and 13.51% for *ALAS2*L+C; and for volunteer 2, 9.23% for *ALAS2*L and 13.12% for *ALAS2*L+C; and for volunteer 3, 14.53% for *ALAS2*L and 12.21% for *ALAS2*L+C.

### STABILITY

Next, the effect of storage conditions on the stability of the *ALAS2* biomarker in DBSs was assessed. In the first experiment, DBSs from 3 volunteers were stored for 1, 2, or 3 weeks at room temperature or 4 °C (as a control). After storage for 1 week, the expression level of *ALAS2*L in the room temperature sample was only 3.24% lower than that in the 4 °C sample (Table 1). However, after storage at room temperature for 2 or 3 weeks, the expression level of *ALAS2*L was reduced by 17.15% or 14.10%, respectively. By contrast, there were relatively small effects of storage time and temperature on the expression level of *ALAS2*L+C (Table 1). Also, different spotted blood volumes on DBS were not observed to affect the ALAS2 ratio (see Fig. 1 in the online Data Supplement).

In the second experiment, DBSs from 2 volunteers were stored at 37 °C or 4 °C (as a control) for 4, 8, or 24 h. When stored at 37 °C, the expression level of *ALAS2*L decreased progressively over 24 h, whereas the *ALAS2*L+C expression level remained remarkably constant and decreased by only 13% after storage for 1 day at 37 °C (Table 1).

### LONGITUDINAL MEASUREMENTS

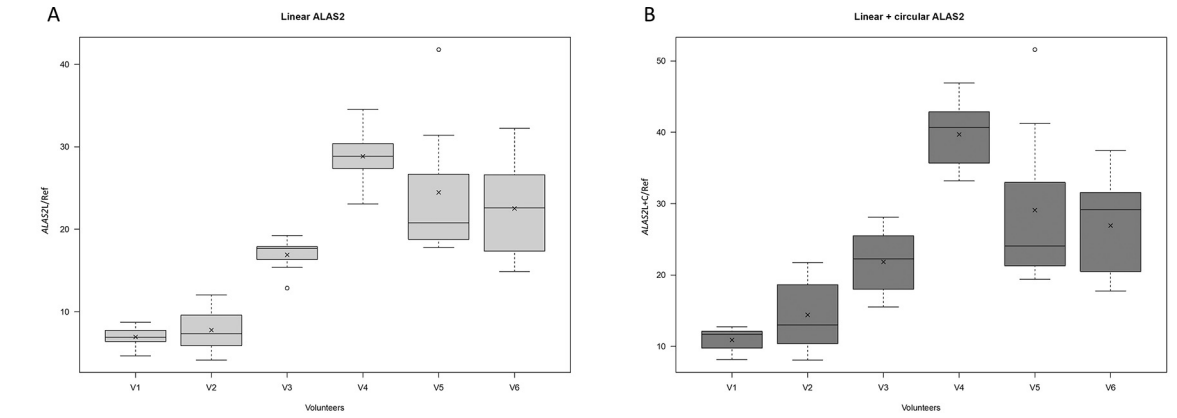
The variability of *ALAS2* expression in DBS was assessed with samples from 6 healthy volunteers collected once per week for 7 weeks. For each sample, the expression levels of *ALAS2*L and *ALAS2*L+C were measured in duplicate to evaluate the stability of these biomarkers over time. The mean relative expression level of *ALAS2*L ranged from 6.9 to 28.9 across the 6 individuals, whereas the intraindividual variability over the 7-week period ranged from 12.5% to 36.5% (Fig. 1A). For *ALAS2*L+C, the mean relative expression level (range, 10.9–39.7) and intraindividual variability (range, 13%–42.4%) were slightly higher than those of *ALAS2*L (Fig. 1B). The interindividual variabilities in the expression levels of *ALAS2*L and *ALAS2*L+C were 54% and 49%, respectively.

### BLOOD WITHDRAWAL CLINICAL STUDY

The reticulocyte percentage (Ret%) increased gradually after blood withdrawal and peaked significantly at day 6 (+31%, $P = 0.042$) (Fig. 2A). A similar response was observed for the immature reticulocyte fraction (IRF); however, the stimulation was more marked for this parameter (+61%, $P = 0.001$ at day 6) (Fig. 2B). Both parameters returned to baseline values after 30 days.

The relative expression levels of *ALAS2*L and *ALAS2*L+C were assessed in whole-blood samples collected in Tempus Blood RNA tubes, which are designed to stabilize genetic material in venous blood. Like the hematological parameters, *ALAS2*L and *ALAS2*L+C expression levels peaked 6 days after blood withdrawal (Fig. 3A). Compared with those in the blood samples collected 1 day before blood withdrawal, the expression levels of *ALAS2*L and *ALAS2*L+C were significantly higher on days 6–15 ($P = 0.0004$, $P = 0.0015$, and $P = 0.0019$) and 3–15 ($P = 0.047$, $P = 0.0013$, $P = 0.0006$, and $P = 0.018$) after withdrawal, respectively, and returned to baseline by day 30. In addition to the increased expression occurring earlier for *ALAS2*L+C than *ALAS2*L, the mean expression level of *ALAS2*L+C was approximately 1.7-fold higher than that of *ALAS2*L across all time points.

Next, the relative expression levels of *ALAS2*L and *ALAS2*L+C were quantified in DBSs collected at the same time points (1 day before and up to 30 days after blood withdrawal). The responses of these parameters to




| *ALAS2*L | V1 | V2 | V3 | V4 | V5 | V6 |
|---|---|---|---|---|---|---|
| n | 7 | 7 | 7 | 7 | 7 | 7 |
| Mean | 6.9 | 7.8 | 16.9 | 28.9 | 24.5 | 22.5 |
| SD | 1.4 | 2.8 | 2.1 | 3.7 | 8.9 | 6.6 |
| %CV | 19.5 | 36.1 | 12.5 | 12.7 | 36.5 | 29.1 |

| *ALAS2*L+C | V1 | V2 | V3 | V4 | V5 | V6 |
|---|---|---|---|---|---|---|
| n | 7 | 7 | 7 | 7 | 7 | 7 |
| Mean | 10.9 | 14.4 | 21.8 | 39.7 | 29.1 | 26.9 |
| SD | 1.8 | 5.3 | 4.8 | 5.2 | 12.3 | 7.5 |
| %CV | 16.4 | 36.4 | 22.0 | 13.0 | 42.4 | 27.7 |

**Fig. 1. Longitudinal monitoring of (A) *ALAS2*L and (B) *ALAS2*L+C expression levels over a 7-week period.**
Volunteers 1–5 were male, and volunteer 6 was female. Data were normalized to the corresponding expression levels of reference genes.

stimulation of erythropoiesis were similar to those in blood samples collected in Tempus tubes (Fig. 3B). However, in the DBS, the *ALAS2*L expression level was only significantly higher ($P = 0.046$) than that in the preblood withdrawal sample on day 9, whereas the level of *ALAS2*L+C was increased significantly on days 6 ($P = 0.0016$), 9 ($P = 0.0007$), and 15 ($P = 0.0069$). Unlike that in the Tempus tube samples, *ALAS2*L+C expression in the DBSs was still increased 30 days after blood withdrawal. Detection sensitivity was also 2-fold higher when the linear and circular forms of *ALAS2* were assessed. Furthermore, there were strong correlations between the expression levels of *ALAS2*L (Pearson $r = 0.7$) and *ALAS2*L+C (Pearson $r = 0.75$) in the Tempus tubes and DBS samples (Fig. 3C, D). Finally, for both matrices, there was no difference of *ALAS2* expression between the 2 groups of the clinical study (placebo or iron), and therefore the general mean is presented.

When measured in Tempus tubes or DBSs, the erythropoietic stimulation-induced increase in *ALAS2* expression (2.7-fold) on day 6 was much more pronounced than the increases in Ret% (1.3-fold) and IRF

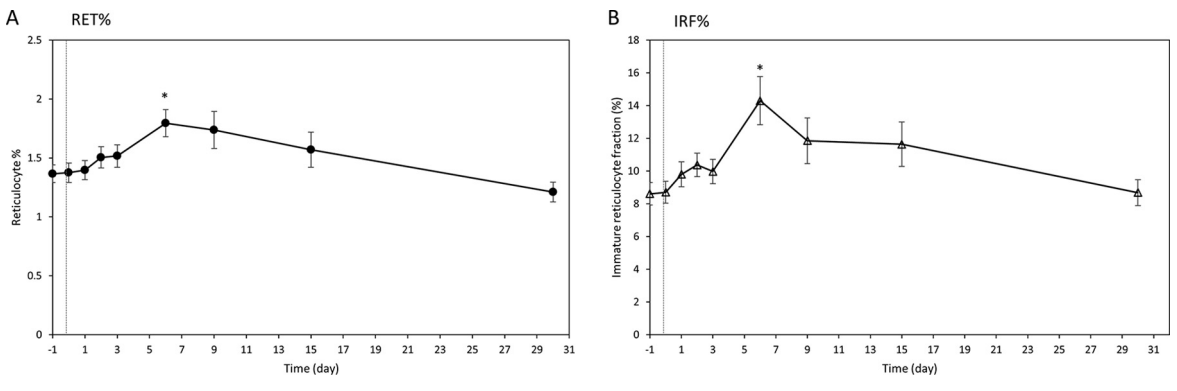



**Fig. 2. The effect of blood withdrawal on (A) Ret% and (B) IRF.**
Blood samples were collected 1 day before (−1) and up to 30 days after blood withdrawal. *$P = 0.042$ (Ret%) and $P = 0.001$ (IRF) versus time −1.

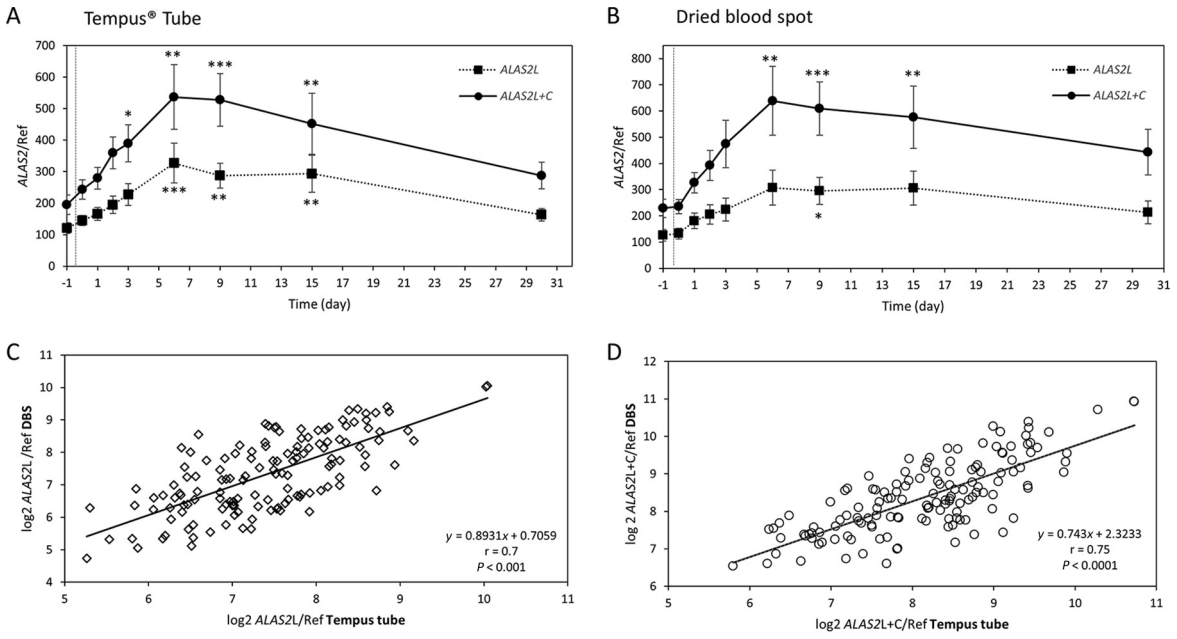



**Fig. 3. Relative expression levels of *ALAS2*L and *ALAS2*L+C after blood withdrawal.**

Relative expression levels of *ALAS2*L and *ALAS2*L+C in Tempus tube (A) and DBS (B) samples collected 1 day before (−1) and up to 30 days after blood withdrawal (n = 16). Dashed line indicates blood withdrawal. Data were normalized to the corresponding levels of reference genes. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with time −1. (C, D) Correlations between *ALAS2*L (C) and *ALAS2*L+C (D) expression levels in Tempus tube and DBS samples (n = 140).

(1.6-fold) at the same time-point and remained high for up to 15 days after blood withdrawal. Finally, the correlation between the level of *ALAS2*L or *ALAS2*L+C (measured in Tempus tube or DBS samples) and IRF was stronger than that between *ALAS2*L/L+C and Ret% (see Fig. 2 in the online Data Supplement).

### CLINICAL STUDY WITH rhEPO ADMINISTRATION

Next, the expression levels of *ALAS2*L and *ALAS2*L+C were assessed in 2 volunteers after exogenous stimulation of erythropoiesis by 3 consecutive injections of therapeutic doses of rhEPO every 48 h over 4 days (Fig. 4). Finger prick DBSs were collected 3 days and immediately before the first injection, and at 1, 2, 3, 4, and 7 days afterward. The expression levels of *ALAS2*L and *ALAS2*L+C started to increase after the second rhEPO injection (day 2), with a 4-fold increase at day 4 (vs 3 days preinjection). The maximal response was reached following the third injection, and a 9-fold increase (vs 3 days preinjection) was seen 7 days after the first injection. In contrast, only a 2-fold increase at 7 days after the first injection was observed for Ret% (see Fig. 3 in the online Data Supplement). The mean expression level of *ALAS2*L+C was 1.7-fold higher than that of *ALAS2*L in both volunteers, which confirmed the previous results observed for blood withdrawal study.

### TAP™ PILOT STUDY

Ten individuals were recruited to compare the expression levels of *ALAS2* in DBS collected by finger prick or the TAP device. For both *ALAS2*L and *ALAS2*L+C, there were strong correlations between the relative levels in DBS originating from finger prick and TAP collections ($r = 0.98$, $P < 0.001$; Fig. 5A, B).

## Discussion

This study describes the potential of DBSs as matrices for detecting stimulated erythropoiesis via monitoring of the *ALAS2* transcriptomic biomarker. DBSs offer the advantage of facilitated blood sample collection and may complement whole-blood samples collected for the ABP. Four to 5 DBSs on the same card could be used to detect exogenous performance-enhancing drugs or measure biomarkers specific to different doping methods. The methodology developed here has low analytical variability that is equivalent to the technical variability accepted for the measurement of Ret% in the hematological module of the ABP *(20)*. The expression level of *ALAS2* RNA in DBS remained remarkably stable when stored for 3 weeks at room temperature or 1 day at 37 °C. Co-analysis of the circular and linear forms of *ALAS2* improved the consistency of the biomarker in DBS stored under non-

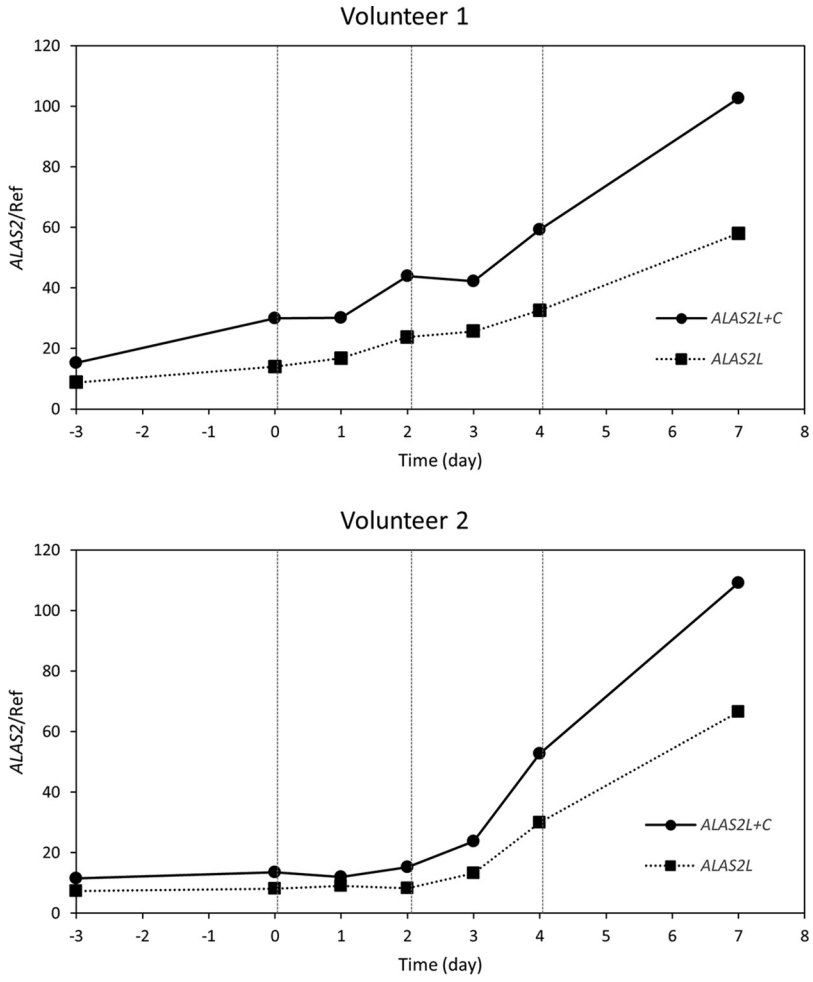



**Fig. 4. Relative expression levels of *ALAS2*L and *ALAS2*L+C in DBS after rhEPO injections.**

Data were normalized to the corresponding expression levels of reference genes. The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

refrigerated conditions (Table 1). These findings illustrate that, contrary to the whole-blood tubes used for the ABP, DBS cards could be transported at room temperature without the risk of *ALAS2* RNA degradation, which would facilitate sample collection, transport, and storage *(21, 22)*.

Detection of blood manipulations via the ABP is based on longitudinal measurements of hematological biomarkers that should remain consistent over time. Across a 7-week analysis of 6 individuals, the intraindividual variabilities of *ALAS2*L and *ALAS2*L+C expression levels were 12.5%–36.5% and 13.0–42.4%, respectively (Fig. 1). By comparison, Ret% varies from 5% to 21% during a competition season *(23)*; although this variability is lower than that of *ALAS2* expression observed here, the responses of *ALAS2*L and *ALAS2*L+C to blood manipulation are more marked than those of Ret% and IRF. Furthermore, the high interindividual variability observed here (54% for *ALAS2*L and 49% for *ALAS2*L+C) reinforces the idea that longitudinal monitoring of individuals should be applied.

In the clinical study, withdrawal of 1 unit of blood from 16 healthy volunteers triggered erythropoiesis and an increase in the expression of *ALAS2*, which peaked 6 days later. The expression levels of *ALAS2*L and *ALAS2*L+C remained high even 30 days following the intervention (Fig. 3B). There was a strong correlation between the results obtained for DBS and blood samples collected in Tempus tubes (Fig. 3C, D). For both sample types, the expression levels of *ALAS2*L and *ALAS2*L+C followed the same kinetics, with a peak at day 6 and increased levels 9 and 15 days after blood withdrawal

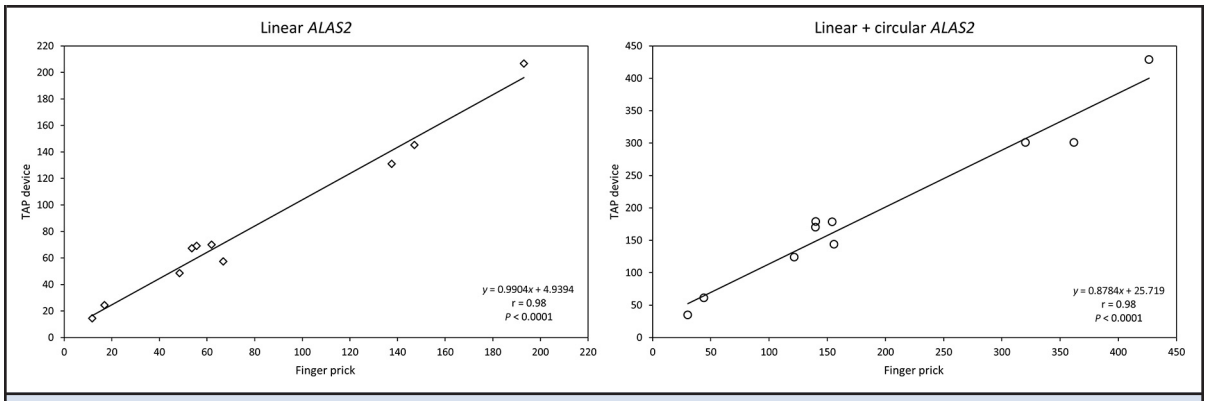



**Fig. 5. Correlations between *ALAS2*L and *ALAS2*L+C levels in finger prick and TAP DBS samples.**

(Fig. 3A). These results show that DBS represent a suitable matrix for monitoring *ALAS2* expression, and that there is no significant difference between venous and capillary blood with regard to *ALAS2* expression.

Moreover, the response observed for *ALAS2*L and *ALAS2*L+C was more marked than for conventional hematological parameters (Ret% and IRF) (Fig. 2). The *ALAS2* expression level was also more correlated with IRF than with Ret%, indicating that *ALAS2* expression levels in immature red blood cells may be a reliable predictor of the bone marrow response to blood withdrawal *(8)*.

*ALAS2*L and *ALAS2*L+C expression levels were quantified in DBS from 2 volunteers who received 3 rhEPO injections over 4 days. *ALAS2* expression increased progressively after the second rhEPO injection and was maximal 7 days after the first injection (3 days after the third injection). As seen in the blood withdrawal clinical study, simultaneous quantification of the linear and circular forms of *ALAS2* RNA had a higher response amplitude than detection of the linear form alone, indicating that *ALAS2*L+C is a suitable biomarker for monitoring erythropoiesis stimulated exogenously.

Blood collection using a finger prick may not always be possible, for example, in athletes who require use of their fingers for their sport. The TAP device offers an alternative painless method of generating DBSs. We demonstrated that the expression levels of *ALAS2* were comparable in finger prick and capillary blood samples collected from the arms of 10 individuals with a TAP device (Figs. 5 and see Fig. 4 in the online Data Supplement). Hence, the TAP system is a promising method of facilitated DBS collection.

This study has several limitations. The rhEPO clinical study included only 2 volunteers, so statistical significance could not be assessed, and DBS collection was performed over 7 days only, the time at which *ALAS2* expression was maximal. Hence, no rebound effect caused by negative feedback could be observed after cessation of rhEPO administration. Furthermore, 3 therapeutic doses were administered in this study, but it would be useful to investigate variations in *ALAS2* RNA expression levels after microdoses of rhEPO. Confounding factors can affect the Ret% and IRF; hence, the effects of these factors on *ALAS2* expression could also be applicable.

In summary, this study describes the measurement of a transcriptomic biomarker in DBS that is stable in samples stored at room temperature. As demonstrated previously *(7)*, *ALAS2* expression was sensitive to both endogenous and exogenous stimulation of erythropoiesis. Longitudinal monitoring of the *ALAS2* RNA expression level in DBS could constitute a complementary approach to classic hematological parameters for the detection of blood doping and could also be used to monitor anemia in the clinical setting without the need for venipuncture. Additional genes affected by erythropoiesis and blood manipulations *(7, 8)* could be added to the method to increase the specificity of the results.

**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

O. Salamin, statistical analysis; E. Gottardo, statistical analysis; C. Schobinger, administrative support; G. Reverter-Branchat, provision of study material or patients; T. Kuuranne, administrative support; N. Leuenberger, statistical analysis, administrative support, provision of study material or patients.

**Authors' Disclosures or Potential Conflicts of Interest:** *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

**Employment or Leadership:** J. Segura, Fundacio IMIM, Barcelona.
**Consultant or Advisory Role:** None declared.
**Stock Ownership:** None declared.
**Honoraria:** None declared.
**Research Funding:** Partnership for Clean Competition (grant: 120500 R318).
**Expert Testimony:** None declared.
**Patents:** None declared.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

**Acknowledgments:** The authors are grateful to Partnership for Clean Competition (grant: 120500 R318) for their financial support. The authors thank Francesco Loria for his excellent technical assistance.

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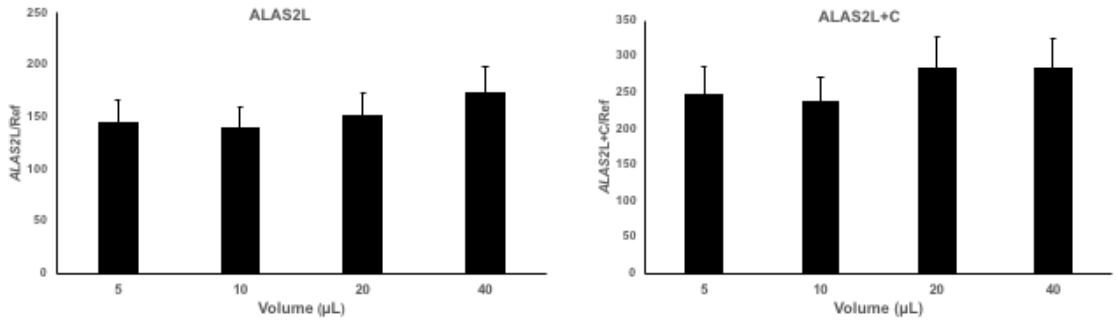
**Supplemental Material**

Supplemental Table 1. Primers for the amplification of targets.

| Genes | Primer type | Primer sequence | Reference |
|---|---|---|---|
| ALAS2 | L-primers[1] | F: TGTGTCCGTCTGGTGTAGTA<br>R: AAACTTACTGGTGCCTGAGA | *(11)* |
| ALAS2 | LC-primers[2] | F: ACCTTCGTGGATGAGGTCCAT<br>R: CACGGGTGCTGGCAATGTAG | *(11)* |
| RGCC | L-primers[1] | F: ACTGTCACTCCTCAGAAAGCTAAA<br>R: GCAGGTCCTCGGAACTTTCT | *(10)* |
| RGCC | C-primers[3] | F: GCTAGAAGCCTTCATTGCTGA<br>R: AGAGAAGAGCTGGGGTAGAGT | *(10)* |
| GAPDH | L-primers[1] | F: CCTGCACCACCAACTGCTTA<br>R: GGCCATCCACAGTCTTCTGAG | |

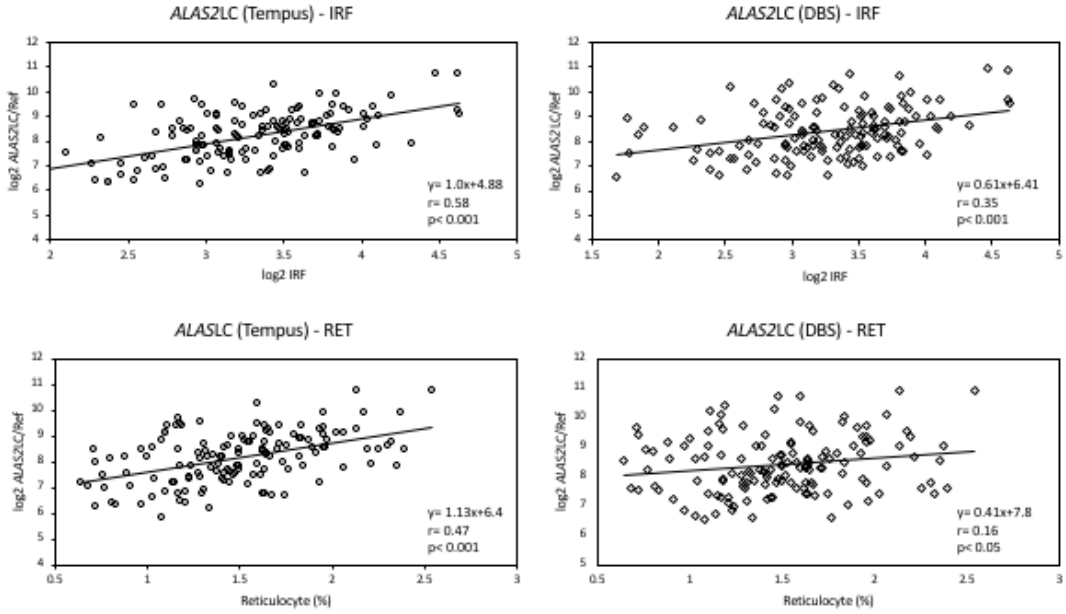
[1]L-primers that mainly amplify linear transcripts.
[2]LC-primers that can simultaneously amplify linear and circular transcripts.
[3]C-primers that mainly amplify circular transcripts.
ALAS2, 5'-aminolevulinate synthase 2; RGCC, regulator of cell cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Figure S1**



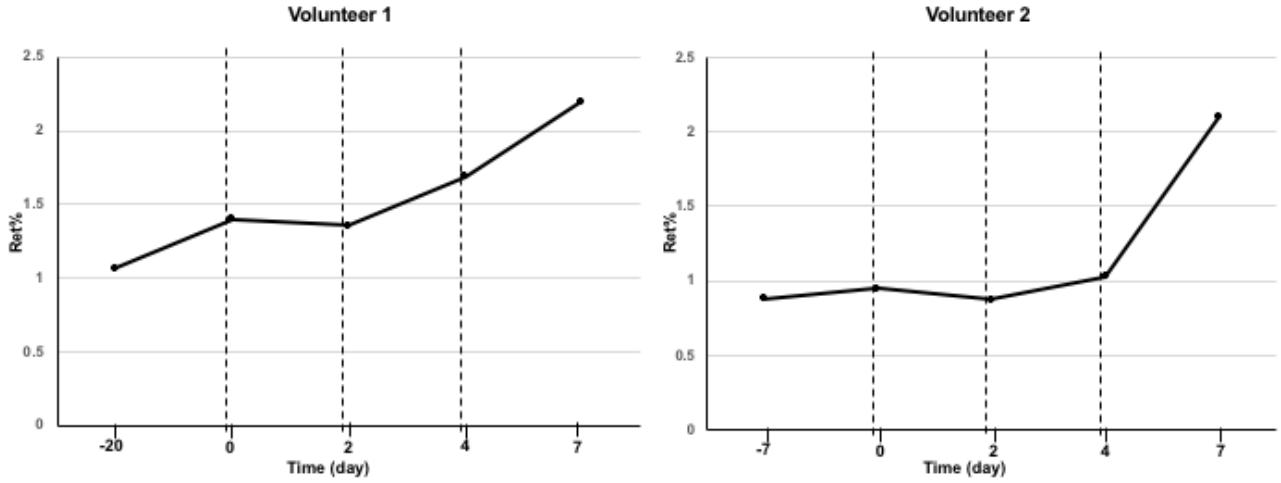

**Supplemental Figure 1. Relative expression levels of *ALAS2*L and *ALAS2*L+C with different whole blood volume spotted on DBS.**
The mean relative expression levels of *ALAS2*L and *ALAS2*L+C for the three volunteers is shown in the figure. Data were normalized to the corresponding levels of reference genes.

**Figure S2**



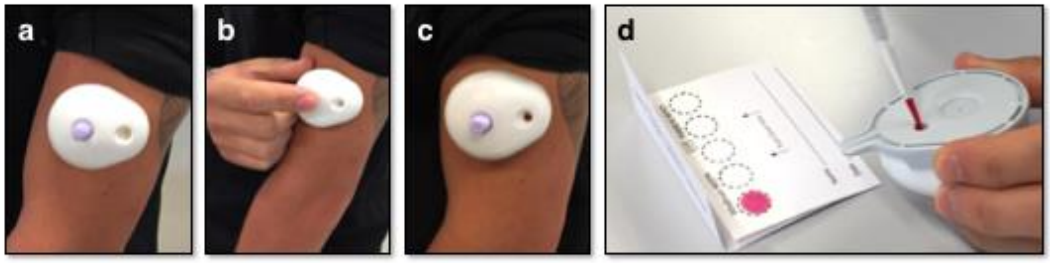

**Supplemental Figure 2. Correlation between ALAS2L+C measured in Tempus and DBS, IRFs and Ret%.**

**Figure S3**




**Supplemental Figure 3. Reticulocyte percentage (Ret%) after three rhEPO injections.**
The dashed lines indicate subcutaneous injections of rhEPO (~50 IU/kg).

Figure S4




**Supplemental Figure 4. The procedure used for TAP™ DBS collection.**

## 2.2 Transcriptomic biomarkers of testosterone administration

### 2.2.1 Article 4: Circulating microRNA-122 as Potential Biomarker for Detection of Testosterone Abuse

Transcriptomics also includes the study of non-coding RNAs such as microRNAs (miRNAs). These transcripts of 20-24 nucleotides regulate gene expression post-transcriptionally by decreasing mRNA stability or by inhibiting translation of mRNA into protein [198]. They are therefore implicated in many biological functions such as differentiation, proliferation, cell signaling, metabolism or apoptosis and exhibit specific expression patterns to a tissue or cell type [199]. In addition, miRNAs are present in many body fluids such as plasma, saliva or tears [200] and these so-called circulating miRNAs have been associated with specific altered physiological conditions such as cancers or cardiovascular diseases [201,202]. Circulating miRNAs are particularly stable in cell-free conditions and resistant to RNases, fluctuations in pH or to multiple freeze-thaw cycles. In the anti-doping context, the use of circulating miRNAs as biomarkers was evaluated for the detection of blood doping [203,204] or growth hormone administration [205].

This article describes the investigation of circulating miRNAs as surrogate transcriptomic biomarkers for the detection of testosterone doping. Plasma samples collected during a testosterone administration (oral and transdermal) study with male subjects were profiled for the relative abundance of 372 miRNAs one day after drug administration using miRNA Ready-to-Use PCR arrays (Exiqon, Vedbaek, Denmark). The identified candidate biomarkers with this screening method were then confirmed using RT-qPCR with a longitudinal monitoring of their levels following testosterone administration. Circulating miR-122, a miRNA primarily expressed in the liver, was significantly upregulated 1 day after drug intake. Given that exogenous testosterone is extensively metabolized by the liver, it suggests that plasma miR-122 might represent a potential biomarker of T doping. However, this finding also implies that circulating miR-122 may lack specificity and that its plasma level could also be impacted by various conditions or medications altering liver function other than testosterone as demonstrated by some studies [206,207]. Therefore, alternative biomarkers from another layer of data (metabolome) had to be investigated for a better sensitivity and specificity in the context of T detection.

PLOS | ONE

RESEARCH ARTICLE

# Circulating microRNA-122 as Potential Biomarker for Detection of Testosterone Abuse

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## Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and thus influence many cellular and physiological processes. miRNAs are also present in cell-free body fluids such as plasma or serum, and these circulating miRNAs are very stable, sensitive, and specific biomarkers of pathophysiological states. In this study, we investigated whether circulating miRNAs could serve as biomarkers of exogenous testosterone administration. Misuse of testosterone as a performance-enhancing drug is thought to be widespread in sports. Detection of testosterone through the urinary steroid profile of the Athlete Biological Passport faces several obstacles, indicating that new biomarkers are required. To this end, we analyzed plasma miRNA levels by high-throughput quantitative real-time PCR. Plasma samples were obtained before and at several time points after transdermal and oral testosterone administration. Screening identified three potential candidate miRNAs that were altered by both routes of testosterone administration. Longitudinal monitoring of these candidates revealed that variation in two of them (miR-150 and miR-342), relative to the corresponding levels in control samples, was testosterone-independent. However, levels of the liver-specific miR-122 increased 3.5-fold 1 day after drug intake. Given that testosterone is metabolized by the liver, this observation suggests that miR-122 in cell-free fluids may be used as a sensitive biomarker of testosterone misuse via multiple dosing routes and could therefore be integrated into a blood-based multiparametric follow-up.



**Citation:** Salamin O, Jaggi L, Baume N, Robinson N, Saugy M, Leuenberger N (2016) Circulating microRNA-122 as Potential Biomarker for Detection of Testosterone Abuse. PLoS ONE 11(5): e0155248. doi:10.1371/journal.pone.0155248

**Editor:** Pierre Busson, Gustave Roussy, FRANCE

**Received:** January 21, 2016

**Accepted:** April 11, 2016

**Published:** May 12, 2016



**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** The authors have no support or funding to report.

**Competing Interests:** The authors have declared that no competing interests exist.

## Introduction

Testosterone, the principal endogenous androgenic anabolic steroid (EAAS), is secreted by endocrine glands. It regulates many physiological processes in adult males, including muscle protein metabolism, sexual and cognitive functions, erythropoiesis, hepatic lipid metabolism, and bone metabolism [1, 2]. Although its use in sports is prohibited by the World Anti-Doping Agency (WADA), testosterone is widely abused by athletes as a performance-enhancing drug [3]. In addition to their anabolic action, small doses of anabolic-androgenic steroids can also

lower fatigue levels, and accelerate recovery, leading to a higher training load and faster increase in physical performance [4].

In doping control, screening for testosterone (T) misuse is primarily performed by GC-MS and GC-MS/MS through assessment of a urinary steroid profile that includes T, its precursors, and its metabolites. For several decades, evaluation of the ratio of testosterone to epitestosterone (T/E) has been the gold standard for screening for T administration. Based on population references, a cut-off value of 4 was established as the threshold for this ratio [5]. When the ratio exceeds the threshold, urine samples are submitted for confirmation analysis with GC-C-IRMS [4]. However, despite its sensitivity and specificity, the T/E biomarker suffers from a short detection window, particularly when T is taken orally, as well as high inter-individual variability due to natural high T/E and genetic polymorphism [6–8]. To overcome these limitations and improve the ability to detect T, the urinary steroidal module of the Athlete Biological Passport (ABP) was recently implemented (January 1, 2014). This module relies on a longitudinal/multiparametric approach using specific markers of altered metabolism of endogenous steroidal hormones, including T/E and other metabolites ratios (Androsterone (A)/T, A/Etiocholanolone (Etio), 5α-Adiol/5β-Adiol), for each athlete using intra-individual references generated by a Bayesian Adaptive Model [9].

However, more than 1 year after its implementation, the efficacy of the steroidal module of the ABP is still in question, principally due to instability of urine samples (bacterial contamination, medications, enzyme induction-inhibition, etc.) and polymorphism of the *UGT2B17* gene, which encodes a protein responsible for testosterone glucuronidation [10–12]. Therefore, new approaches and potential biomarkers of T abuse must be investigated to improve the discriminative performance of the ABP.

MicroRNAs (miRNAs), a class of small non-coding RNAs about 22 nucleotides in length [13], regulate gene expression post-transcriptionally by decreasing mRNA stability or inhibiting translation of mRNA into protein. Consequently, miRNAs are implicated in many cellular processes such as differentiation, proliferation, metabolism, and apoptosis [14, 15]. Recently, miRNAs were detected in various body fluids, including urine, plasma, serum, saliva, and tears [16]. Although the physiological functions and regulation of these so-called 'circulating miRNAs' are largely unknown, they are associated with specific pathophysiological states such as cancers and cardiovascular diseases [17–19]. In an anti-doping context, multiple groups reported associations between altered plasma miRNA expression profiles and doping interventions such as erythropoiesis-stimulating agents (ESA), autologous blood transfusion, and recombinant growth hormone (rhGH) administration [20–23]. In comparison with proteins, circulating miRNAs offer non negligible advantages as non-invasive biomarkers. miRNAs are highly stable in body fluids, and are resistant to RNases, fluctuations in pH, and to multiple-freeze/thaw cycles [24, 25]. In addition, expression of many miRNAs is specific to a tissue or cell type [26], and their levels can be easily measured using common laboratory techniques, including amplification-associated methods that require very small sample volumes [27].

Therefore, in this study we investigated whether circulating miRNAs can be used as surrogate biomarkers of testosterone abuse via various dosing routes. We compared the resultant data with both commonly measured parameters and emerging parameters.

## Material and Methods

### Clinical study

All samples were derived from a previous clinical study that included 19 healthy male volunteers aged 19–28 (mean 24.3 ± 2.7 years) with BMI between 18.3 and 27.2 kg/m$^2$ (mean 23.1 ± 2.4) and different *UGT2B17* genotypes (ins/ins, ins/del, and del/del) [6]. All subjects

gave signed consent form and the protocol was authorized by the Ethical Commission for the Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Switzerland) and Swissmedic (Protocol no. 155/11). The clinical trial took place over 5 weeks, divided into four major phases (S1 Fig): the first week was dedicated to collection of control samples (no treatment), followed by the administration of two transdermal systems that delivered 2.4 mg/24 h (Testopatch®, Pierre Fabre Pharma GMBH, Freiburg, Germany) during the second week. After a wash-out period of 2 weeks, two 40 mg testosterone undecanoate (TU) tablets (Andriol Testocaps®, Essex Chemie AG, Luzern, Switzerland) were ingested by each volunteer. First blood collection was performed at 07:00 AM. Plasma and serum samples were centrifuged at 1500 x g for 15 min after 15 min stabilization at room temperature. Plasma and serum aliquots were kept at -20°C until analyses as described in [6].

## miRNA quantitative real-time PCR (qRT-PCR)

Total RNA, including miRNAs, was isolated from 200 μl of plasma using the miRCURY RNA Isolation Kit—Biofluids (Exiqon, Vedbaek, Denmark) with minor modifications. Briefly, before RNA extraction, all plasma or serum samples were thawed completely, followed by centrifugation at 16,000 × g for 10 min to remove remaining cell debris and platelets [28, 29]. During the treatment with lysis buffer, 1 μl of spike-in mix containing synthetic UniSp2, UniSp4, and Unisp5 and 1 μl of cel-miR-39-3p (RNA Spike-in Kit, Exiqon) were added to the samples as a control for extraction efficiency. After protein precipitation, supernatant was loaded onto spin columns. Finally, the purified RNA was eluted with 35 μL RNAse free-water.

For miRNA profiling, 4 μL of eluted RNA was used in a 20-μL reverse transcription (RT) reaction. UniSp6 Spike-in miRNA was added to the RT reaction mix. The resulting cDNA was diluted 110 times and profiled for the relative abundance of 372 miRNAs using miRNA Ready-to-Use PCR, Human panel I, V4.R quantitative real-time PCR (qRT-PCR) arrays (Exiqon), as described in [21]. Raw data were analyzed using the LightCycler 480 software (version 1.5.0). All primer sequences are available on the Exiqon web site (http://www.exiqon.com/mirna-pcr-primer).

## Hematology, clinical chemistry, and immunology

Blood parameters were analyzed in EDTA samples using a fully automated hematology analyzer (Sysmex XT-2100i, Sysmex, Norderstedt, Germany). Free T was measured in serum using Immulite technology (Siemens AG, Munich, Germany). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and C-reactive protein (CRP) serum levels were measured using a Dimension EXL200 automated system (Siemens Healthcare Diagnostic SA, Zurich, Switzerland).

## Statistics

Unless specified otherwise, data are expressed as means ± SEM versus baseline. Statistical comparisons were performed using the two-tailed Student's t-test (for free T) or non-parametric Wilcoxon signed-rank test (for miRNA levels). Differentially expressed miRNAs were identified using the R package LIMMA as described in [21]. $P < 0.05$ was considered to represent a statistically significant difference. Average Ct corresponds to the mean of Ct values at 0 and 24 h. Fold-Change (FC) was calculated from Ct values. Conditions were compared by one-way ANOVA (the *aov* function in R) and post-hoc pairwise comparisons were performed with Tukey's Honestly Significant Difference (the *TukeyHSD* function in R). Descriptive and statistical comparisons were performed using various softwares (Microsoft Office Excel 2007, StataIC, StataCorp and R).

## Results

To investigate the utility of circulating miRNAs as biomarkers of testosterone misuse, miRNA profiling was performed by RT-PCR in samples obtained before (0 h) and at various time points after (12 and 24 h) oral and transdermal T administration (S1 Table). Data were analyzed using a linear model that paired values by subjects. Out of 372 miRNAs tested, 134 (including controls) were detected after ingestion of two TU tablets, and 143 were detected after application of diffusion patches. This screen enabled to identify the circulating miRNAs that were most affected by T administered by different routes (Table 1 and S2 Table). These candidates were selected for further investigations.

Significant variations in the levels of candidate miRNAs were only observed 24 h after testosterone intake via either route. Most circulating miRNAs decreased >2-fold at 24 h versus 0 h after administration; of these, miR-342 and miR-150 were the only miRNAs that were significantly affected by oral and transdermal T administration. By contrast, miR-122 was the only miRNA whose abundance increased under both conditions (2.3-fold on average). These miRNAs were selected for further analyses because of their abundance, statistical significance and fold change.

Profiling of circulating miRNAs as biomarkers of T also permitted to identify miRNAs that were not responsive to the treatment (S3 Table). Among these, miR-486-5p exhibited a low coefficient of variation across all samples under both conditions and was present at high levels in plasma. Therefore, miR-486-5p was selected as an internal control for RT-PCR validation.

The results of the screen were confirmed by individual assays throughout the timeline, with special emphasis on the three miRNAs identified as candidate markers of T administration. Measurements of miR-342 and miR-150 at the various time points confirmed the screening results: the levels of both miRNAs decreased significantly 24 h after T administration relative to their levels at 0 h (prior to T administration) (Fig 1). For miR-342, the decrease was also significant 2 and 8 h after transdermal T application (Fig 1A). However, when observing the kinetics of these two miRNAs during the control period, we noticed that the curves resembled those of the T periods. The three conditions differed primarily in regard to the baseline levels of miR-342 and miR-150.

T administration induced miR-122 with a peak occurring 24 h after drug intake (Fig 2). Consistent with the screening results, the increase in plasma miR-122 level was evident only at 24 h after T administration. Exogenous testosterone increased the levels of this circulating miRNA by an average of 4-fold. The amplitude of variation between baseline and 24 h was larger after ingestion of TU than after application of diffusion patches (Fig 2). During the control phase, the plasma level of miR-122 did not change significantly.

Circulating miR-122 level was integrated in an individual longitudinal follow-up setting subject-based threshold (Fig 3). For each subject, the threshold was calculated as the mean + three standard deviations (SD) of the values during the control phase [6, 7, 30]. Fig 3 shows measurements of miR-122 levels over time in two volunteers with different *UGT2B17* genotypes following oral T administration, along with their individual thresholds.

Because miR-122 is highly expressed by the liver, the activity of hepatocellular enzymes (ALT/AST) was also determined in the serum of volunteers. In these analyses, the focus was placed on samples that exhibited a large increase of plasma miR-122 following T administration. However, longitudinal monitoring revealed no significant changes in either enzyme over time (S2 Fig). Likewise, no significant change was observed in CRP, a biochemical marker of leukocytes, after T administration (S2 Fig).

Because intake of exogenous T is thought to affect endogenous T concentration, serum free T concentrations of volunteers were measured by immunoassay under each condition (Fig 4).

**Table 1. miRNAs most affected by testosterone.** List of the 12 most affected miRNAs 1 day after ingestion of two TU tablets or application of two Testo-patches, identified by miRNA expression profiling.

| | Oral Testosterone | | | | | | Transdermal Testosterone | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| miRNA | Fold-Change (FC) | FC. low | FC. high | Average Ct | P.value | miRNA | Fold-Change (FC) | FC. low | FC. high | Average Ct | P.value |
| **hsa-miR-122-5p** | **2.2** | **-1.07** | **5.17** | **30.24** | **0.015168** | **hsa-miR-122-5p** | **2.3** | **1.34** | **3.95** | **30.45** | **0.000812** |
| hsa-miR-146b-5p | -2.1 | -3.66 | -1.21 | 35.08 | 0.002894 | hsa-miR-425-5p | -1.65 | -2.63 | -1.04 | 30.52 | 0.006124 |
| hsa-miR-629-5p | -2.39 | -4.04 | -1.41 | 34.15 | 0.002396 | hsa-miR-324-3p | -1.76 | -2.48 | -1.25 | 32.74 | 0.002622 |
| hsa-miR-29b-3p | -2.51 | -4.5 | -1.4 | 33.91 | 0.001766 | hsa-miR-582-5p | -1.97 | -3.76 | -1.04 | 34.76 | 0.005832 |
| hsa-miR-29c-3p | -2.64 | -5.76 | -1.21 | 32.1 | 0.003952 | hsa-let-7c-5p | -2 | -3.92 | -1.02 | 34.68 | 0.007064 |
| hsa-miR-874-3p | -2.93 | -6.3 | -1.36 | 33.99 | 0.002421 | hsa-let-7g-5p | -2.1 | -4.56 | 1.03 | 28.93 | 0.009684 |
| hsa-miR-361-5p | -2.99 | -5.58 | -1.6 | 31.47 | 0.002397 | hsa-miR-423-5p | -2.13 | -3.57 | -1.27 | 31.59 | 0.001973 |
| hsa-miR-140-3p | -3.16 | -9.05 | -1.11 | 28.91 | 0.006288 | hsa-miR-29a-3p | -2.95 | -9.75 | 1.12 | 30.99 | 0.009417 |
| hsa-miR-423-3p | -3.21 | -8.84 | -1.17 | 31.83 | 0.010688 | **hsa-miR-342-3p** | **-2.99** | **-9.08** | **1.02** | **28.86** | **0.005837** |
| hsa-miR-29a-3p | -3.59 | -8.82 | -1.46 | 31.36 | 0.000905 | hsa-miR-338-3p | -3.07 | -7.59 | -1.24 | 33.78 | 0.001644 |
| **hsa-miR-342-3p** | **-5.7** | **-13.93** | **-2.33** | **28.15** | **0.000228** | hsa-miR-424-5p | -3.36 | -9.89 | -1.14 | 32.7 | 0.002452 |
| **hsa-miR-150-5p** | **-6.87** | **-21.08** | **-2.24** | **27.23** | **0.000295** | **hsa-miR-150-5p** | **-3.96** | **-15.47** | **-1.02** | **26.85** | **0.005019** |
| hsa-miR-486-5p | -1.29 | -2.3 | 1.39 | 27.35 | 0.3626 | hsa-miR-486-5p | -1.14 | -1.7 | 1.31 | 27.78 | 0.5245 |

*miRNAs selected for individual assays are indicated in bold and miR-486-5p was chosen as an endogenous control for data normalization.

doi:10.1371/journal.pone.0155248.t001

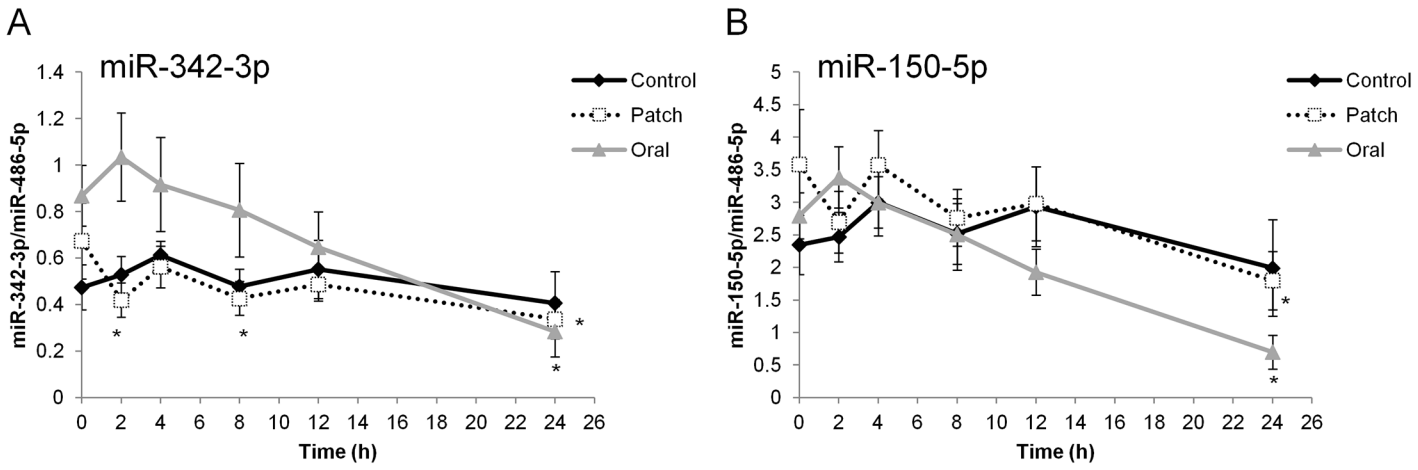


**Fig 1. Circulating miRNA levels after oral and transdermal administration of T and during the control period.** Levels of miR-342 (A) and miR-150 (B) in plasma samples collected from healthy volunteers after administration of oral (gray line) or transdermal (dashed line) T or in the control phase (black line). Collection time points are indicated on the x-axis. Data were normalized against the corresponding levels of endogenous miR-486-5p. Values are expressed as means (±SE) of 19 independent samples. (*) indicates statistically significant difference relative to time = 0 h.

doi:10.1371/journal.pone.0155248.g001

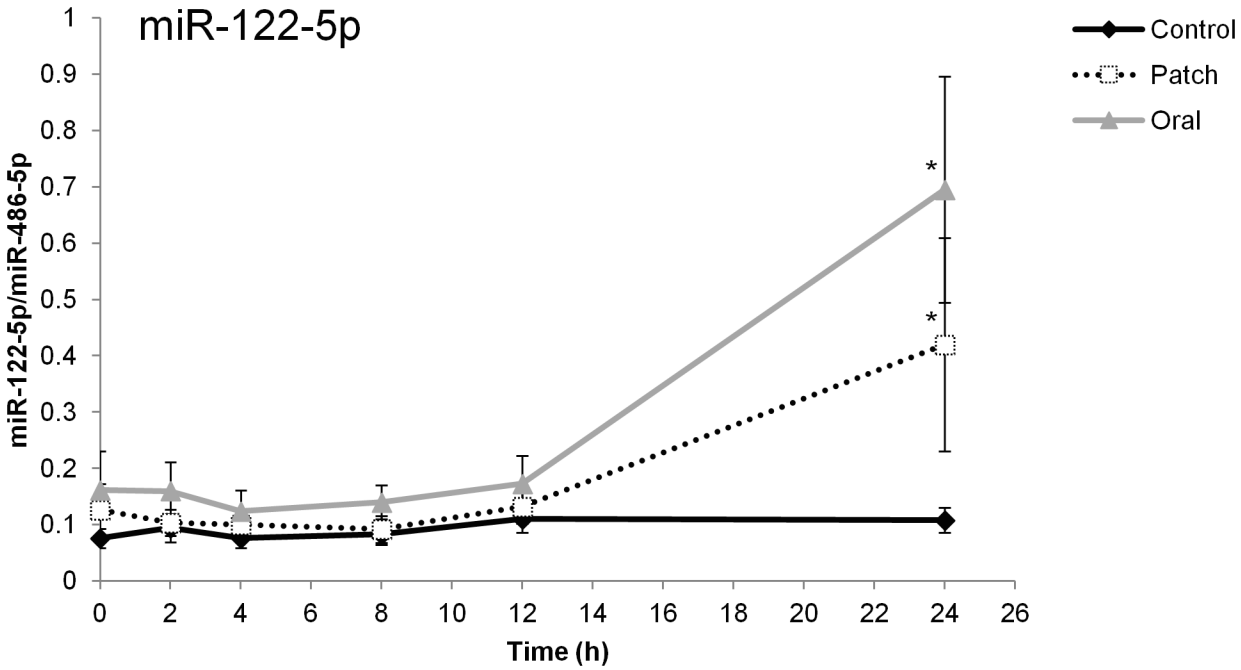


**Fig 2. Circulating miR-122 levels after oral or transdermal T administration and during the control period.** Level of plasma miR-122 after ingestion of two TU tablets (gray line) and application of two Testopatches (dashed line) or in the control period (black line). Data were normalized against the corresponding levels of endogenous miR-486-5p Collection time points are indicated on the x-axis. Values are expressed as means (±SE) of 19 independent samples. (*) indicates statistically significant difference relative to time = 0 h and the control.

doi:10.1371/journal.pone.0155248.g002

A clear increase in serum free T was apparent from 8 to 24 h after transdermal T administration. However, ingestion of oral T did not affect serum free T, which followed the same pattern as during the control period (significant decrease at 8 and 12 h, returning to baseline value at 24 h).

## Discussion

In this study, we investigated whether circulating miRNAs could be used to detect testosterone abuse. Our key finding was that an exogenous source of testosterone triggered an increase in the level of a specific miRNA relative to a control period during which no treatment was administered. This observation suggests that this circulating miRNAs may serve as biomarkers to detect exogenous testosterone intake.

Our screen for potential circulating miRNAs whose levels were altered by testosterone identified three candidates that might serve as efficient biomarkers of testosterone administration via different dosage routes. These candidates were confirmed by individual qPCR assays, and their kinetics were compared with the control results.

Administration of T via either of two routes triggered a significant decrease in the levels of miR-342 and miR-150. These miRNAs are mainly expressed in white blood cells, and miR-150 is particularly abundant in mature B and T lymphocytes [31, 32]. Levels of both miRNAs decreased markedly relative to baseline (0 h) 1 day after T administration. WBC count did not change significantly over the course of the study, eliminating this parameter as an explanatory variable. One potential explanation for this decrease is the immunosuppressive effect of T, which might decrease the rate of release of miRNAs from WBCs [33–35]. However, the kinetics of natural variations in levels of miR-342 and miR-150 (control) were very similar to the

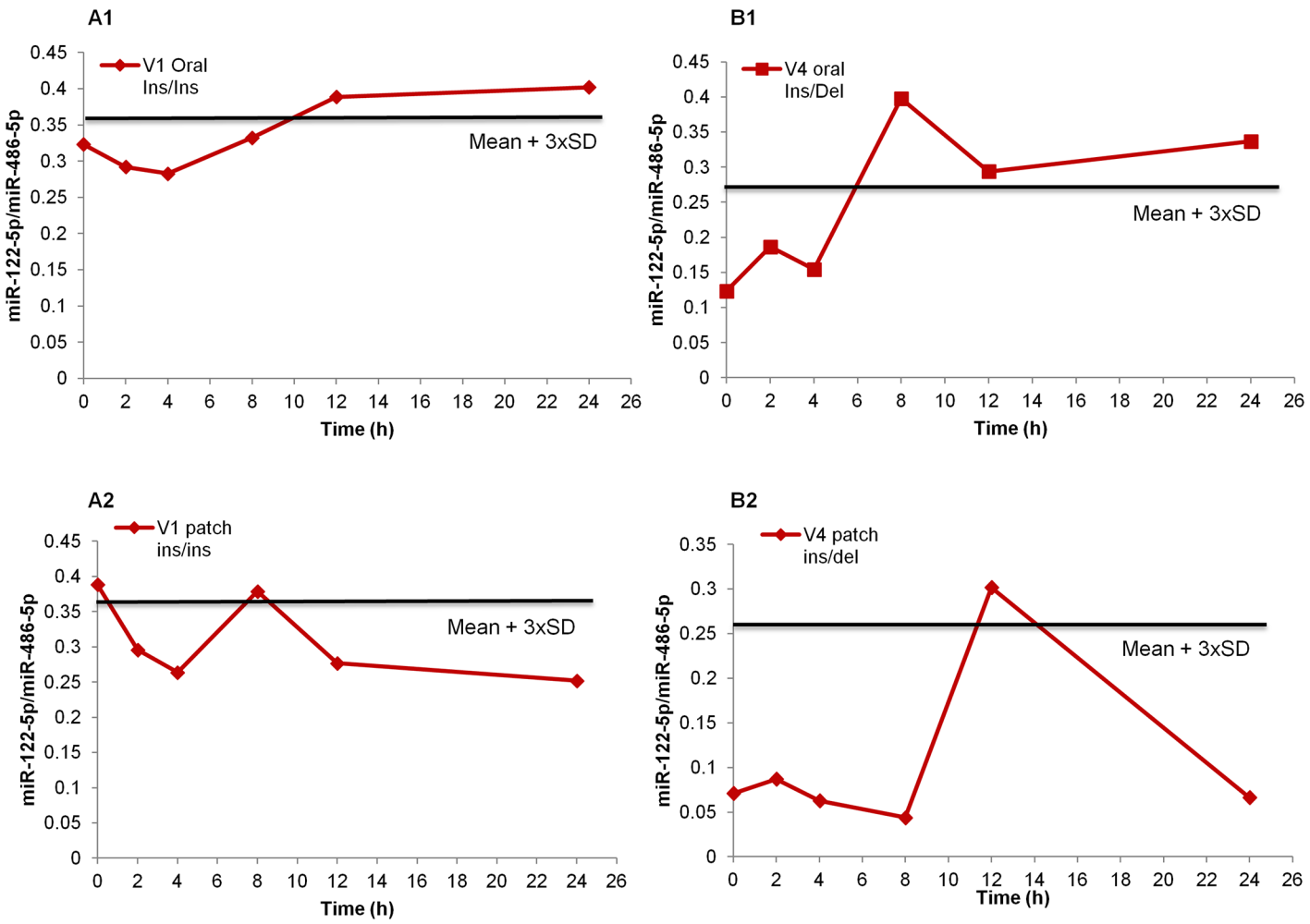


**Fig 3. Examples of individual follow-up of miR-122 with personalized threshold. (A and B)** Two examples of longitudinal monitoring of plasma miR-122 with personalized threshold (indicated by continuous black line) for two volunteers with different *UGT2B17* genotypes (ins/del and ins/ins) after oral and transdermal intake of T. Thresholds are calculated as the mean of the control phase results + 3 × SD. In these examples, longitudinal monitoring of plasma miR-122 levels after oral T administration is more sensitive than of transdermal T administration. Data were normalized against the corresponding levels of endogenous miR-486-5p.

doi:10.1371/journal.pone.0155248.g003

kinetics of variation during the testosterone phases, especially between 12 and 24 h. The control and testosterone phases differed primarily in regard to the baseline levels of miR-342 and miR-150, which were higher during both intervention periods. Therefore, we cannot conclude that the decrease in both miRNAs was testosterone-dependent, so they cannot serve as biomarkers for the detection of testosterone. Further investigations should be conducted to characterize the natural variations in the plasma levels of these miRNAs over periods longer than 24 h.

Intake of testosterone, either orally or topically, increased the plasma level of only one miRNA, which was miR-122. Unlike miR-342 and miR-150, its expression level remained constant throughout the day during the control period, supporting the idea that the peak induction of plasma miR-122 1 day after T intake was specific to the performance-enhancing drug rather than the circadian rhythm. miR-122 is expressed primarily in the liver, where it regulates lipid metabolism [26, 36, 37], and it has already been investigated as a potential biomarker of drug-induced liver injury [26, 37].

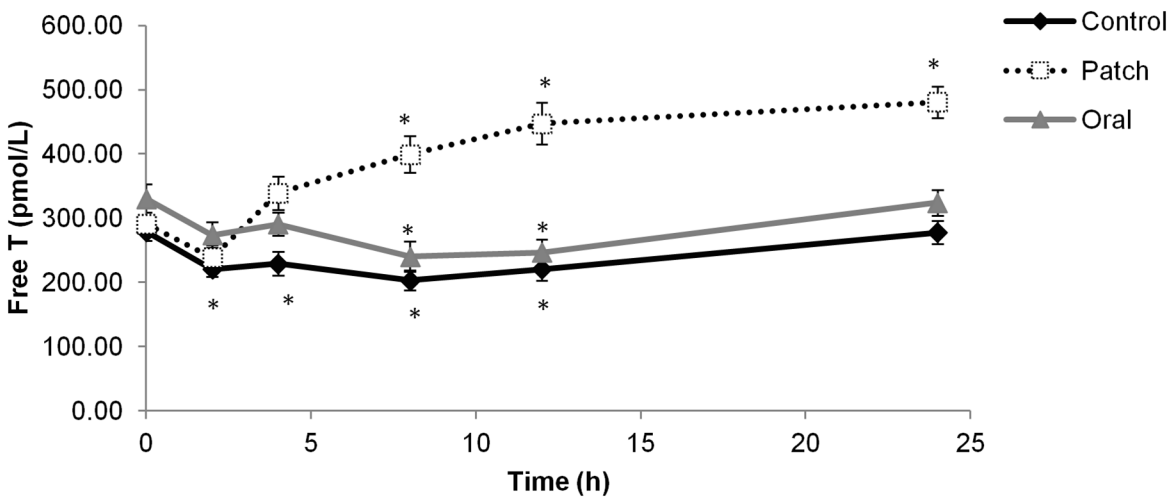


**Fig 4. Serum free T concentrations after oral or transdermal T administration and during the control period.** Mean (±SE) serum free T after oral (gray line) or transdermal (dashed line) T administration or in the control phase (black line). Values represent the average of 19 independent samples at each time point. (*) indicates statistically significant difference relative to time = 0 h.

doi:10.1371/journal.pone.0155248.g004

The liver is a target organ of testosterone, which influences liver metabolism, suppresses hepatic protective responses, and promotes hepatocellular carcinoma [38–40]. However, very little information is available regarding the effect T on hepatic miRNA expression. Delic et al reported upregulation of six miRNAs, including miR-122, in female mouse liver following 3 weeks of T treatment [41]. *In silico* analysis identified an androgen response element (ARE) in the promoter region of miR-122, suggesting that T-induced upregulation of miR-122 is mediated by an interaction between the T-AR complex and its cognate response element. However, a more detailed understanding of the regulation and function of cell-free miRNAs is required before we can safely conclude that the increase in plasma miR-122 is the result of direct transcriptional activation by androgen nuclear receptor.

Alternatively, the increase of plasma miR-122 may be the result of T-induced hepatotoxicity, particularly when the drug is taken orally. However, CRP and AST/ALT levels did not vary significantly, eliminating hepatic inflammation as an explanatory factor. Instead, given that circulating miRNAs may be byproducts of hepatic cells, an increase in cellular activity might underlie the alterations in release of miRNAs by the liver [21, 42, 43]. Consistent with this idea, T administered by various routes is metabolized in the liver, increasing hepatocytes activity.

Even though a clear induction of circulating miR-122 was observed 1 day after oral or transdermal T administration, the sensitivity of this method was compromised by the high inter-individual variability, reflecting the heterogeneity of the volunteers in response to the intervention. Based on the idea of a transcriptomic passport, one appropriate approach would involve integration of miR-122 level into an individual longitudinal follow-up with a personalized threshold. To this end, we applied a method that previously yielded successful results in steroid profiling [6, 7, 30]. In this method, the mean ± 3 × SD of the control values for a given volunteer was established as his personalized threshold.

This method has several advantages over previous approaches. First, in contrast to classical urine biomarkers such as the T/E ratio, commonly used in accredited laboratories, it is independent of the *UGT2B17* genotype of the subject. Second, expression of plasma miR-122 is independent of blood-cell count [36]. Third, the detection window is also longer than that of individual monitoring of typical urinary metabolites, especially in the context of oral T

administration, which is between 2 h and 12 h. However, because a single biomarker is not sufficiently powerful, circulating miR-122 should be combined with several other biomarkers to increase the sensitivity of testosterone screening.

Recently, a new approach based on individual follow-up of blood steroid hormones by LC-MS/MS was proposed as a complement to urinary steroid profiling [44]. This method, which is also independent of *UGT2B17* genotype, has the additional advantage that blood matrix is more stable and less susceptible to confounding factors than urine. However, although LC-MS/MS effectively detected T after transdermal application, longitudinal monitoring after oral TU misuse was less sensitive.

In agreement with this finding, no variation of serum free T was observed after oral TU ingestion in our study. This surprising observation could be explained by a rapid metabolism of free T into 5α-dihydrotestosterone, androstenedione and estradiol [45]. Indeed, two studies reported T retro-conversion to androstenedione following 50 mg oral DHEA or 20 mg androstenedione sublingual ciclodextrin tablet in young males [46, 47]. Finally, food has a particular importance on the bioavailability of oral TU [48].

On the other side, oral TU ingestion triggered a clear increase in plasma miR-122 with a larger amplitude than that observed after transdermal administration and a detection window longer than those of typical urinary biomarkers. The difference between both routes of administration could be explained by the first-pass effect after oral T administration, associated with a great increase of hepatocytes activity and miR-122 secretion. In contrast, transdermal systems slowly deliver T in the body leading to a weaker activity of the liver and a less important secretion of miR-122.

This finding opens the door to using the blood steroid profile in combination with circulating miRNA signatures to increase the sensitivity of the approach [44]. This integration of multiple parameters, transcriptomic and metabolomic, in a longitudinal follow-up can improve the discriminatory power of T detection, and thus represents a powerful and efficient tool for targeting athlete samples for GC-C-IRMS testing. Because both methods require blood samples, the collection process is simplified, and no additional sample material must to be taken from the athlete.

To address issues of reproducibility and robustness, we chose an endogenous control to counteract the effects of physiological variation in miRNA levels between individuals. To this end, we identified the least variable miRNAs by screening; miR-486-5p was detected as a circulating miRNAs whose abundance was not altered by testosterone via either route of administration. This miRNA is highly expressed in RBCs [36], is present at high levels in plasma (27.5 Ct), and was already shown to be an efficient endogenous control in different studies [21, 49]. Therefore, we chose miR-486-5p as an endogenous control for data normalization of relative expression.

Variations between studies are also related to choice of blood medium, potentially due to intercellular miRNA trafficking during the coagulation process [19]. In our study, results were inconsistent between plasma and serum. Expression levels were particularly low in serum, and our profiling screen was unable to identify any miRNAs that were differentially expressed after oral ingestion of TU (S4 Table).

The study had several non-negligible limitations. To integrate circulating miRNAs as biomarkers into the adaptive model of the ABP, intrinsic and extrinsic factors that might affect measurements of cell-free miRNAs must be fully characterized. In addition, the influence of confounding factors such as sex, age, high altitude, or physical exercise remains to be determined. Long-term longitudinal measurement of miR-122 in elite athletes and controls is also required before this circulating miRNA can be used in an anti-doping context. Monitoring of plasma miR-122 for a longer period than 24 h would also have been of particular interest.

In summary, our results suggest that transcriptomic biomarkers may be used for detection of T administered either orally or transdermally. This steroid induced an increase of a specific

miRNA whose origin was related to the liver. Nevertheless, due to high inter-individual variability, longitudinal and individual measurement was the optimal strategy. When using the Athlete Biological Passport, a suspicion of doping is based on a combination of several biomarkers. Therefore, the addition of circulating miRNAs to the adaptive model has the potential to enhance its discriminatory performance. Our study thus provides the basis for using a combination of steroidomic and transcriptomic biomarkers as a new approach that is complementary to urine-based methods for detection of testosterone and steroids.

## Supporting Information

**S1 Fig. Study design.** Samples were collected at different time points after application of two Testopatches (patch phase) at week 2 and the ingestion of two TU tablets (oral phase) at week 5 after a wash-out period of 2 weeks. During the first week of the study, samples were also gathered at the same time points with no treatment (control phase). Circulating miRNAs were extracted from plasma or serum, reverse transcribed into cDNA and quantitated by RT-qPCR. Time points selected for the screening are indicated in bold.
(TIF)

**S2 Fig. AST/ALT and CRP levels after oral or transdermal T administration or during the control period. (A and B)**. Mean (±SE) ALT and AST activity (U/I) at the indicated time points after testosterone administration. Values represent the average of seven independent samples (for patch phase) or ten independent samples (for oral phase). **(C)** Mean (±SE) CRP (mg/dl) at the indicated time points during each phase. Values represent the average of 19 independent samples at each time point.
(TIF)

**S1 Table. Ct values of plasma miRNAs at 0 h and 24 h for each subject in both intervention periods.**
(XLSX)

**S2 Table. Total affected plasma miRNAs by oral and transdermal T administration.**
(XLSX)

**S3 Table. Least variable miRNAs.** List of circulating miRNAs whose levels were invariant following oral or transdermal T administration.
(PDF)

**S4 Table. Ct values of serum miRNAs at 0 h and 24 h for each subject in oral period.**
(XLSX)

## Acknowledgments

We acknowledge Dr. Flavia Badoud for the management of the clinical study and free testosterone quantification in serum and Charlotte Brunner for its excellent technical assistance. Dr. Sylvain Pradervand is acknowledged for the statistical analyses regarding miRNAs profiling. We are grateful to Prof. Vincent Mooser for the critical reading of the manuscript.

## Author Contributions

Conceived and designed the experiments: OS NL. Performed the experiments: OS LJ. Analyzed the data: OS LJ NL NR. Contributed reagents/materials/analysis tools: NB. Wrote the paper: OS NL. Obtained ethical approval: MS.

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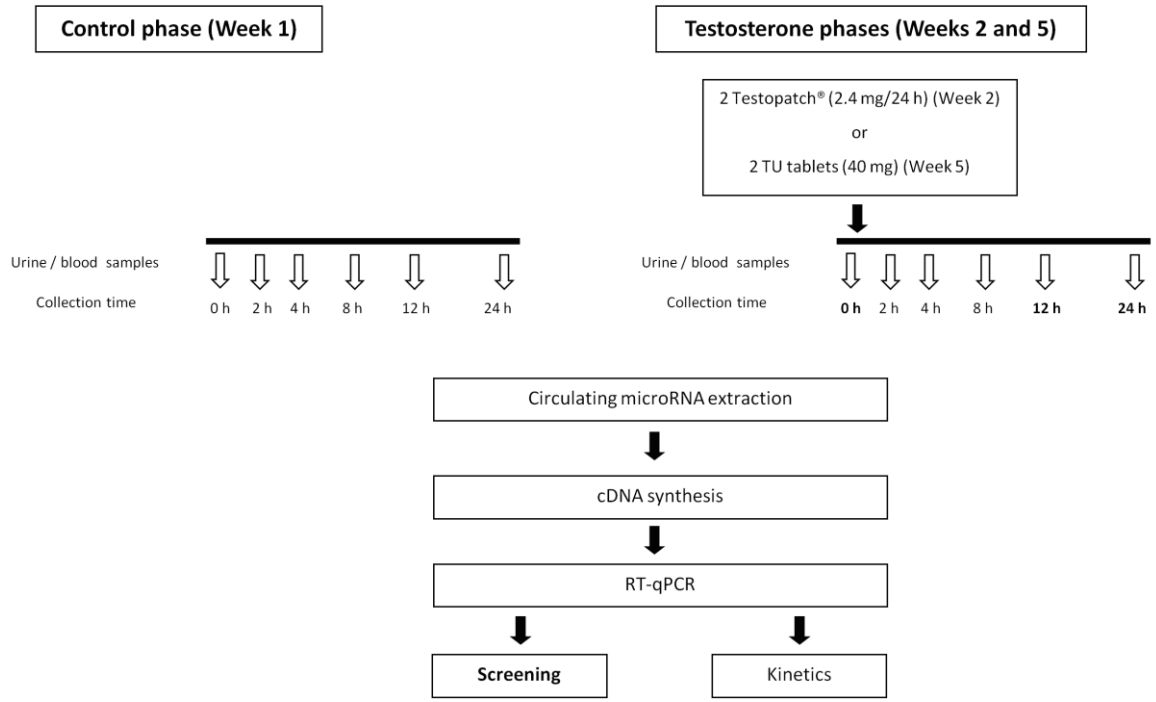
# Supplemental Material

## Supplementary figures

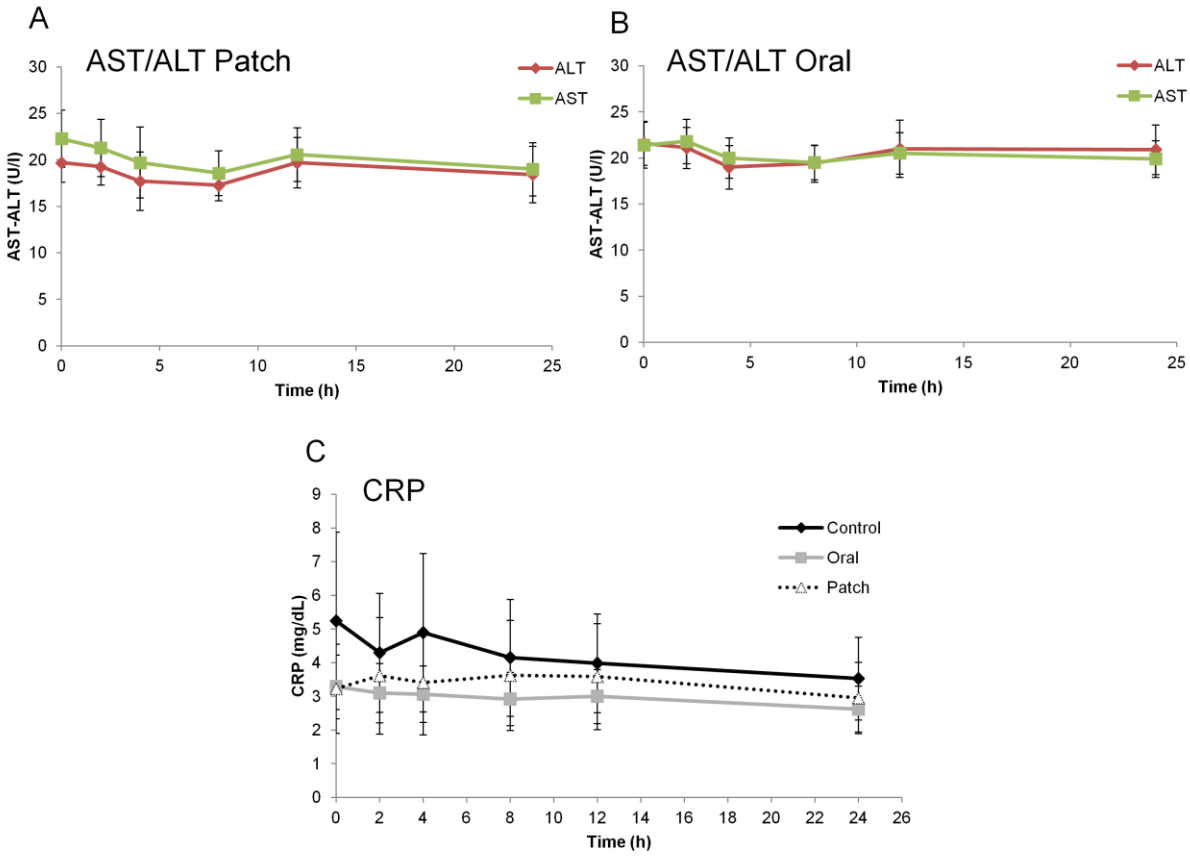
### S1 Fig. Study design.

Samples were collected at different time points after application of two Testopatches (patch phase) at week 2 and the ingestion of two TU tablets (oral phase) at week 5 after a wash-out period of 2 weeks. During the first week of the study, samples were also gathered at the same time points with no treatment (control phase). Circulating miRNAs were extracted from plasma or serum, reverse transcribed into cDNA and quantitated by RT-qPCR. Time points selected for the screening are indicated in bold.



**S2 Fig. AST/ALT and CRP levels after oral or transdermal T administration or during the control period.**

(A and B). Mean (±SE) ALT and AST activity (U/l) at the indicated time points after testosterone administration. Values represent the average of seven independent samples (for patch phase) or ten independent samples (for oral phase). (C) Mean (±SE) CRP (mg/dl) at the indicated time points during each phase. Values represent the average of 19 independent samples at each time point.



**Supplementary tables**

**S1 Table. Ct values of plasma miRNAs at 0 h and 24 h for each subject in both intervention periods.**

Available at: https://doi.org/10.1371/journal.pone.0155248.s003

(XLSX)

**S2 Table. Total affected plasma miRNAs by oral and transdermal T administration.**

Available at: https://doi.org/10.1371/journal.pone.0155248.s004

(XLSX)

**S3 Table. Least variable miRNAs.**

List of circulating miRNAs whose levels were invariant following oral or transdermal T administration.

Available at: https://doi.org/10.1371/journal.pone.0155248.s005

(PDF)

**S4 Table. Ct values of serum miRNAs at 0 h and 24 h for each subject in oral period.**

Available at: https://doi.org/10.1371/journal.pone.0155248.s006

(XLSX)

# Chapter III

## Blood steroid profiling (steroidomics) for T detection

# 3. Monitoring of endogenous steroids in blood

## 3.1 Introduction

The quantitative analysis of endogenous steroids in blood matrix is an essential clinical tool for the screening, diagnosis or monitoring of endocrine-related disorders. Initially, this analysis was mainly performed with immunoassays allowing high-throughput. However, this method is subject to certain limitations such as cross-reactivity reactions which may generate overestimated values [208]. Furthermore, the analysis is often limited to the measurement of a single or few compounds instead of a flexible panel of substances, which may lead to larger sample waste and longer analysis time in case of quantification of multiple analytes. Actually, clinical laboratories rather use LC- tandem MS (MS/MS) as gold standard method to measure steroid hormones as it offers the possibility of quantifying multiple compounds simultaneously with higher specificity and selectivity [209].

In the anti-doping research context, our group developed sensitive approaches using ultra-high performance LC (UHPLC)-MS/MS or high resolution mass spectrometry (HRMS) methods for the determination of endogenous steroids in serum as a complementary strategy to the urinary steroid module of ABP (Federico Ponzetto thesis, 2019) [210,211]. Notably, the longitudinal monitoring of serum T and DHT concentrations resulted in improved detection of testosterone administration in male subjects, especially for the *del/del* genotype for UGT2B17 enzyme and with a longer detection window after transdermal T administration.

However, this approach was only applied to male subjects while it may be particularly efficient for females, which are more sensitive to T administration due to lower basal levels. Although many administration studies have been performed for the investigation of steroid metabolism in male subjects, there is a lack of knowledge on the steroid metabolism in females either at basal level during a menstrual cycle or after an exposition to T.

## 3.2 Article 5: Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle

To overcome this lack of knowledge, a clinical trial involving eumenorrheic healthy women and T gel administration was performed between 2019 and 2020 (entitled 'TestoFem', ISRCTN10122130). This article describes the longitudinal evaluation of multiple biomarkers for the detection of T gel administration in these female subjects. The trial was divided into three 4-weeks phases corresponding to three consecutive menstrual cycles. The first cycle corresponded to the control phase, followed by the treatment phase during which 10-mg T gel was administered daily and a third post-treatment phase. The sensitivity of the current urinary and hematological markers of ABP as well as serum steroid biomarkers was investigated for the monitoring of the 28-day T gel treatment combined with endogenous fluctuations of the menstrual cycle. Furthermore, a subset of urinary samples was analyzed with GC/C/IRMS to assess the sensitivity of the targeting method as well as the sensitivity of the method itself. The most affected urinary biomarker ratios were T/E and 5αAdiol/E, while in serum the concentrations of T and DHT increased significantly during the treatment. The detection capability of both urinary biomarkers was deeply influenced by fluctuations of E concentration observed within the menstrual cycle and resulted in a reduced sensitivity of the urinary steroidal ABP module. On the contrary, the longitudinal monitoring of T and DHT serum concentrations, along with the newly proposed T/androstenedione ratio, showed improved sensitivity to detect T administration but also targeting for confirmatory GC/C/IRMS analysis. The confirmatory IRMS results demonstrated moderate sensitivity with less than one third of the tested urine samples fulfilling the criteria for positivity. Finally, the hematological parameters were not affected by neither the menstrual cycle nor T administration.

Received: 12 February 2021 | Revised: 16 March 2021 | Accepted: 29 March 2021

DOI: 10.1002/dta.3040

SPECIAL ISSUE - RESEARCH ARTICLE

WILEY

# Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle

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**Funding information**
World Anti-Doping Agency, Grant/Award Number: 17D18MS

## Abstract

In women, hormonal fluctuations related to the menstrual cycle may impose a great source of variability for some biomarkers of testosterone (T) administration, which can ultimately disrupt the sensitivity of their longitudinal monitoring. In this study, the sensitivity of the current urinary and haematological markers of the Athlete Biological Passport (ABP), as well as serum steroid biomarkers, was investigated for the monitoring of a 28-day T gel treatment combined with endogenous fluctuation of the menstrual cycle in 14 healthy female subjects. Additionally, the analysis of urinary target compounds was performed on a subset of samples for endogenous/exogenous origin via isotope ratio mass spectrometry (IRMS). In serum, concentrations of T and dihydrotestosterone (DHT) increased significantly during the treatment, whereas in urine matrix the most affected biomarkers were found to be the ratios of testosterone/epitestosterone (T/E) and 5α-androstane-3α,17β-diol/epitestosterone (5αAdiol/E). The detection capability of both urinary biomarkers was heavily influenced by [E], which fluctuated depending on the menstrual cycle, and resulted in low sensitivity of the urinary steroidal ABP module. On the contrary, an alternative approach by the longitudinal monitoring of serum T and DHT concentrations with the newly proposed T/androstenedione ratio showed higher sensitivity. The confirmatory IRMS results demonstrated that less than one third of the tested urine samples fulfilled the criteria for positivity. Results from this study demonstrated that the 'blood steroid profile' represents a powerful complementary approach to the 'urinary module' and underlines the importance of gathering bundle of evidence to support the scenario of an endogenous prohibited substance administration.

**KEYWORDS**
Athlete Biological Passport, menstrual cycle, steroid profile, testosterone gel, women

## 1 | INTRODUCTION

Testosterone (T) is the main endogenous steroidal hormone responsible for the development of male secondary sex characteristics (androgenic) as well as the increase of muscular mass, physical strength or aggressiveness. In men, it is synthesized in testes under the regulation of the hypothalamic–pituitary–gonadal (HPG) axis. In females, T is produced in small amounts by the ovaries and adrenal

glands, while 50% is synthesized by conversion in peripheral tissues.[1] On average, circulating testosterone level of an adult men is seven to eight times higher than in women at approximately 20 times greater daily amount of production.[2,3]

In addition to its therapeutic use for hypogonadism in men and in women for sexual disorders in rare occasions, T and its analogues (anabolic steroids) still represent the most important class of prohibited substances abused by athletes, as also reported by antidoping laboratories.[4] Exogenous T can be administered by various routes, with transdermal application gaining more and more popularity in the treatment of hypogonadic men. This pharmaceutical formulation is particularly preferred as doping preparation owing to its low absorbed doses and regular diffusion through the skin, which result in low peak of concentrations and further to complex detectability.[5] In addition to its anabolic action, T administered in small and repeated doses can reduce fatigue, stimulate the erythropoiesis and consequently, improve recovery.[6,7] In particular, females are more sensitive to testosterone than males,[8] and hence, due to the existing low basal concentration, an increase in the concentration of T is proposed to lead to a significant benefit in performance. Notably, a study has recently demonstrated that a moderate increase of testosterone levels in young women, following a 10-week treatment with 10-mg transdermal testosterone, improved significantly physical performance and increased total lean mass, type II fibre size and capillarization.[9,10]

In antidoping context, the endogenous nature of T makes it particularly difficult to regulate and identify, as the endogenous form shall be distinguished from its synthetic counterpart in urine sample. Since 2014, the steroidal module of the Athlete Biological Passport (ABP) has been used for targeting synthetic forms of endogenous androgenic anabolic steroids (EAAS) such as testosterone, in urine samples. The steroidal ABP is composed of the urinary concentrations of free and glucuro-conjugated fractions of T, its isomer epitestosterone (E) and phase I metabolites androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) measured by gas chromatography (GC)–(tandem) mass spectrometry (MS(/MS)). These concentrations are combined into five specific ratios (T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E) corresponding to the biomarkers which are plotted and longitudinally evaluated in the ABP. The ABP software is based on an adaptive Bayesian model in which statistical inferences are applied to each sample, progressing from initial population-based limits to individual reference ranges.[11] The T/E ratio is the primary parameter of the ABP and triggers automatically a confirmatory GC–combustion–isotope ratio mass spectrometry (GC/C/IRMS) analysis request in case of the confirmed outlying parameter. The other ratios are secondary parameters that are evaluated by the Athlete Biological Passport Management Unit (APMU), who can instruct the laboratory to conduct further analyses/actions. Among these, GC/C/IRMS analysis is performed to detect the potential exogenous origin of testosterone and its metabolites by determining their isotopic $^{13}C/^{12}C$-composition. A recent study also highlighted the complementary data from the longitudinal monitoring of haematological parameters as a tool to trigger GC/C/IRMS analysis due to the alterations induced by testosterone administration.[12]

The urinary steroid profile may be influenced by various confounding factors, which could be categorized as exogenous (alcohol consumption, medication, bacterial contamination, etc.) or endogenous (sex, age and enzyme polymorphism), and which can have an impact on the apparent profile and its interpretation.[13] In particular, the T/E ratio is significantly affected by a deletion polymorphism of UGT2B17 enzyme, responsible for the glucuronide conjugation of T. The *del/del* genotype results in low urinary T levels, often below the limit of quantification (LOQ) of the GC–MS(/MS), and therefore lower T/E values.[14] Similar analytical issues can be encountered with urine samples of female athletes due to generally lower urinary T and other related EAAS concentrations compared with males.[15]

To further improve the steroidal module, more sensitive urinary biomarkers with wider windows of opportunity have been investigated using other GC–MS(/MS)-based methods and liquid chromatography (LC) in combination with MS or high-resolution MS (HRMS).[16–18] Alternatively, serum was proposed as a complementary biological matrix for the steroidal module.[19] This matrix has lower risks of bacterial contamination or manipulation, and it could allow for easier interpretation of pharmacokinetic information. In previous studies, longitudinal monitoring of serum T and dihydrotestosterone (DHT) concentrations has resulted in improved detection of both oral and transdermal T administration in men volunteers, especially in the cases associated to UGT2B17 polymorphism.[19] This approach has already been proven particularly effective for female athletes who are not subject to negative T and E feedback by the HPG axis.[20,21] The additional information from serum steroid profiling may thus not only improve the targeting of direct GC/C/IRMS analysis in urine samples but also provide further evidence of the 'Use or Attempted Use of a Prohibited Substance or a Prohibited Method' (Article 2.2 Anti-Doping Rule Violations).[22]

In comparison with men, women have more complex and fluctuating hormonal metabolism. The menstrual cycle is under the control of a well-regulated hormonal machinery driven by pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus and imposes a great source of variability, notably with three distinct phases.[23] The first part of the cycle (follicular phase) starts the day of menstruation and the maturation of the follicle occurs under the control of the follicle-stimulating hormone (FSH). The sex hormone concentrations are usually low until the dominant follicle starts to rapidly produce high amounts of oestrogen, which generates the luteinizing hormone (LH) surge by a positive feedback that further stimulates ovulation (ovulatory phase). During the luteal phase, LH and FSH levels decrease, the ruptured follicle transformed into corpus luteum produces progesterone, and oestrogen increase at the same time. In case of nonfertilization, the corpus luteum degenerates and no longer produces progesterone. Oestrogen level also decreases, and a new menstrual cycle begins. These cyclic variations may affect the longitudinal follow-up of biomarkers of the ABP and lead to more complex individual profiles for interpretation. Indeed, women exhibit large intraindividual variations in urinary ratios monitored in ABP due to either hormonal fluctuations throughout the menstrual cycle or low

urinary concentrations of some steroidal metabolites, which are often close to the detection limit of the analytical method.[24] In particular, E concentration is influenced by the menstrual cycle, impacting consequently T/E and 5αAdiol/E ratios.[24,25] Not only steroidal but also the haematological variables may be influenced by the menstrual cycle, notably the reticulocyte percentage (RET%).[26]

The aim of this study was to investigate the impact of T gel administration on the ABP biomarkers (steroidal and haematological module) and on serum steroid biomarkers in females. A direct comparison was performed between urinary and blood steroid profiling for the detection of T gel administration based on their sensitivity either for longitudinal evaluation or for targeting GC/C/IRMS analyses. In addition, the influence of the menstrual cycle on the fluctuations of the selected biomarkers combined to T gel administration was also thoroughly monitored in this study.

## 2 | MATERIAL AND METHODS

### 2.1 | Study cohort

Fourteen healthy women volunteers aged 22–37 years (median 27.5 years), with a body mass index (BMI) 19–24 kg/m$^2$ (median 20.77 kg/m$^2$) and with regular menstruation cycle (26–32 days) were recruited. In addition to regular menstrual cycles, inclusion criteria were age (20–40 years), BMI (18–30 kg/m$^2$), haemoglobin concentration (HGB) 12–16 g/dl, basal testosterone concentration (≤2 nmol/L), negative pregnancy test at screening, commitment to use highly efficient non-hormonal contraception during the study period and normal hormonal (LH, FSH, SHBG, free thyroxine and prolactin) and biological (full blood count, hepatic function [AST/ALT], CRP and kidney function [creatinine]) results at the screening. Exclusion criteria were the presence of cardiovascular, liver, biliary or renal disease; endocrine or metabolic disorder; hypertension; acne or hirsutism; dyslipidaemia; treatment with ketoconazole, ACTH, corticoids or anticoagulants; intake of hormonal contraception within 2 months prior to the study; pregnancy considered within 6 months after study completion; breastfeeding; eating disorder; and blood donation within 3 months prior to the study. Female subjects agreed not to participate in any sports competition during the study period in order not to violate antidoping rules. Subjects were permitted to moderately consume alcohol beverages (≤3 units/week) and to use over the counter medication and instructed to report any at the time of the study visit. All subjects provided written informed consent prior to any study procedures, and the open-label trial was approved by the local Ethical Committee of the Canton de Vaud in Switzerland (2018–02106, SNCTP000003264) and Swissmedic (2018DR1168), registered on www.isrctn.com (ISRCTN10122130) and conducted in accordance with the Declaration of Helsinki.

### 2.2 | Study design

The study was an open-label trial with each subject being her own control. The protocol was divided into three 28-day phases corresponding to three consecutive menstrual cycles for a total of 12 weeks (Figure 1). For each subject, the protocol started at early follicular phase (on the Monday following the start of menses). Phase 1 corresponded to the control period during which no treatment was administered. During the first week, urine and serum samples were collected the first 4 days (Monday to Thursday) whereas in the second and third week, samples were collected on Monday, Wednesday and Friday, and in the fourth week, collection occurred from Monday to Friday. Phase 2 corresponded to the treatment during which testosterone gel 10 mg (Tostran®) was self-administered every morning on the upper thigh or abdomen for 28 days. Samples were collected just before the application of the treatment, corresponding approximately to 24 h after gel administration, and with the sampling scheme similar to the first phase. In case of delay of menses, subjects were instructed to wait for 1 week until menstruation before starting the treatment. Phase 3 corresponded to the post-treatment period and was similar to first phase. In addition to urine and serum samples, also whole blood was collected once a week (Wednesday) for haematological analysis according to guidelines of the World Anti-Doping Agency (WADA).[27] Serum samples were collected in 8.5-ml BD Vacutainer® SST™ II Advance tubes and whole blood in 4-ml BD Vacutainer® K2EDTA tubes whereas 40- to 80-ml urine was collected into sterile cups. Subjects were instructed that the urine sample should normally correspond to the first urine of the day and that it should preferably be collected less than 1 h before study visit. Study visits involving sample collection took place at the Clinical Trial Unit (CTU) of Centre Hospitalier Universitaire Vaudois (CHUV) approximately at the same hour for each subject between 7 and 11 in the morning.

### 2.3 | Serum steroid profile

Serum samples were analysed using a previously described and validated ultra-high-performance LC (UHPLC)–MS/MS method for the quantification of 14 different steroid hormones (testosterone, epitestosterone, androstenedione [A4], progesterone [P], 17α-hydroxyprogesterone [17αOHP], DHEA, DHT, corticosterone, cortisol, deoxycorticosterone, 11-deoxycortisol, oestrone [E1], oestradiol [E2] and oestriol [E3]).[19] Steroid hormones from a 200-µl serum aliquot spiked with 20 µl of an internal standard (IS) mix were extracted using supported liquid extraction (SLE) on ISOLUTE SLE+ (Biotage, Uppsala, Sweden) 400-µl 96-well plates.[19] The dried eluate was reconstituted with 100 µl of MeOH:$H_2O$ 50:50 solution, and 10 µl of each extract was injected into UHPLC–MS/MS system for analyses. Calibration was performed using an 8-point linear calibration model (weighting 1/*x*) in spiked depleted serum for each compound, prepared freshly within each batch.

Chromatographic separation was performed on a UPLC system (Waters, Milford, MA, USA) equipped with an Ethylene Bridged Hybrid (BEH) $C_{18}$ column (100 × 2.1 mm, 1.7 µm; Waters) with a precolumn and set at 30°C. The mobile phases were composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (ACN; B) with a flow rate set at 400 µl/min. The gradient started

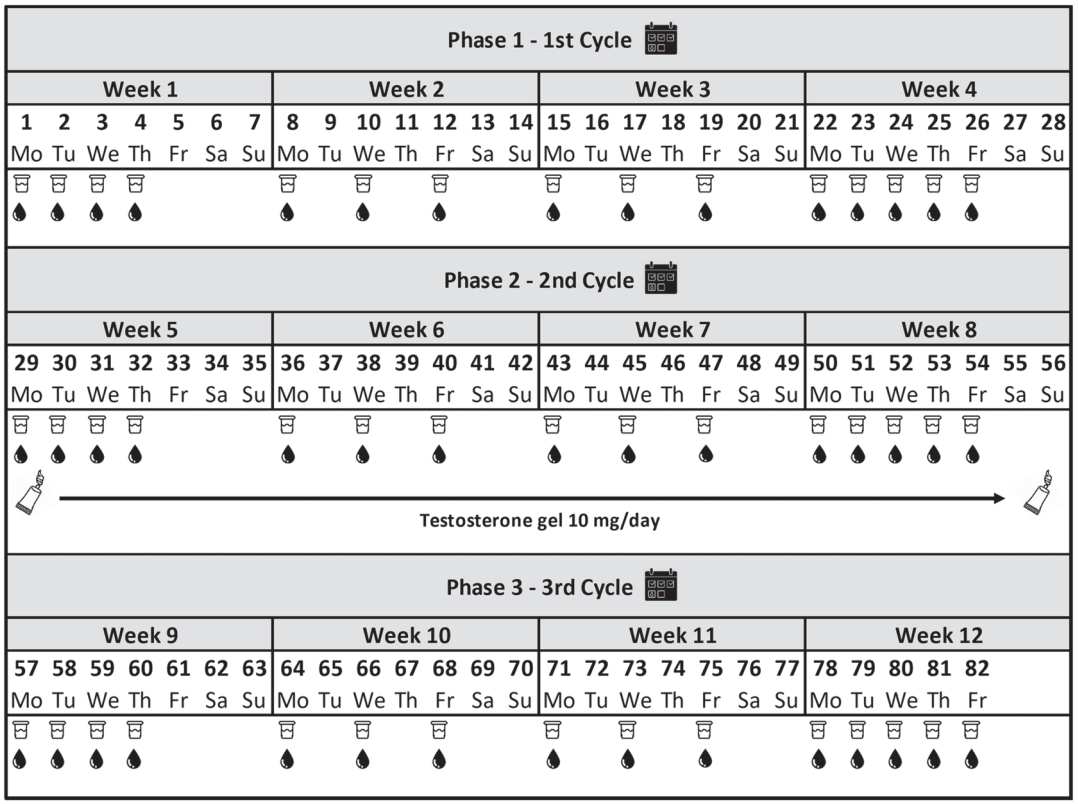



**FIGURE 1** Study design

linearly from 2% to 25% B over 0.5 min, followed by an increase to 58% over 5.5 min and by a further increase to 98% over 2 min; the column was then re-equilibrated for 3 min at initial conditions.

The UPLC system was coupled to a Xevo-TQ S triple quadrupole MS/MS system (Waters, Milford, MA, USA) operating in positive ionization mode and multiple reaction monitoring (MRM) mode. The detailed instrumental UHPLC–MS/MS conditions (MRM transitions, ESI conditions, cone voltages and collision energies) are described in Ponzetto et al.[19] For quantification of oestrogens (E1, E2 and E3), the samples were injected a second time with mobile phases composed of water and ACN and MS/MS system operating in negative ionization mode.

Serum LH and FSH were measured by direct chemiluminescence using Siemens ADVIA® Centaur™ Immunoassay System. LH peak was considered to denote mid-cycle, whereas the mid-luteal phase was marked by a peak in systemic P. Other phases of the cycle were extrapolated from these two time points in addition to the day of the start of menstruation.

## 2.4 | Urinary steroid profile

Urinary steroid profiles were determined using GC–MS with a validated screening method in accordance with WADA technical document TD2018EAAS.[28] Specific gravity was measured using an automatic digital refractometer (ATAGO RX-5000α, Tokyo, Japan). A 2.5-ml urine aliquot spiked with 20 μl of IS mixture was hydrolysed using β-glucuronidase enzyme (Roche Diagnostics, Mannheim, Germany) and extracted using liquid–liquid extraction (LLE) with *n*-pentane at alkaline pH. The steroids were derivatized with a mixture of *N*-trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA), ethanethiol and ammonium iodide. The derivatized extracts were transferred to microvials and analysed on GC–MS (Agilent 7890B/5977B). Separation was performed using an Agilent HP-1 column (17 m × 0.2 mm, 0.11 μm). The inlet was operated in split mode (1:10) with an injection volume of 1 μl and the temperature was set at 300°C. The GC oven temperature program was initiated at 181°C, increased by 3°C/min to 230°C, followed by 120°C/min to 310°C with a hold time of 3 min. The MS data were acquired by operating single quadrupole mass spectrometer in selected ion monitoring (SIM) mode. The system operation, data acquisition and data analysis were performed using MassHunter software. The limits of detection (LODs) were 0.2 ng/ml for T, 0.3 ng/ml for E, 1 ng/ml for 5αAdiol and 5βAdiol and 20 ng/ml for A and Etio, and the LOQs were 0.5 ng/ml for T, 1 ng/ml for E, 2 ng/ml for 5αAdiol and 5βAdiol and 50 ng/ml for A and Etio. Presence of microbial contamination markers was also evaluated in the same analytical GC–MS batch in compliance with the TD2018EAAS.

The presence of confounding factors in urine samples such as ethylglucuronide (EtG), finasteride and ketoconazole was analysed by dilute-and-shoot UHPLC–MS/MS operating in both positive and negative ionization mode.

## 2.5 | Carbon isotope mass spectrometry

The carbon isotopic ratios of the urinary steroids were determined as described in Piper et al,[29] in accordance to WADA requirements (TD2019IRMS). Depending on the steroid concentration, 5 to 30 ml of urine aliquot was purified using preconditioned BondElut C18 SPE cartridges (Agilent Technologies, Waldbronn, Germany). The purified extract was then hydrolysed for 60 min at 50°C using *Escherichia coli* β-glucuronidase in phosphate buffer prior to two consecutive LLE with 4-ml *tert*-butyl methyl ether (TBME). The organic fraction was collected from both steps and evaporated to dryness. The residue was reconstituted with a mixture of water and ACN, and the samples were first submitted to chromatographic purification with an Agilent 1260 Infinity HPLC with a DAD and an automated fraction collector. The steroids were separated on a XBridge Shield RP18 (4.6 × 250 mm, 5 μm) column from Waters (Milford, USA) with water and ACN as solvents using the same gradient as described in Piper et al.[30] The collected fractions were dried and acetylated with 50-μl acetic anhydride and 50-μl pyridine followed by a 60 min incubation at 70°C. After evaporation to dryness under a stream of air, the dried residue was reconstituted and transferred to either LC autosampler vials. The fractions containing multiple steroids (fractions III and IV) and the testosterone fraction (fraction II) were further purified by a second HPLC fractionation using a different gradient as described in Piper et al.[30] The final extracts of acetylated steroids were dried and reconstituted with cyclohexane according to their concentration. For all samples, the target compounds (TCs) T, 5αAdiol, 5βAdiol and A, as well as the two endogenous reference compounds (ERC) pregnanediol (PD) and 16-androstenol (16-EN), were analysed. The GC/C/IRMS analysis was carried out on a Trace 1310 GC coupled to a Delta V Plus isotope ratio mass spectrometer with an IsoLink II and a Conflo IV from Thermo Scientific. The separation was performed on an Agilent J&W DB17-MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm) with a constant helium flow of 1.4 ml/min. The inlet was operated at 280°C in splitless mode with 1.5 min of purge. The oven was set to 70°C for the first 2 min, increased at 30°C/min up to 270°C, then at 2°C/min to 300°C and kept for 5 min. Raw data evaluation was performed with IsoDat software.

## 2.6 | UGT2B17 genotyping

DNA was extracted from a 200-μl whole blood aliquot using the Maxwell® RSC whole blood DNA kit on an automated Maxwell® RSC instrument (Promega, Switzerland). Resulting DNA concentration was measured using a Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The UGT2B17 copy number variation analysis was performed using a premade copy number assay (HS03185327_CN, Life Technologies, Carlsbad, CA, USA) as previously described using a Roche LightCycler 480 real-time PCR system.[31] The human RNase P gene was used as internal positive control to monitor reaction quality. Raw data and normalization were performed with LightCycler 480 software (release 1.5.0). The absence of UGT2B17 amplification in samples associated with RNase P signals was considered as *del/del*. No discrimination between UGT2B17 allele carriers (*ins/ins* and *ins/del*) was investigated.

## 2.7 | Haematological analyses

Blood samples were collected and analysed following WADA requirements. The participants remained in a normal seated position for 10 min prior to collection of blood samples into anticoagulated EDTA tubes from antecubital vein. Blood samples were transported to the laboratory in a refrigerated box, stored at 4°C upon receipt and analysed within 24 h at the Swiss Laboratory for Doping Analyses. Samples were first homogenized for 15 min at room temperature and analysed for the full blood count in accordance to WADA technical document for blood analytical requirements (TD2019BAR[32]) using an automated haematology analyser Sysmex XN-1000 (Sysmex, Japan). Abnormal blood profile score (ABPS) was calculated using the publicly available R package.[33]

## 2.8 | ABP evaluation

The Anti-Doping Administration and Management System (ADAMS) training environment hosted by WADA was used to establish individual haematological and urinary steroidal passport profiles. The website access to this training platform was generously provided by WADA in the framework of the WADA-funded project.

For the steroidal module, the quantitative data of T, E, A, Etio, 5αAdiol and 5βAdiol as well as specific gravity were entered into the ADAMS training module for each sample to simulate an individual steroid profile with the ratios T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E as biomarkers for each study subject. Upper and lower limits for the ratios were determined using baseline samples of the first phase and were considered the study subjects' 'true' individual thresholds. In order not to compromise the sensitivity of the model by 'positive' samples, the calculated limits for the sample of the first day of treatment were applied to all future samples without adaptation. Potential confounding factors, such as concentrations of EtG higher than 5 μg/ml, were also registered. Due to lack of an adaptive model for serum steroid biomarkers, the longitudinal monitoring was performed using individual limits calculated as mean ± 3 × standard deviation (SD) of the baseline samples (Phase 1) corresponding to the 99% specificity level.

For the haematological module, individual values of the 13 haematological variables[32] were entered into the model to produce longitudinal profiles for HGB, RET%, OFF score (computational parameter based on HGB and RET%) and ABPS. The individual limits were not adjusted, and the thresholds were set at the 99% specificity level. A passport with at least one of the urinary steroidal ratios, serum concentrations or HGB and/or OFF score flagging an abnormality was considered an atypical passport finding (ATPF).

### 2.9 | Statistical analysis

All urinary concentrations were corrected for specific gravity to normalize the results between urine samples. Unless otherwise specified, data are expressed as the mean (±SD). Normality of data was determined with the Shapiro test. In cases of non-normality, non-parametric test was used. One-way repeated ANOVAs followed by post hoc pairwise comparisons (Tukey's honestly significant difference) or Kruskal–Wallis' tests followed by Dunn's multiple comparison test (if normality non respected) were used to test differences between phases of the menstrual cycle. Correlations were calculated using Spearman's rank order method. A $p$ value < 0.05 was considered statistically significant. Statistical analyses were performed with R software and receiver operating characteristic (ROC) curves were generated using SPSS software. With the aim of highlighting biomarkers of T administration, parallel factor analysis (PARAFAC) multiway modelling was performed on the dataset obtained from the urinary and serum steroid analyses of clinical study samples. PARAFAC models were computed under the MATLAB 8 environment (The MathWorks, Natick, MA, USA) with routines implemented in the N-Way Toolbox (version 3.1). The CORCONDIA criterion based on resampling was used to assess model size.[34]

## 3 | RESULTS AND DISCUSSION

The design of the clinical study was adapted for the discovery and evaluation of present and novel biomarkers of doping practises.[35] Indeed, it was set up so that robust baseline sampling and measurement of within-subject changes in the different biomarkers evaluated over time using longitudinal approach could be obtained. The first study phase corresponding to the control phase allowed establishing basal levels of the various biomarkers with 15 sample collections over a 4-week period. The similar sample collection design between the three phases enabled to overlay data for multivariate analysis and allowed discriminating between natural within-subject variability and treatment-induced variability of the evaluated biomarkers. In this manner, each subject was individually evaluated, and the changes in biomarker pattern were assessed at the individual level.

### 3.1 | Multivariate data analysis

Monitoring multiple parameters over time in a cohort of individuals inherently leads to a three-mode tensor data arrangement. Multiway approaches offer adequate tools to efficiently handle these structures and highlight relevant trends in the data. Among them, the PARAFAC algorithm constitutes an unsupervised decomposition method that can be seen as an extension of standard principal component analysis (PCA) to higher order data. It preserves links between the different modes, for example, variations of parameters over time and between volunteers, thus avoiding potential loss of information. Data from urinary and serum steroids quantification were analysed separately with the unsupervised multiway PARAFAC algorithm. Both three-way tensors (time × subjects × variables) obtained from the data from three study phases by GC–MS and UHPLC–MS/MS were centred across the time mode whereas unit variance scaling was applied within the variables mode. All serum steroid concentrations, except oestriol for which concentration was mostly below lower LOQ (LLOQ), were included in data analysis in addition to a previously suggested T/A4 ratio as indicator of androgen excess.[36] For the urinary matrix, concentrations of the six variables (adjusted for specific gravity) as well as the five ABP ratios were included for data analysis.

#### 3.1.1 | Serum steroid profile

When performing PARAFAC multiway analysis, two components were highlighted as consistent, thus leading to a model summarizing 25.7% of the total variance. By these means, two distinct trends were clearly revealed as a function of time (Figure 2A,B). The first component could be related to the administration of T gel during the second study phase when the score of each time point was plotted chronologically with a massive contribution from T, DHT and T/A4 (Figure 2D). The time score started to increase already 1 day after the first T gel application (Day 30), reached a peak at Day 47 at the end of the third treatment week, slightly declined during the fourth week of treatment and returned to baseline levels within 2 days after cessation of T gel. The second component highlighted a periodic fluctuation observed across the three study phases, which corresponded to menstrual cycle variations. The evaluation of the variables mode revealed that these fluctuations were mainly associated with oestrogens (E1 and E2), P and 17αOHP in addition to deoxycorticosterone (Figure 2D). When the subject scores for each component were plotted against each other, the response to the T gel administration (Component 1) was relatively homogenous whereas few dissimilarities between subjects could be observed in length of menstrual cycle (Component 2) (Figure 2C). Notably, Subject 2 who had longer menstrual cycles than the other subjects was the only volunteer associated with a negative score on the second component.

#### 3.1.2 | Urine steroid profile

Unsupervised PARAFAC modelling was applied similarly to urine steroid data, and the model explained 31% of the total variance with two components found as consistent. The first component was highly related to T administration when the scores were plotted against the time points of sample collection (Figure 3A). T/E, 5αAdiol/E ratios and 5αAdiol concentration contributed largely to this pattern, and Subjects 6 and 15 were the major contributors of the fluctuations (Figure 3C, D). It could be suggested that these two subjects are high responders to T administration. While the time score of the first component increased immediately after the start of T gel treatment, it rapidly decreased between the second and third week of treatment (Day 36 to Day 47) before returning to higher values during the fourth and

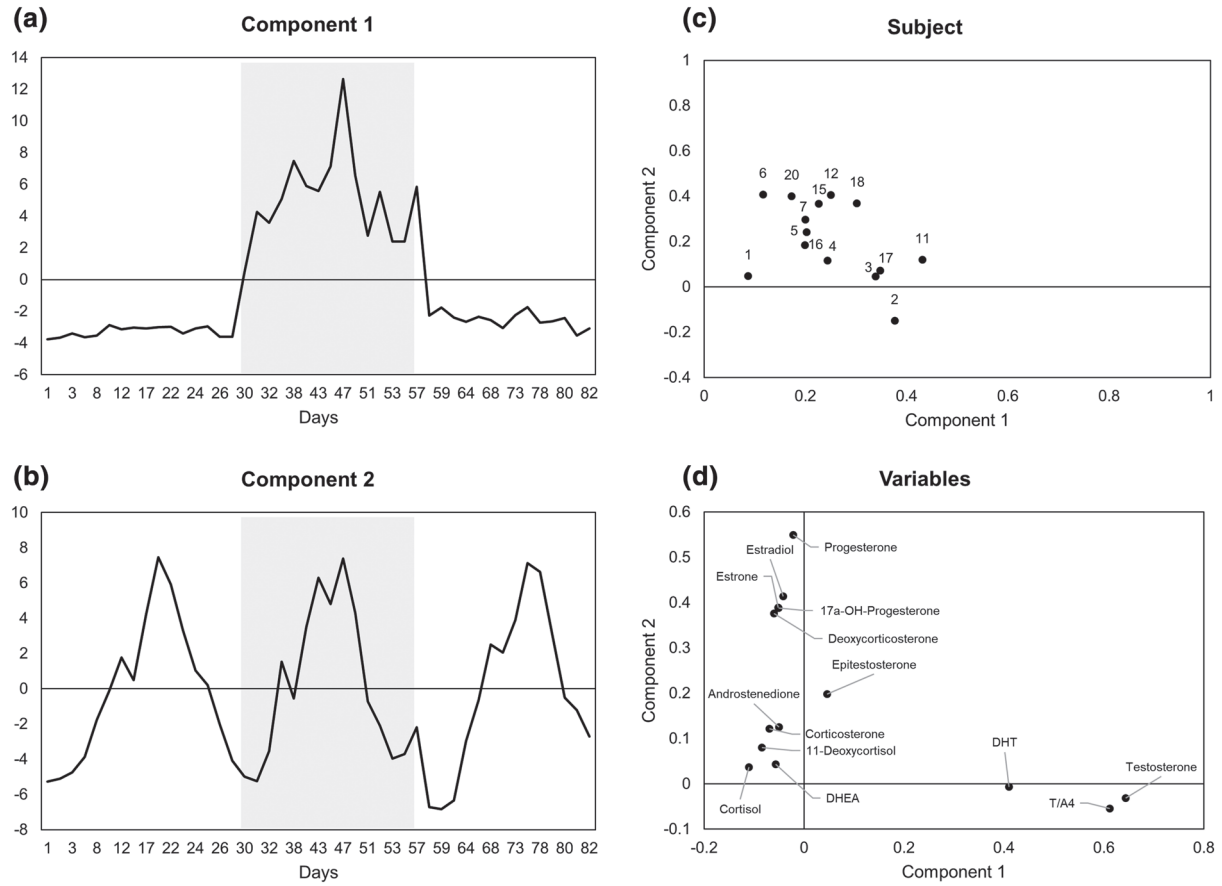



**FIGURE 2** Score plots of time mode of Components 1 (A) and 2 (B) obtained from PARAFAC modelling on serum steroid data. Score plots of Components 1 and 2 for subjects (C) and variables (D)

last week of T gel administration. It is to be noted that the variables with the highest scores for Component 1 were the two ratios dependent of E concentration. Component 2 seemed also to be related to T administration with the same altered biomarkers as Component 1 in addition to T (Figure 3B). Contrary to Component 1, the time score increased between the second and the third week of treatment but returned to baseline during the fourth week of T administration. However, this pattern was largely associated with Subject 2 (80.7% of the total component inertia), with a longer menstrual cycle, whereas the scores for the remaining subjects were low for the Component 2. We may therefore hypothesize that the decrease in Component 1 during treatment is linked to the effect of menstrual cycle on E-dependent ratios and that this decrease is pronounced during the fourth week of treatment (Day 50 to Day 54) for Component 2 due to the shift in the menstrual cycle for Subject 2. These phenomena might originate from some subjects with heterogeneous profiles or profiles shifted in the time.

## 3.2 | Evaluation of menstrual cycle and T administration

The PARAFAC models highlighted patterns related to T administration as well as menstrual fluctuations for variables originating from serum and urine matrices. Both aspects were therefore evaluated in deeper details by monitoring the mean values of the biomarkers across time.

The fluctuations related to the menstrual cycle are of high importance in longitudinal evaluation of biomarkers in healthy eumenorrheic women. The impact of the menstrual cycle phases was therefore investigated based on the multiple variables using data from the control phase (Phase 1). Among the six urinary parameters included into the steroidal module of ABP, E concentration was the only one significantly influenced by the phases of the menstrual cycle as reported in previous studies.[24,25,37] Median concentration was greatly higher in the ovulatory and luteal phases compared with the follicular phase ($p < 0.0001$) (Figure 4A). Notably, the E concentration in the luteal phase was more than twice as high as in the follicular phase, and it demonstrated the highest relative standard deviation (RSD) in function of the menstrual cycle. Consequently, E-dependent ratios (T/E and 5αAdiol/E) were conversely influenced with higher values in the follicular phase compared with the ovulatory and luteal phases, and the E-dependent ratios T/E and 5αAdiol/E had the highest variability among the ratio biomarkers longitudinally monitored in the ABP (Table 1). There was no association between low T or E concentrations and elevated RSDs suggesting that the variability was rather explained by endogenous variation than analytical performance. Finally, the results showed that other urinary concentrations or ratios were not significantly affected by the phases of the menstrual cycle.

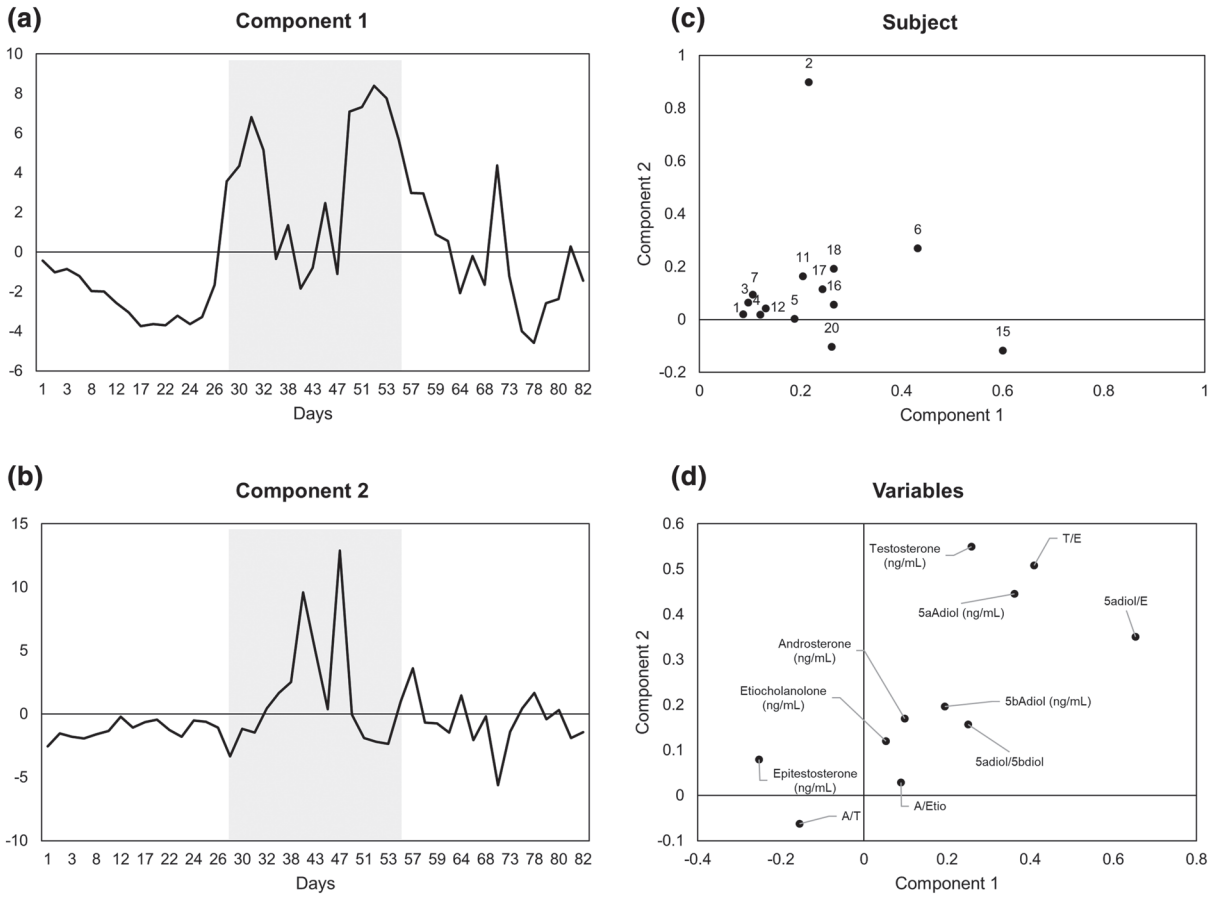



**FIGURE 3** Score plots of time mode of Components (A) 1 and (B) 2 obtained from PARAFAC modelling on urine steroid data. Score plots of Components 1 and 2 for (C) subjects and (D) variables

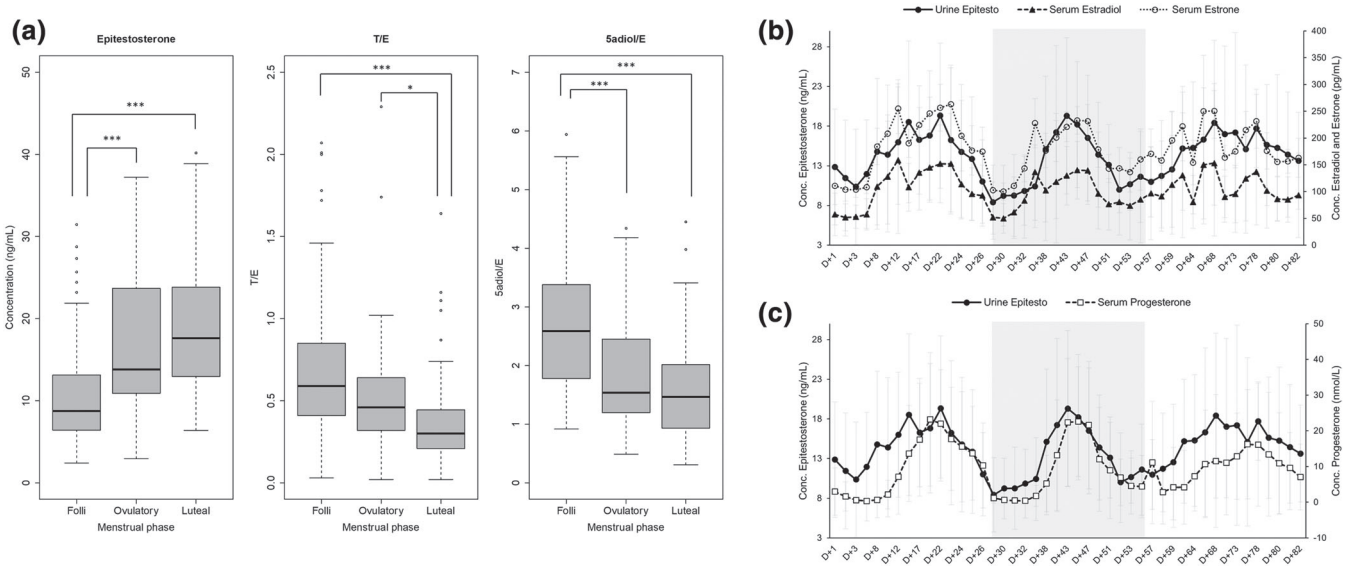



**FIGURE 4** (A) Boxplots of [E], T/E and 5αAdiol/E according to the menstrual phase of the subjects during Phase 1 (follicular $n = 98$; ovulatory $n = 34$; luteal $n = 72$) $^{*}p < 0.05$; $^{***}p < 0.001$. (B) Mean (SD) serum concentrations of oestradiol and oestrone in comparison with urinary epitestosterone and (C) mean (SD) serum concentration of progesterone in comparison with urinary epitestosterone during the whole study period. The grey area corresponds to the treatment phase and divides the three study phases

**TABLE 1** Relative standard deviation of the urinary T and E concentrations, T/E and 5αAdiol/E and of serum T, DHT concentrations and T/A4 during one menstrual cycle

| Subject | Testosterone (urine) | Epitestosterone | T/E | 5αAdiol/E | Testosterone (serum) | DHT | T/A4 |
|---|---|---|---|---|---|---|---|
| S1 | 20% | 41% | 26% | 25% | 11% | 20% | 8% |
| S2 | 35% | 58% | 25% | 37% | 30% | 38% | 14% |
| S3 | 35% | 32% | 20% | 26% | 19% | 24% | 12% |
| S4 | 25% | 39% | 36% | 48% | 12% | 18% | 12% |
| S5 | 19% | 41% | 39% | 38% | 11% | 16% | 11% |
| S6 | 20% | 49% | 48% | 43% | 16% | 31% | 9% |
| S7 | 35% | 33% | 31% | 35% | 22% | 17% | 20% |
| S11 | 22% | 48% | 45% | 47% | 19% | 64% | 11% |
| S12 | 39% | 35% | 28% | 33% | 14% | 9% | 10% |
| S15 | 48% | 39% | 29% | 26% | 16% | 15% | 10% |
| S16 | 50% | 40% | 27% | 30% | 23% | 19% | 15% |
| S17 | 49% | 35% | 57% | 29% | 15% | 16% | 8% |
| S18 | 23% | 46% | 34% | 39% | 17% | 15% | 11% |
| S20 | 29% | 25% | 24% | 21% | 16% | 12% | 12% |
| Median | 32% | 40% | 30% | 34% | 16% | 18% | 11% |

As illustrated on Component 2 with serum data, the major female sex hormones, namely, E1, E2, P and 17αOHP, were highly related to menstrual fluctuations with consistent variations between the three study phases. Among the other serum steroid hormones quantified by UHPLC–MS/MS, few androgens were also influenced by the phases of the menstrual cycle. For example, T concentration was slightly higher in the ovulatory (median 1.21 nmol/L) and luteal phase (median 1.05 nmol/L) compared with the follicular phase (median 0.93 nmol/L). The same was also observed for A4 concentration (ovulatory: 6.38 nmol/L; luteal: 5.09 nmol/L; follicular: 4.73 nmol/L). This increase originates from theca cells and is subsequent to LH peak and progesterone increase in the ovulatory and luteal phase. Accordingly, LH correlated significantly with A4 concentrations throughout the study period ($r = 0.5$; $p < 0.001$). As observed in the PARAFAC model, T and/or A4 do not contribute to the fluctuations observed in Component 2, and we can therefore argue that the effect of the menstrual cycle on these biomarkers is low and negligible. Furthermore, the proposed association of T and A4 in a ratio would also attenuate this increase.

Similarly to urinary parameters, the intraindividual variability of the serum steroids concentration was also evaluated based on the data from the first menstrual cycle (Table 1). For potential biomarkers such as T, DHT and A4, concentrations remained stable throughout the cycle with RSD of 16% for T and 18% for DHT. The combination of T with A4 in a T/A4 ratio had even lower RSD of 11%. Thus, in comparison with urine, the variability observed in serum parameters is decreased by almost factor of two. It also reinforced the fact that the influence of menstrual cycle on T or A4 is only scarce and should not cause variability issues for longitudinal monitoring of an individual.

The fluctuations of the main menstrual hormones correlated well with urinary E concentration, and they were repeatable over the three consecutive menstrual cycles (Figure 4B,C). These findings support the earlier results stating the E metabolism as independent from T[38] and suggest that E concentration is not influenced by exogenous T administration due to the absence of negative feedback in females. The strongest correlation to E was observed for E1 ($r = 0.56$; $p < 0.001$), whereas similar values were observed for E2 ($r = 0.45$; $p < 0.001$) and P ($r = 0.49$; $p < 0.001$). A significant but weaker correlation was also featured with LH ($r = 0.28$; $p < 0.001$), probably explained by the variability of the LH peaks between the subjects. While the variations between both oestrogens and E concentrations seemed temporally related, a slight shift of few days was noted for the relation progesterone–epitestosterone. Indeed, it seems that the increase of urinary E occurs before the elevation of P levels. These results suggest a possible control of E production or excretion by the HPG axis. For example, pregnant women demonstrate high level of urinary E within the first trimester concordant with large levels of oestrogens and P highlighting the hypothetical antiandrogenic effect of E.[39] Furthermore, women with polycystic ovary syndrome (PCOS), which is characterized by increased pulsatile release of GnRH and consecutive increased LH/FSH ratio, displayed higher levels of urinary E compared with women with normal menstrual cycle as reported in a previous study.[24] From the clinical perspective, it is also to be noted that a 28-day T gel treatment did not significantly disturb the menstrual status of the subjects as they continued to ovulate, and the female sex hormones fluctuations appeared not affected by the increased serum T of the second phase.

The influence of menstrual cycle combined with T administration was further studied for the most relevant urinary and serum biomarkers throughout the study. The mean serum T and DHT concentration corroborated with results of PARAFAC (Figure 5A,B). T and DHT started to increase 1 day after the first T gel application and

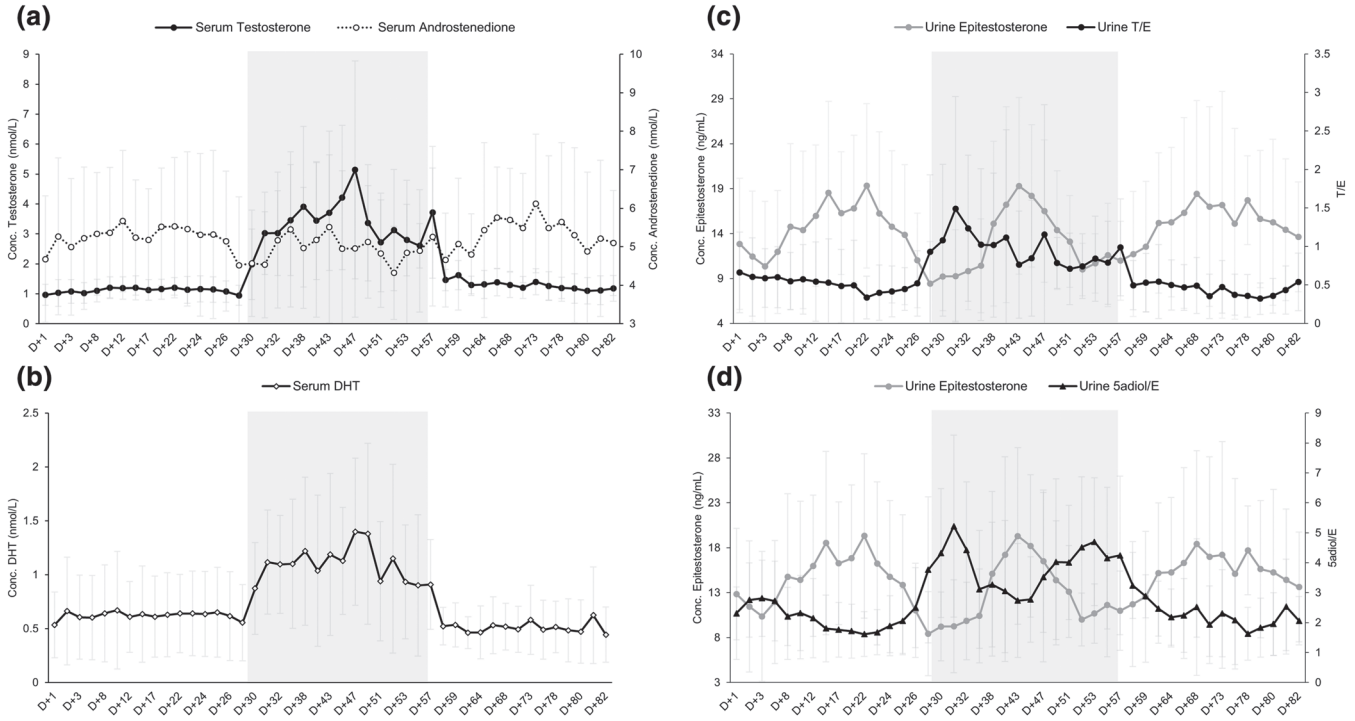



**FIGURE 5** (A) Mean (SD) serum T and androstenedione concentrations and (B) mean serum DHT concentration over the study period. (C) Mean (SD) urinary T/E and (D) 5αAdiol/E in comparison with E concentration over the study period. The grey area corresponds to the treatment phase and divides the three study phases

progressively continued to increase until reaching a global maximum at the end of the third week of treatment (Day 47). At this peak, the mean increase among all subjects was fivefold for T and 2.3-fold for DHT. Both concentrations slightly decreased during the last week of treatment and returned to baseline within 2–3 days. As expected, due to the direct metabolic connection, fluctuations of T and DHT were highly correlated ($p < 0.001$; $r = 0.57$) over the whole study period. A wider increase was expected for serum DHT due to the high expression of 5α-reductase in the skin and as reported in previous studies with male volunteers administered with either T patch or T gel.[5,19] The discrepancy might be explained by a rapid absorption of the gel through the skin due to the hydro-alcoholic origin of the gel or by lower 5α-reductase levels in women compared with men. When evaluating the effect of the study phase on serum steroid concentrations, population mean A4 was significantly lower ($p < 0.05$) in the second phase (treatment period) than in the first phase. However, when the factor 'day' was applied for comparison between the collection days, no significant difference was observed, which supports the hypothesis of the absence of negative feedback of T in women. Although a subtle decrease was observed in the population mean plot during Phase 2 (Figure 5A), A4 level was relatively stable throughout the study phases. Consequently, T/A4 ratio was proposed here as an additional biomarker for longitudinal monitoring in addition to T and DHT. This ratio has already been used in clinical endocrinology to characterize the excess of androgens.[36]

For urinary biomarkers, both T/E and 5αAdiol/E ratios peaked during the first week of treatment after four doses of T gel. T/E ratio progressively decreased during the 28-day treatment with lower values during the third and fourth week of treatment (Figure 5C). For 5αAdiol/E ratio, a decrease was visible between the second and third week of treatment before returning to higher values in the fourth week (Figure 5D). Both biomarkers returned to baseline values within 2–3 days after the cessation of T administration. Across the three study phases, a decrease in both ratios was observed in the mid-end of the phase reflecting the effect of menstrual cycle on E concentration. Higher E levels and consecutive lower T/E and 5αAdiol/E values can therefore explain the decrease observed along Component 1 of the PARAFAC model during treatment as highlighted in Figure 3A. Whereas T/E is the most traditional diagnostic biomarker of ABP for T detection with various routes of administration, 5αAdiol/E appears to be particularly inclusive for the detection of transdermal testosterone.[5,40] Indeed, the high presence of 5α-reductase in the skin may favour this route of metabolism. Hence, the concentrations of A and 5αAdiol during the treatment were higher than 5βAdiol or Etio (Figure S1), and the urinary T concentration demonstrated high correlation with urinary 5αAdiol ($r = 0.7$; $p < 0.001$). The other urinary mean values are available in Figure S1.

The urinary steroid concentrations were also investigated for possible associations with serum hormones over the course of the study. Urinary 5αAdiol concentration was found to be significantly correlated with serum DHT ($r = 0.45$; $p < 0.001$) and to a lesser extent with serum T ($r = 0.35$; $p < 0.001$), highlighting the preferred 5α-metabolic pathway for exogenous transdermal T biotransformation.

### 3.3 | Individual longitudinal monitoring

Because the use of biomarkers in antidoping is intended to be personalized and sequential, individual and longitudinal monitoring of the affected urinary and serum steroid biomarkers was performed. The urinary steroid profile of each volunteer was evaluated using the ADAMS training adaptive model with the specificity set at 99%. By these means, steroid profile ratios were evaluated against baseline measurements, and the results for the treatment period and first week post-treatment are summarized in Table 2 (individual and complete profiles for T/E and 5αAdiol/E are available in Figure S2 and S3). Using the adaptive longitudinal model, all subjects of the study had at least one urine sample that was flagged as atypical during the T treatment phase. Data were also evaluated for the Suspicious Steroid Profile (SSP) criteria defined in the WADA technical document,[28] which were met in total by five samples from four different subjects. Regarding T/E > 4, the profiles were flagged for Subject 2 at Days 40 (with T > 50 ng/ml) and 47 (with T > 50 ng/ml, 5αAdiol > 150 ng/ml combined with 5αAdiol/E > 10) and for Subject 16 at Day 31. Two additional samples containing a concentration of ethyl glucuronide (EtG) above 5 μg/ml also met the criteria for A/T < 20 (Subject 3 at Day 31) and for T > 50 ng/ml (Subject 20 at Day 71). To support the ABP evaluation, UGT2B17 genotyping was performed and two individuals (Subjects 1 and 17) were identified as bearing the *del/del* genotype for UGT2B17 among the 14 subjects. Notably, the phenotypical properties (low T/E values and T concentrations) already anticipated the genotype of both individuals.

Overall, the T/E and 5αAdiol/E ratios returned the most ATPFs and were considered as the most robust diagnostic parameters for the identification of atypical samples linked to T administration as observed using unsupervised multiway analysis. Most of the time, both parameters were flagged simultaneously. While 5αAdiol/E returned the most of all ATPFs and increased the detection window for some subjects (3, 6, 7), it is also the biomarker with the most non-specific ATPFs which could be linked to E fluctuations during the menstrual cycle or alcohol consumption. This suggests and reinforces the idea that an abnormal 5αAdiol/E alone may not be specific enough to target a GC/C/IRMS confirmation and that the combination with an abnormal T/E is preferred for a better sensitivity and specificity, except in the situation of a subject with low T excretion. On average, the adaptive model flagged approximately 50% of the samples during the treatment period. The majority occurred during the first and the last week of the administration phase, highlighting once again the impact of E fluctuations during the menstrual cycle. Low T excretors such as Subject 1 (*del/del*), 6 or 17 (*del/del*) did not show difference of sensitivity between T/E and 5αAdiol/E as reported in male subjects.[41] This low sensitivity could either be explained by a rapid elimination of exogenous T, as urine samples were collected 24 h after gel application, or by elevated urinary E levels in the middle end of the menstrual cycle, which consequently decreased T/E and 5αAdiol/E ratios that

**TABLE 2** Atypical findings (ATPFs) of the urinary steroid module at each time point for each subject during the treatment phase as highlighted in light grey

| | | Urine steroid profile | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Subject | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 11 | 12 | 15 | 16 | 17 | 18 | 20 |
| Admin (day) | 30 | | A;B | | | | A;B;D | A;B | | | A;B | A;B | | A;B | B |
| | 31 | | A;B | A;B;C | | | A;B;D | A;B | | | A;B | A;B | A | A;B | A;B |
| | 32 | A | A;B | | | | A;B;D | A;B | A;B | | A;B | | A | A | B |
| | 36 | | A;B | | | | | A;B | A;B;D | | | A | A | | |
| | 38 | | A;B | | | | B;D | A;B | A;B | | | A | A | | |
| | 40 | | A;B | | | | B;D | | A;B | A | | | | A | |
| | 43 | | A;B | | | | A;B;D | | A;B;D;E | A | | | A | | |
| | 45 | A | | | | | B;D | | A;B | | A | | A | A;B | |
| | 47 | A;B | A;B;C | A;B | | | B;D | | E | A | | | A;B | A | |
| | 50 | | | | | | A;B;D | | | | | | A;B | A | |
| | 51 | B | | | | A;B | A;B;D | | | | | | A;B;D;E | A;B | |
| | 52 | B | A | | A;B;D | | A;B;D | | | | | | A;B;D | | A;B |
| | 53 | A;B | | | A;B | A;B | A;B;D | | | A;B | | | A;B | | A;B |
| | 54 | | A | | | B | A;B;D | | | A;B | | | A;B;D | A;B | |
| | 57 | | A | | | | A;B;D | A;B | | | | | A | | A;B |
| Washout (day) | 58 | | | | B | B | A;B | A;B | | | | | A | | |
| | 59 | | | | | | B | B | | | | | | | |
| | 60 | | | B | | | B | A;B | | | | | | | |

*Note*: The T gel administration occurred between Day 29 and Day 56. A: T/E; B: 5αAdiol/E; C: A/T; D: 5αAdiol/5βAdiol; E: A/Etio. The dark grey indicates a noncollected sample.

returned within normal individual ranges. These results also suggest that negative feedback of E production following low doses of transdermal T administration do not occur in women. Moreover, both ratios demonstrated the highest RSD of the five biomarkers during a regular menstrual cycle. This caused much wider individual tolerance ranges calculated by the adaptive model and therefore complicated the interpretation of the profile as well as the detection of T administration.

Although subjects were told to restrain their alcohol consumption during the study, EtG above 5 μg/ml (5–40 μg/ml) was reported in 16 of 515 (approximately 3%) urine samples collected. Among these, four samples were collected during treatment phase. While for their majority, urinary concentrations were not significantly affected by alcohol consumption, the sample collected at Day 31 for Subject 3 and containing a moderate level of EtG (37 μg/ml) exhibited a strong increase of T and 5αAdiol concentrations, which masked the response to T administration. For Subject 6, the presence of EtG (5, 6 and 9 μg/ml, respectively) in samples collected at the end of the study (Days 78, 80 and 82) coincided with atypically high 5αAdiol/E ratio, whereas a 40 μg/ml concentration of EtG in sample collected at Day 71 for Subject 20 generated high T/E, 5αAdiol/E and low T/A.

PARAFAC modelling highlighted T, DHT and T/A4 as possible biomarkers of T administration, and the potential of each marker for detecting T gel was therefore evaluated using individual thresholds. Because no adaptive model is not yet available for blood matrix, longitudinal monitoring of T, DHT, T/A4 was performed using individual limits calculated as mean ± 3SD of the baseline samples (Phase 1) as they were considered as the most relevant biomarkers. This approach corresponds to the specificity at the 99% level and was already used in several previous studies.[5,18,19] ATPFs were determined as the time points exceeding individual threshold, and results from both treatment phase and the first week post-treatment are summarized in Table 3 (individual profiles are available in Figure S4, S5 and S6).

Using the longitudinal approach, all subjects had at least one serum sample that was flagged as atypical during the treatment phase. T and consequently T/A4 returned the most ATPFs whereas atypical DHT concentrations were always associated with ATPFs for T or T/A4. Indeed, for few volunteers, DHT concentration never exceeded the individual threshold suggesting a potential polymorphism in the 5α-reductase activity. Moreover, it indicates that DHT may serve as secondary biomarker, similarly to 5αAdiol/E. The combination of T and A4 improved the detection (20% more ATPFs than for T) and more interestingly increased the window of opportunity to detect T after the end of the treatment. Overall, during the whole treatment period, values above individual threshold for T, DHT and/or T/A4 were reported for 92% of the samples. The few samples that were not flagged based on the serum steroid concentrations were the ones collected 1 day after the first application of T gel (Day 30). The sensitivity was maximal during the third week of T gel application when all samples of each volunteer demonstrated abnormal values. The response of the three biomarkers was independent from basal T concentrations, and we also can speculate that the effect of menstrual cycle on T could be neglected for longitudinal monitoring. However, because the samples were all collected in the morning, the effect of the circadian rhythm could not be taken into account as some hormones were reported to demonstrate diurnal variation with elevated levels in the morning.[42] Because antidoping samples may be collected at different times of the day, this factor needs to be considered for interpretation. As both T and A4 exhibit a similar diurnal variation, the ratio T/A4 may compensate for this effect.[19] No significant difference between low T excretors and the other subjects could be observed in the number of ATPFs. We may therefore suggest that T and T/A4 would be used as primary biomarkers, whereas DHT would serve as secondary biomarker.

For serum samples collected at the same time as urine samples containing EtG, there was no sign of alteration of concentrations for T or DHT suggesting that the presence of EtG in urine samples seems not to be associated with significant alterations of serum steroid concentrations. Indeed, the influence of ethanol consumption on the regulation of serum steroids is probably more transient than on urinary concentrations. Moreover, it is hypothesized that the mechanism behind alteration of urinary steroid concentrations is related to a possible competition of ethanol with enzymes involved in the phase I or II metabolism,[43,44] which would be independent from free steroid in serum.

### 3.4 | ROC curve analysis

ROC curve analyses were performed to evaluate the overall accuracy of the measured parameters for the discrimination between control samples and sample collected during and after T administration. For that purpose, true positive rate (sensitivity) and false positive rate (1 – specificity) were assessed at different thresholds and reported as performance measurement on the ROC graph.

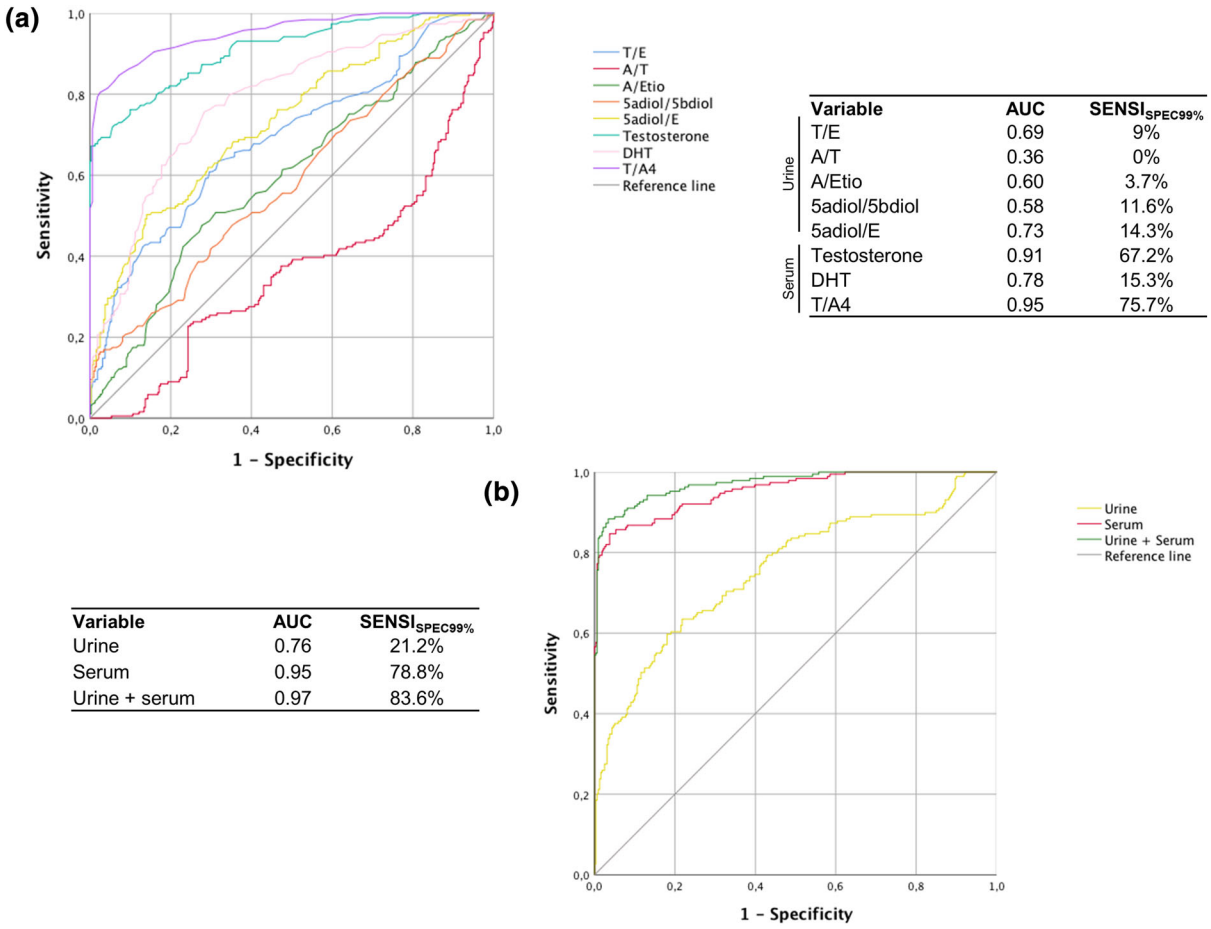
ROC curves were built for the five urinary ratios and for T, DHT and T/A4 (Figure 6A). Similar ROC curves were also performed for the combination of biomarkers, either the combination of the five urinary ratios or combination of serum T, DHT and T/A4 and combination of both combinations (Figure 6B). To do so, binary logistic regression was performed to obtain the probability, which was then used as the test parameter in the ROC curve.

Regarding evaluation of individual biomarkers, T/E and 5αAdiol/E were the most predictive parameters among all urinary ratios, despite demonstrating low sensitivity at 99% specificity ($SENSI_{SPEC99\%}$ = 9% and 14.3%), as all other ratios had lower discrimination power. The classification was much more accurate using serum biomarkers with T/A4 having the highest sensitivity of all tested biomarkers. The combination of multiple biomarkers further improved the general predictive accuracy, reaching a sensitivity of 83.6% at 99% specificity for the combination of urinary ratios and serum concentrations (Figure 6B). This finding underlines the great benefit of combining multiple evidence to increase the likelihood ratio that the observed fluctuations results from the use of a prohibited substance rather than from physiological or pathological condition.

**TABLE 3** Atypical findings (ATPFs) of the serum steroid module at each time point for each subject during the treatment phase as highlighted in light grey

| | | Serum steroid profile | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Subject | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 11 | 12 | 15 | 16 | 17 | 18 | 20 |
| Admin (day) | 30 | | Y | | | | | Y;Z | X;Y;Z | | X;Y;Z | X;Y | X;Y;Z | X;Y;Z | X;Y;Z |
| | 31 | | X;Y | X;Y;Z | X;Y;Z | X;Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y | X;Y;Z | X;Y | X;Y;Z | X;Y;Z | X;Y;Z |
| | 32 | X;Y | | X;Y;Z | X;Y;Z | X;Y | X;Z | X;Y;Z | X;Y;Z | X;Y | X;Y;Z | X;Y | X;Y;Z | X;Y;Z | X;Y;Z |
| | 36 | X;Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y | | X;Y;Z | X;Y;Z | X;Y | | X;Y;Z | X;Y;Z | | X;Y;Z |
| | 38 | X;Y | X;Y;Z | X;Y;Z | X;Y | X;Y | X | X;Y;Z | X;Y;Z | X;Y | Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y |
| | 40 | Y | X;Y | Y | X;Y | X;Y | X;Z | | X;Y;Z | X;Y;Z | X;Y | X;Y;Z | X;Y | X;Y;Z | X;Y;Z |
| | 43 | X;Y | X;Y | X;Y;Z | X;Y | X;Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | Y | | X;Z | X;Y;Z | X;Y |
| | 45 | X;Y | X;Y;Z | Y | X;Y | X;Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y | X;Y;Z | | X;Y;Z | X;Y;Z | X;Y;Z |
| | 47 | Y | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | X;Y;Z | Y | | X;Y;Z | X;Y;Z | X;Y |
| | 50 | | | X;Y;Z | X;Y | Y | X;Y;Z | X;Y;Z | | X;Y;Z | | | X;Y;Z | X;Y;Z | X;Y |
| | 51 | Y | Y | X;Y;Z | X;Y | X;Y | X;Y;Z | X;Y | | X;Y | | | X;Y | X;Y;Z | X;Y;Z |
| | 52 | Y | X;Y | X;Y;Z | X;Y | | X;Y;Z | X;Y;Z | | | | | X;Y;Z | Y | X;Y;Z |
| | 53 | X;Y | Y | Y | X;Y | X;Y | X;Y;Z | X;Y;Z | | X;Y;Z | | | X;Z | X;Y;Z | X;Y |
| | 54 | Y | X;Y | X;Y | | X;Y | Y | X;Y;Z | | X;Y;Z | | | X;Z | | X;Y;Z |
| | 57 | Y | X;Y | X;Y;Z | | | X;Y;Z | X;Y;Z | X;Y;Z | | | | X;Y;Z | | X;Y;Z |
| Washout (day) | 58 | Y | Y | | | Y | Y | Y | | | | | X;Y;Z | | |
| | 59 | Y | | | X | Y | Y | X;Y | | | | | X;Y;Z | | X;Y |
| | 60 | Y | | | | | | | | | | | Y | | Y |

*Note*: The T gel administration occurred between Day 29 and Day 56. X: T; Y: T/A4; Z: DHT. The dark grey indicates missing samples.




| | Variable | AUC | SENSI$_{SPEC99\%}$ |
|---|---|---|---|
| Urine | T/E | 0.69 | 9% |
| | A/T | 0.36 | 0% |
| | A/Etio | 0.60 | 3.7% |
| | 5adiol/5bdiol | 0.58 | 11.6% |
| | 5adiol/E | 0.73 | 14.3% |
| Serum | Testosterone | 0.91 | 67.2% |
| | DHT | 0.78 | 15.3% |
| | T/A4 | 0.95 | 75.7% |

| Variable | AUC | SENSI$_{SPEC99\%}$ |
|---|---|---|
| Urine | 0.76 | 21.2% |
| Serum | 0.95 | 78.8% |
| Urine + serum | 0.97 | 83.6% |

**FIGURE 6** Receiver operating characteristic (ROC) curves analysis of the urinary and serum steroid profile biomarkers. (A) Analysis of single biomarkers. (B) Analysis of combined biomarkers. Urine: combination of T/E, A/T, A/Etio, 5αAdiol/5βAdiol and 5αAdiol/E; serum: combination of T, DHT and T/A4. AUC, area under the curve [Colour figure can be viewed at wileyonlinelibrary.com]

## 3.5 | GC/C/IRMS analyses

In the antidoping context, GC/C/IRMS analysis is used as a confirmatory procedure for the detection of exogenous administration of EAAS, such as testosterone, and this analysis is usually triggered by ATPFs received from the evaluation of the adaptive model of the ABP. In this study, a subset of urine samples were intentionally selected for IRMS analyses. One urine sample from each week of the treatment phase (each Wednesday), as well as samples from the first post-treatment week, was selected for eight different subjects leading to a total of 40 samples analysed by GC/C/IRMS. The results were evaluated according to the criteria of the WADA technical document (TD2019IRMS[45]) and presented in Table 4. Overall, more than half of the tested urine samples did not fulfil the criteria for an adverse analytical finding (AAF; 'positive test result'). Negative results mainly occurred during the first week of treatment, corresponding to samples collected after only two doses of gel, and during the first week post-treatment corresponding to samples collected 3 days after the last T administration. It suggests that on one side, the administered dose was probably too low to significantly affect the $\Delta^{13}$C values of the TCs after two doses, and on the other side that $\Delta^{13}$C-values of TC returned to baseline levels very quickly following treatment cessation, even after a 28-day administration period. Nevertheless, among urine samples associated with GC/C/IRMS results not meeting the positivity criteria, several would still be reported as inconclusive (ATF; 'atypical test results') because in the laboratory's opinion, they are not consistent with the endogenous origin of the TC(s) (highlighted by an asterisk in Table 4). On the contrary, the number of positive and atypical samples increased as the treatment period progressed with most being detected during the third and fourth week of treatment, respectively. Positive results were mainly due to the combination of T and Adiols, especially 5αAdiol (criteria i), or the combination of both Adiols (criteria ii). All subjects had at least one sample fulfilling the positivity criteria, except for Subject 1 who is a UGT2B17 *del/del* individual. Whereas 5αAdiol $\Delta^{13}$C value was above 3‰ in one sample, T $\delta^{13}$C value was outside the linearity range and therefore too low to be reported, leading to an inconclusive overall result.

Among urine samples tested positive or atypical by GC/C/IRMS, the urinary steroid profile was flagged for half of them, whereas the serum steroid profile was flagged in all corresponding serum samples. Concerning samples evaluated as negative by GC/C/IRMS, 42% produced ATPFs for the urinary monitoring whereas almost 80% of

**TABLE 4** Summary of the GC/C/IRMS results according to the TD2019IRMS for a subset of urine samples of eight different subjects

| | | Subject | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Day** | **S1** | **S2** | **S3** | **S4** | **S5** | **S6** | **S7** | **S20** |
| Admin (day) Phase 2 | 31 | Negative | Negative* | Negative | Atypical (T > 3‰) | Atypical (T > 3‰) | Positive (both Adiols > 3‰) | Negative* | Atypical (T > 3‰) |
| | 38 | Atypical (5αAdiol > 3‰) | Positive (T > 3‰ and both Adiols > 3‰) | Positive (5αAdiol > 4‰ and 5α < −27‰) | Negative* | Negative* | Positive (both Adiols > 3‰) | Positive (T > 3‰ and both Adiols > 3‰) | Negative* |
| | 45 | Negative* | Positive (T > 3‰ and 5αAdiol > 3‰) | Negative | Positive (T > 3‰ and 5αAdiol > 3‰) | Positive (T > 3‰ and 5αAdiol > 3‰) | Positive (both Adiols > 3‰) | Positive (T > 3‰ and both Adiols > 3‰) | Positive (T = 3‰ and both Adiols > 3‰) |
| | 52 | Negative | Atypical (T > 3‰) | Atypical (T > 3‰) | Negative* | Atypical (T > 3‰) | Positive (both Adiols > 3‰) | Atypical (5αAdiol > 3‰) | Atypical (5βAdiol > 3‰) |
| Washout (day) Phase 3 | 59 | Negative | Negative | Negative | Negative | Negative* | Negative | Negative | Negative |

*Note*: Light grey indicates that concordant serum sample was above individual ranges, and dark grey indicates that both urine and serum samples were outside individual thresholds for the longitudinal profile. The T gel administration occurred between Day 29 and Day 56. * signifies that the GC/C/IRMS results are inconclusive because they do not meet the positivity criteria, but in the laboratory's opinion, they are not consistent with the endogenous origin of the target compound(s).

**TABLE 5** Mean values of the haematological parameters in different menstrual phases monitored for three menstrual cycles

| | Follicular | | | Ovulatory | | | Luteal | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | RSD | Mean | SD | RSD | Mean | SD | RSD |
| HCT (%) | 40.20 | 2.40 | 6.0% | 40.41 | 2.05 | 5.1% | 40.93 | 2.26 | 5.5% |
| HGB (g/dl) | 13.23 | 0.86 | 6.5% | 13.31 | 0.75 | 5.6% | 13.50 | 0.80 | 5.9% |
| IRF (%) | 7.97 | 2.89 | 36.3% | 8.52 | 2.91 | 34.1% | 8.17 | 2.83 | 34.7% |
| MCH (pg) | 29.75 | 1.05 | 3.5% | 29.73 | 1.09 | 3.7% | 29.88 | 1.14 | 3.8% |
| MCHC (pg/dl) | 32.91 | 0.48 | 1.5% | 32.94 | 0.53 | 1.6% | 32.97 | 0.51 | 1.6% |
| MCV (fl) | 90.38 | 3.09 | 3.4% | 90.24 | 3.13 | 3.5% | 90.67 | 3.72 | 4.1% |
| OFF score | 56.89 | 12.54 | 22.0% | 57.12 | 13.45 | 23.5% | 58.47 | 14.19 | 24.3% |
| PLT ($10^3$/μl) | 277.26 | 55.44 | 20.0% | 297.21 | 51.87 | 17.5% | 271.40 | 53.34 | 19.7% |
| RBC ($10^6$/μl) | 4.45 | 0.23 | 5.2% | 4.48 | 0.20 | 4.5% | 4.52 | 0.21 | 4.6% |
| RDW-SD (fl) | 41.46 | 2.66 | 6.4% | 40.95 | 2.45 | 6.0% | 41.43 | 2.96 | 7.1% |
| RET# ($10^6$/μl) | 0.07 | 0.02 | 33.3% | 0.07 | 0.02 | 27.0% | 0.07 | 0.02 | 28.9% |
| RET% (%) | 1.62 | 0.51 | 31.7% | 1.63 | 0.47 | 28.7% | 1.66 | 0.49 | 29.4% |
| WBC ($10^3$/μl) | 6.51 | 1.59 | 24.5% | 7.29 | 1.78 | 24.4% | 7.12 | 1.70 | 23.9% |
| ABPS | −0.95 | 0.86 | 10% | −0.91 | 0.82 | 9% | −0.98 | 0.97 | 11% |

*Note*: RSD indicates the interindividual relative standard deviations.

serum sample profiles exceeded individual thresholds. These observations suggest not only the applicability of serum steroid profiling to target GC/C/IRMS analysis in urine but also the great benefit of combining urinary and serum steroid profile data to obtain a stronger evidence of exogenous T administration, independent from GC/C/IRMS results. Finally, in situation of inconclusive GC/C/IRMS results consistent with the endogenous origin of the TCs (Negative* in Table 4), information originating from the urinary and blood steroid profile would be of particular value for further target testing.

## 3.6 | Haematological passport

The effects of the menstrual cycle and T gel administration on the haematological parameters of ABP were also evaluated. For this purpose, a whole blood sample was collected once a week during each phase of the clinical study.

The influence of the treatment was evaluated by comparing the three phases. A significant difference between the three menstrual cycles was only observed for RET% ($p = 0.03$) and consequently for OFF score ($p = 0.01$). RET% was more elevated in Phase 2 corresponding to the administration phase compared with the third phase ($p = 0.029$) and was close to significance in comparison with Phase 1 ($p = 0.05$). As this factor is dependent of reticulocytes, OFF score was also significantly lower in Phase 2 compared with Phase 3 but did not differ from the first phase.

Potential differences between the menstrual phases were also assessed, but no significant difference between follicular, ovulatory and luteal phases was observed for any of the variables (Table 5). These findings differ from the study conducted by Mullen et al. in which higher RET% was observed in the ovulatory and luteal phases.[26]

The interindividual variability (expressed as RSD) was more important for RET%, reticulocyte number (RET#) and immature reticulocyte fraction (IRF). The intraindividual variability of the haematological parameters was also assessed for each menstrual cycle of the study and for each subject. The median intraindividual RSD was low for HGB (3%) but more important for IRF (23%), RET% (14%) and therefore OFF score (13%) as well. There was no difference in the median CVs between the three menstrual cycles, indicating that the variability within a menstrual cycle is repeatable.

Haematological data were also investigated using the adaptive Bayesian model to generate individual profiles. In total, the model generated four ATPFs for three subjects. Subject 2 displayed an atypical high OFF score in the last sample mainly due to low RET%, whereas HGB and ABPS were stable and within individual ranges. Subject 16 displayed an atypical sequence OFF score on two consecutive weeks mainly caused by a significant RET% increase. In the case report file, an iron perfusion was reported a week before the increase of RET% providing a suggestive explanation for this abnormal status. Finally, Subject 18 demonstrated an atypically high HGB and OFF score in the last sample, which were probably caused by plasma volume fluctuations because RET% and ABPS were observed normal and within tolerance ranges. It is to be noted that for this particular sample, blood collection was problematic, and the procedure had to be repeated several times. Other subjects did not display any ATPF. Their parameters remained relatively stable during the whole study period and subjects supplied with oral iron due to low ferritin level did not show any significant deviation in the haematological variables. The absence of abnormal value related to T gel administration was most probably due to the low administered dose, insufficient to stimulate erythropoiesis compared with other studies.[5,12]

## 4 | CONCLUSION

To our knowledge, this is the first time that a study investigates the combined effects of the menstrual cycle and T gel administration on doping biomarkers. It confirms results from previous studies showing that urinary E level is deeply impacted by the hormonal fluctuations during the cycle with higher concentration at the mid-end of the cycle and with important correlation with female sex hormones. This cyclic fluctuation has a major impact on the E-dependent ratios, namely, T/E and 5αAdiol/E, and therefore on the capacity of these biomarkers to detect testosterone doping. Indeed, while these two biomarkers were most affected by transdermal T administration, elevated E levels during the menstrual cycle resulted in ratios within individual ranges for some subjects and therefore attenuated the response to T treatment. This phenomenon, combined with the rapid metabolism of exogenous T, decreased the sensitivity of the urinary biomarkers used in the prevailing ABP. Serum T and DHT were highlighted as the most promising blood biomarkers of T administration and were much less affected than urinary ratios by the menstrual cycle. A serum T/A4 ratio has been proposed as possible biomarker for antidoping purposes, as well as for the control of sudden androgen excess and association of data to correct gender in clinical context. Longitudinal monitoring of steroid hormones in serum including T, DHT and T/A4 demonstrated high sensitivity/specificity and were proven as powerful alternative and complementary approach to urinary steroid profile either for targeting confirmatory GC/C/IRMS analysis or as a single evidence of T doping. The information of combined blood and urinary steroid profile reached even higher sensitivity, underlining the importance of gathering a bundle of evidence to support the scenario of the administration of an endogenous prohibited substance.

### ACKNOWLEDGEMENTS

The study was carried out with financial support from the World Anti-Doping Agency (Grant 17D18MS). The authors would like to thank all subjects for participating in the study and are grateful to the research nurses at the Clinical Trial Unit of the CHUV Hospital for sample collection and logistical support. Céline Schobinger and Francesco Loria are also acknowledged for their technical assistance in the management of samples.

### CONFLICT OF INTEREST

None of the authors declare any financial conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Salamin O, Nicoli R, Langer T, et al. Longitudinal evaluation of multiple biomarkers for the detection of testosterone gel administration in women with normal menstrual cycle. *Drug Test Anal*. 2021;1–18. https://doi.org/10.1002/dta.3040

*Drug Testing and Analysis*

# Electronic Supplementary Material

# Longitudinal evaluation of multiple biomarkers for the detection of transdermal testosterone administration in women with normal menstrual cycle

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collected until the end of the Phase 1 period (n=15). The blue line indicates the [DHT] for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period and y-axis concentration in nmol/L

**Figure S6** – Longitudinal monitoring of T/A4 ratio. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by mean ± 3xSD of the baseline samples collected until the end of the Phase 1 period (n=15). The blue line indicates the T/A4 for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period and y-axis T/A4 values.

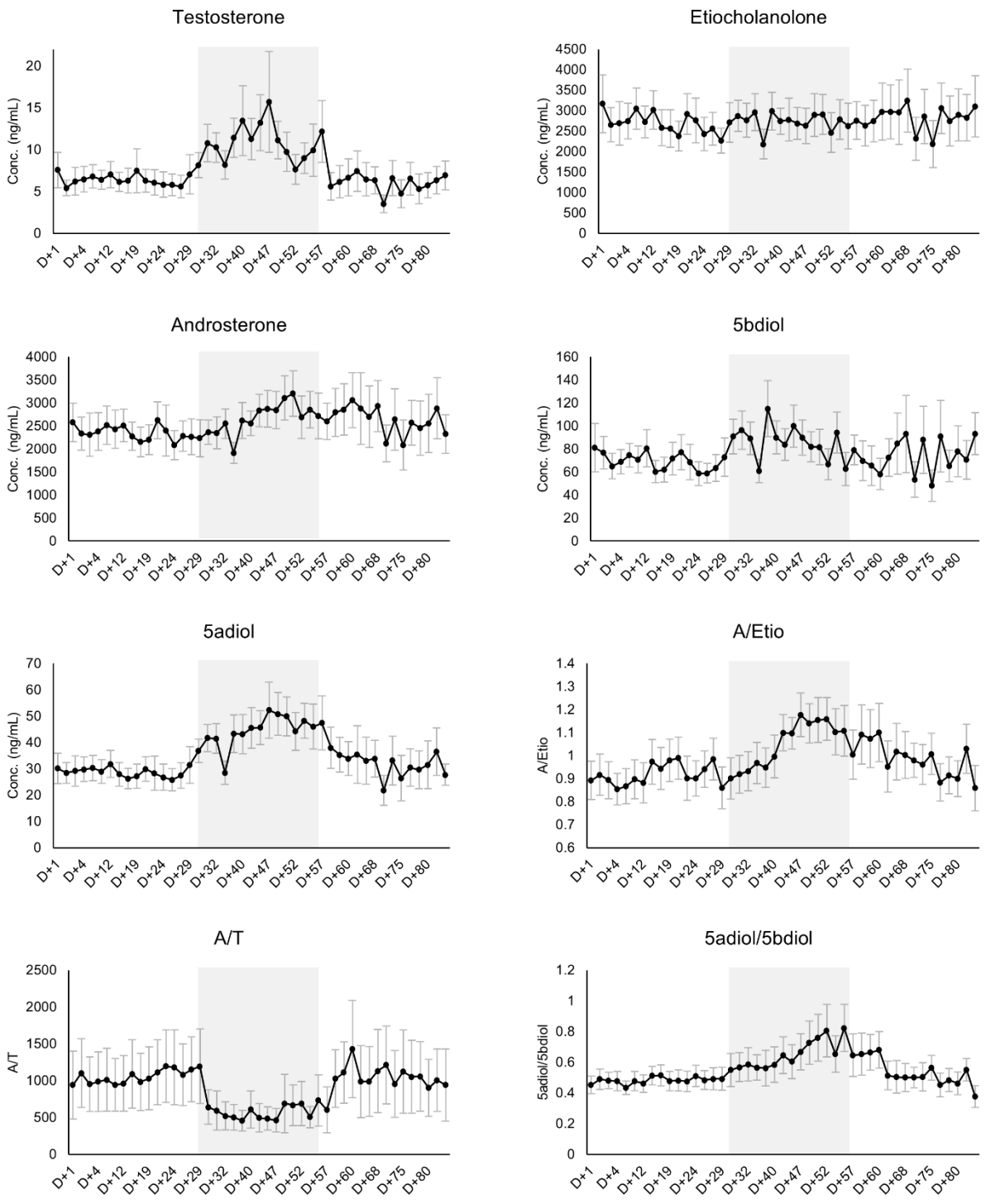


**Figure S1.** Mean of urinary concentrations and ratios over the study period. The grey area corresponds to the treatment phase and divides the three study phases.

**T/E (urine)**

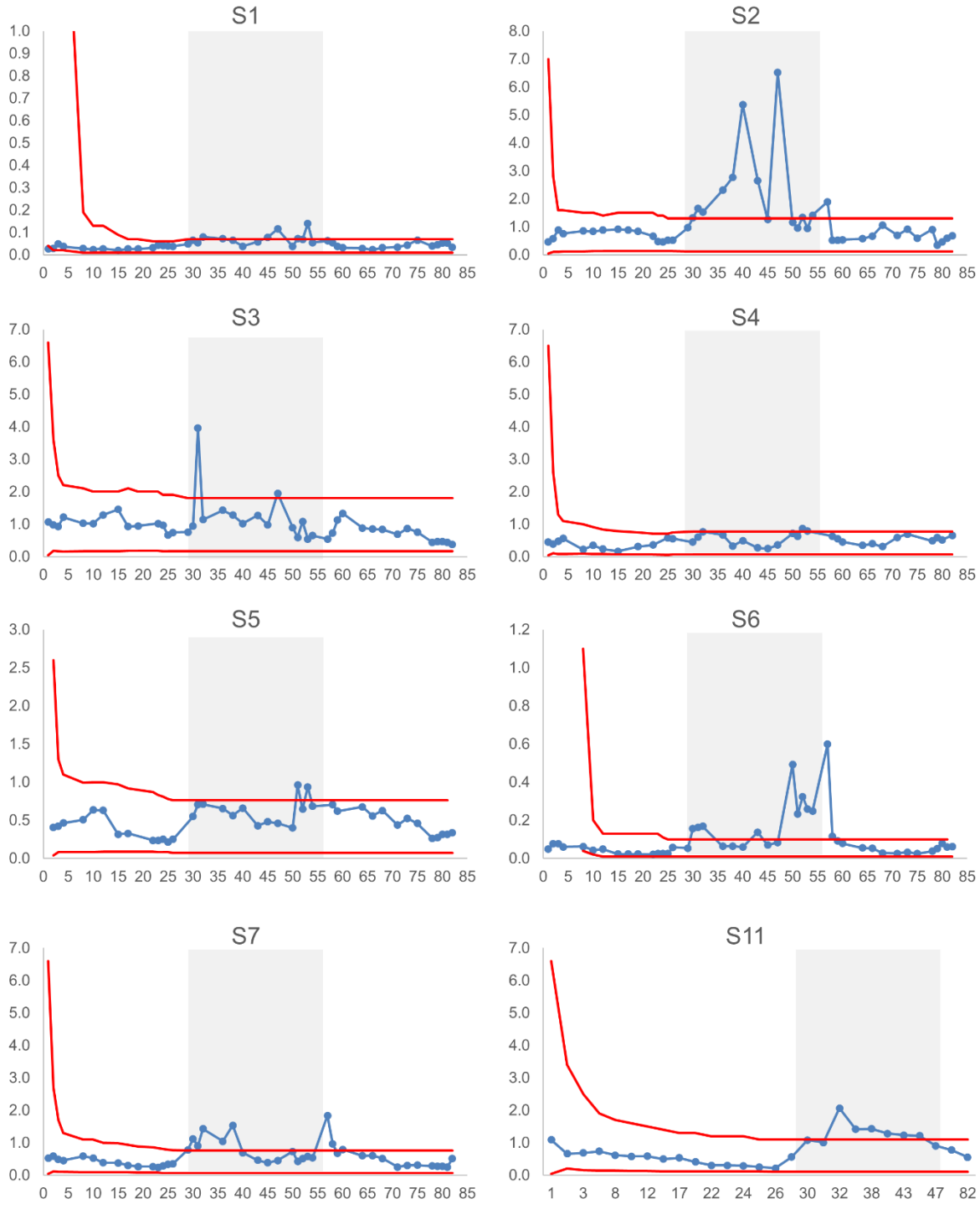


**Figure S2**. Longitudinal monitoring of T/E with ADAMS adaptive model. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by the adaptive model until the end of the Phase 1 period (n=15). The blue line indicates the T/E for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period.

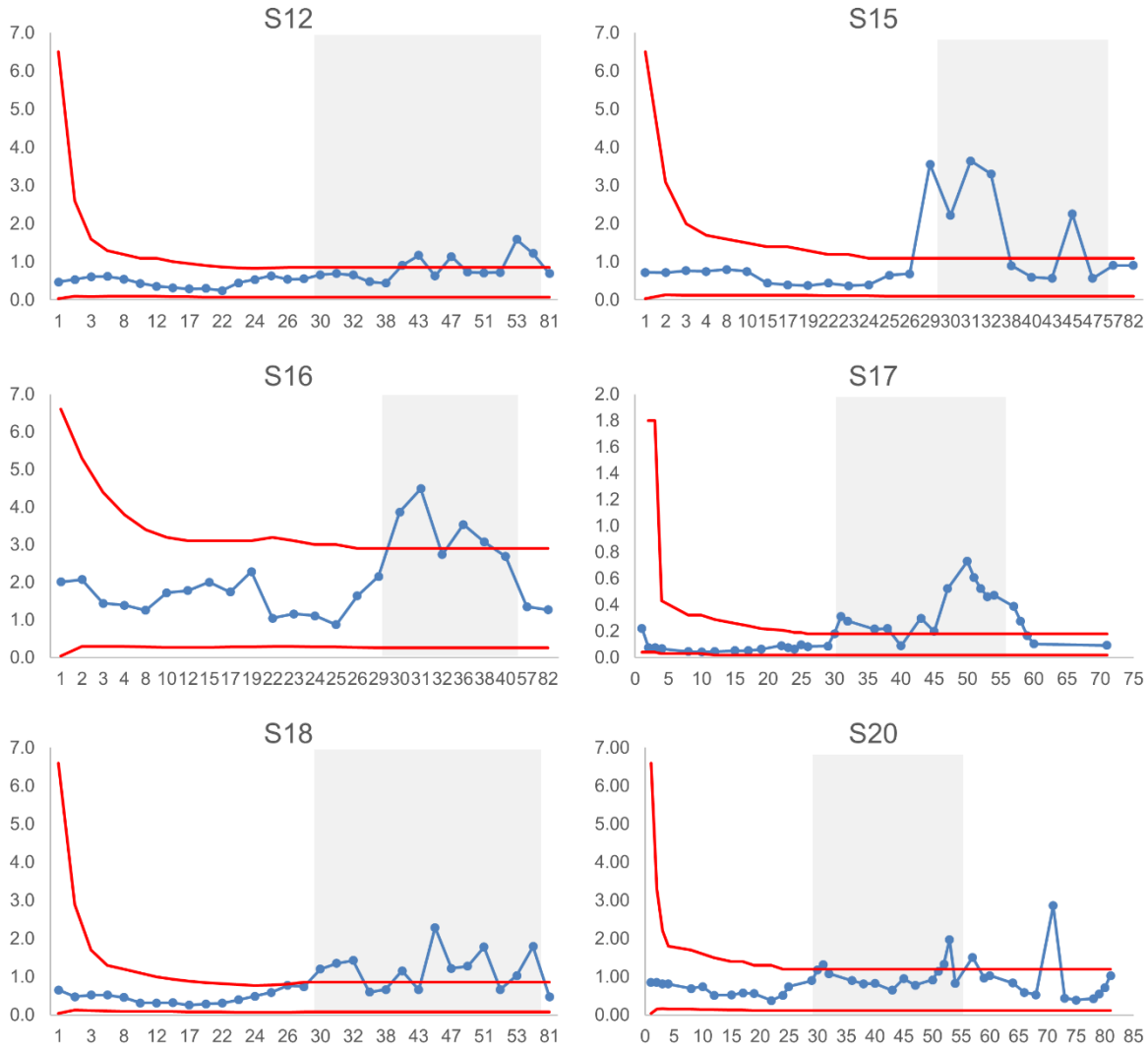


**Figure S2** (continued)

**5αAdiol/E (urine)**

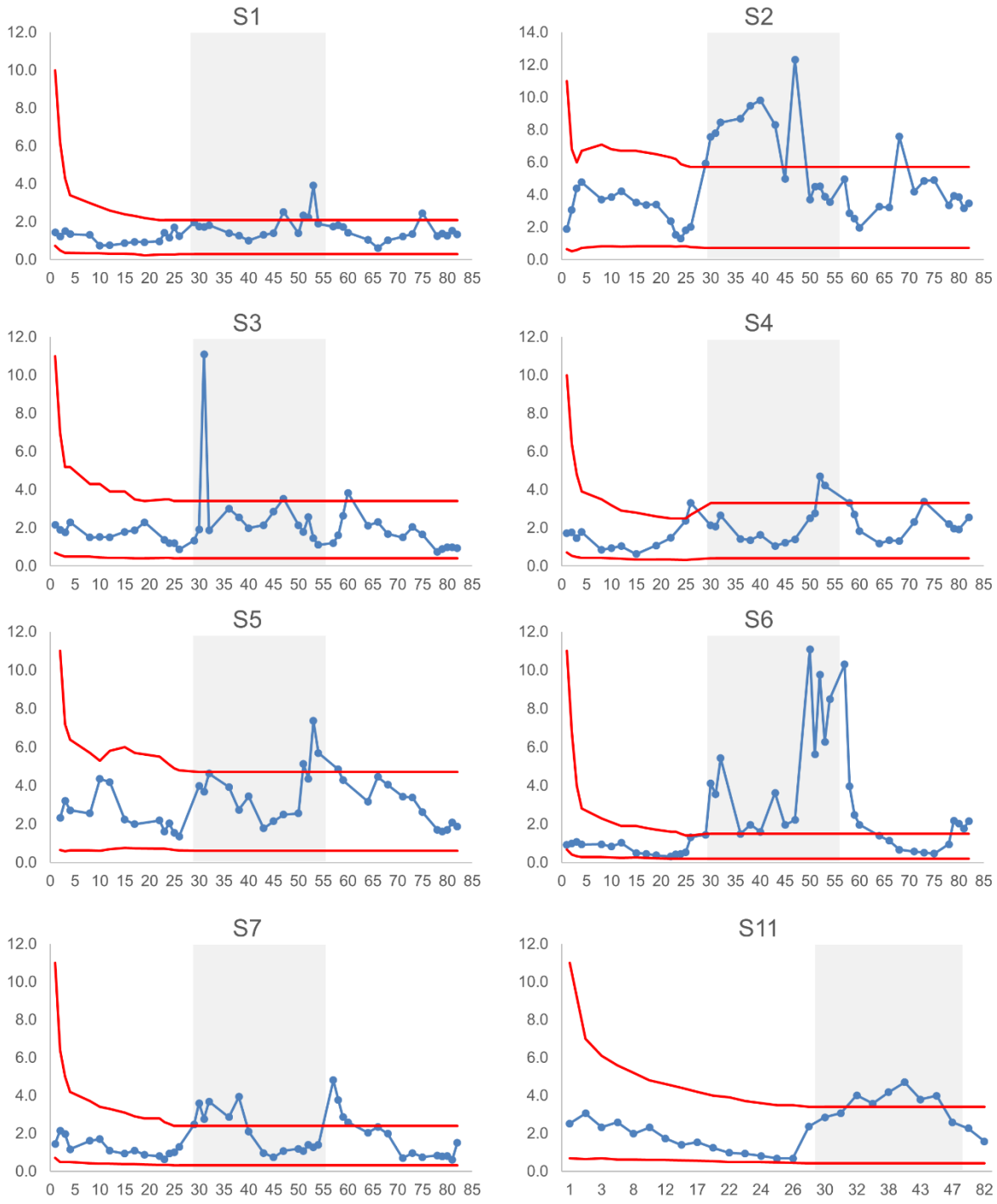


**Figure S3**. Longitudinal monitoring of 5adiol/E with ADAMS adaptive model. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by the adaptive model until the end of the Phase 1 period (n=15). The blue line indicates the 5adiol/E for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period.

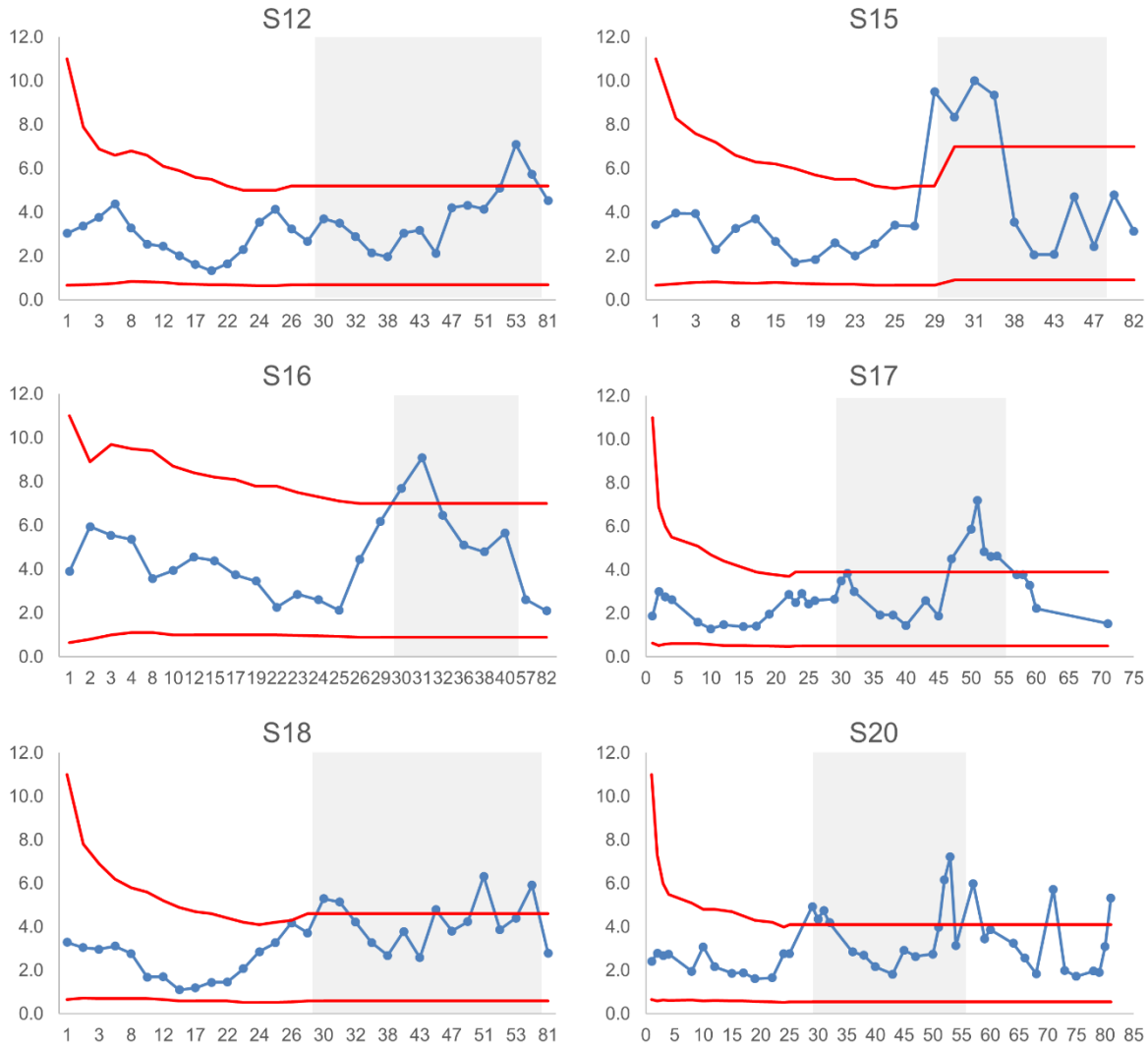


**Figure S3** (continued)

**Testosterone concentration (serum)**

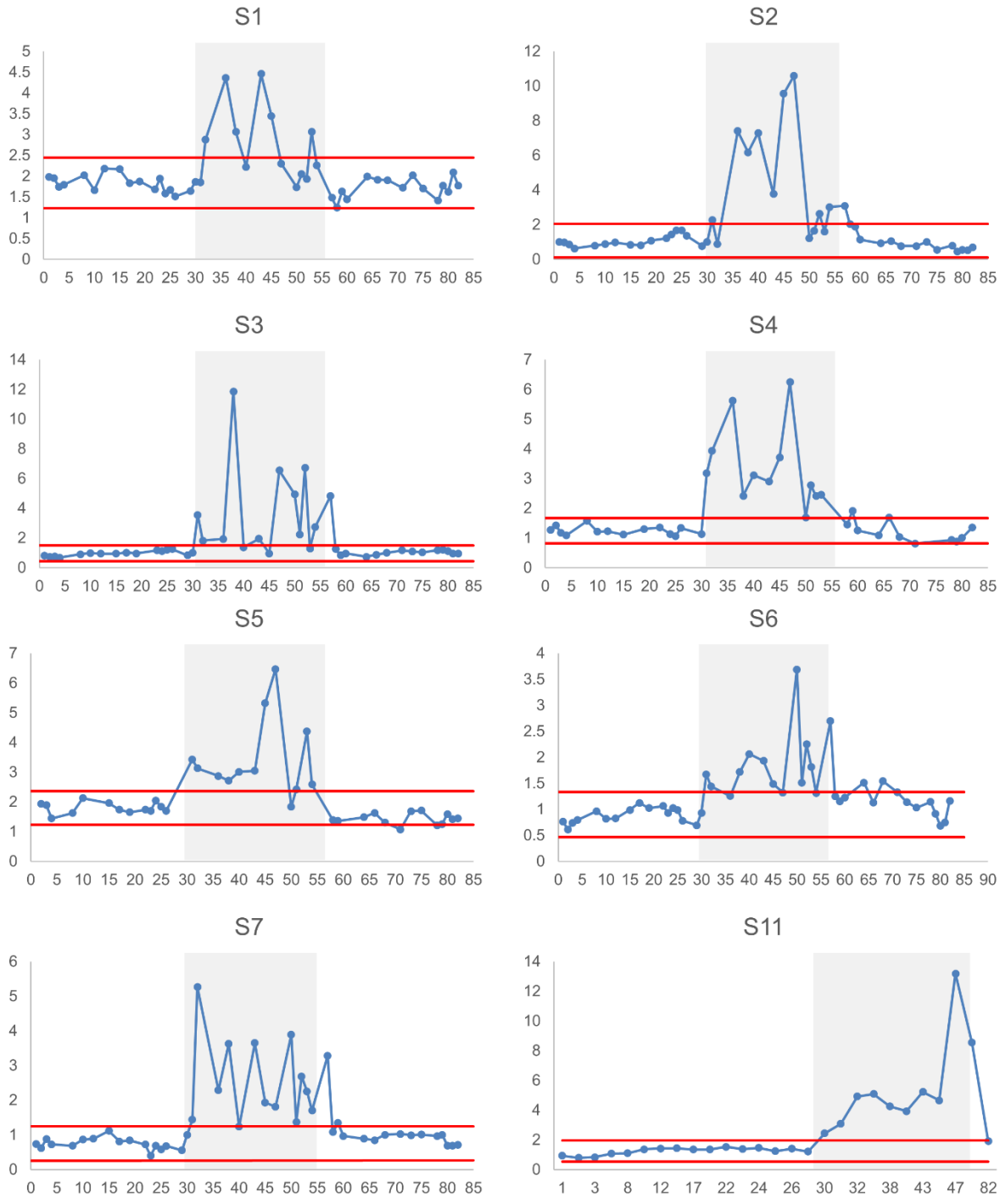


**Figure S4.** Longitudinal monitoring of T concentrations. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by mean ± 3xSD of the baseline samples collected until the end of the Phase 1 period (n=15). The blue line indicates the [T] for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period and y-axis concentration in nmol/L.


**Figure S4** (continued)

**DHT concentration (serum)**

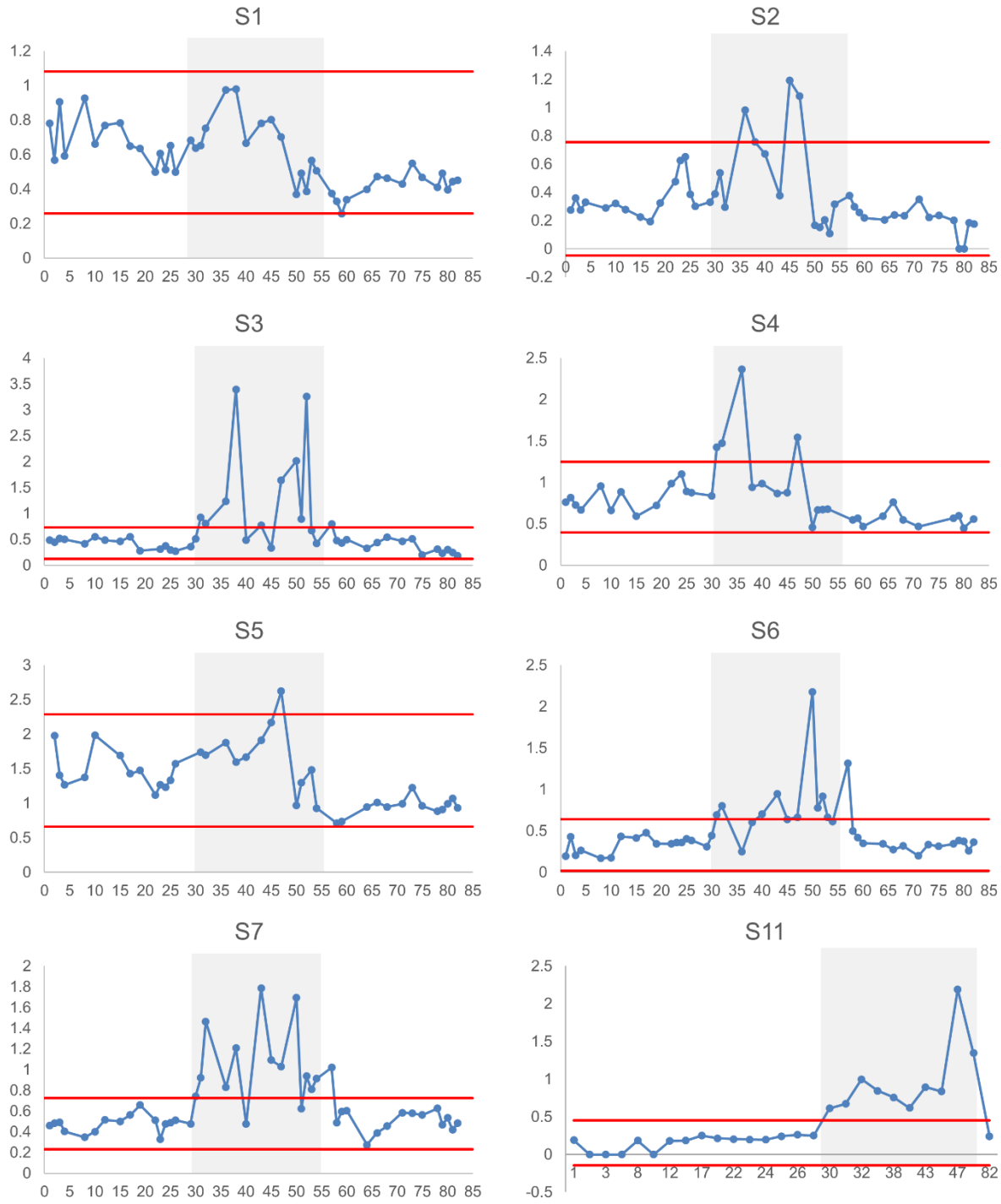


**Figure S5.** Longitudinal monitoring of DHT concentrations. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by mean ± 3xSD of the baseline samples collected until the end of the Phase 1 period (n=15). The blue line indicates the [DHT] for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period and y-axis concentration in nmol/L.

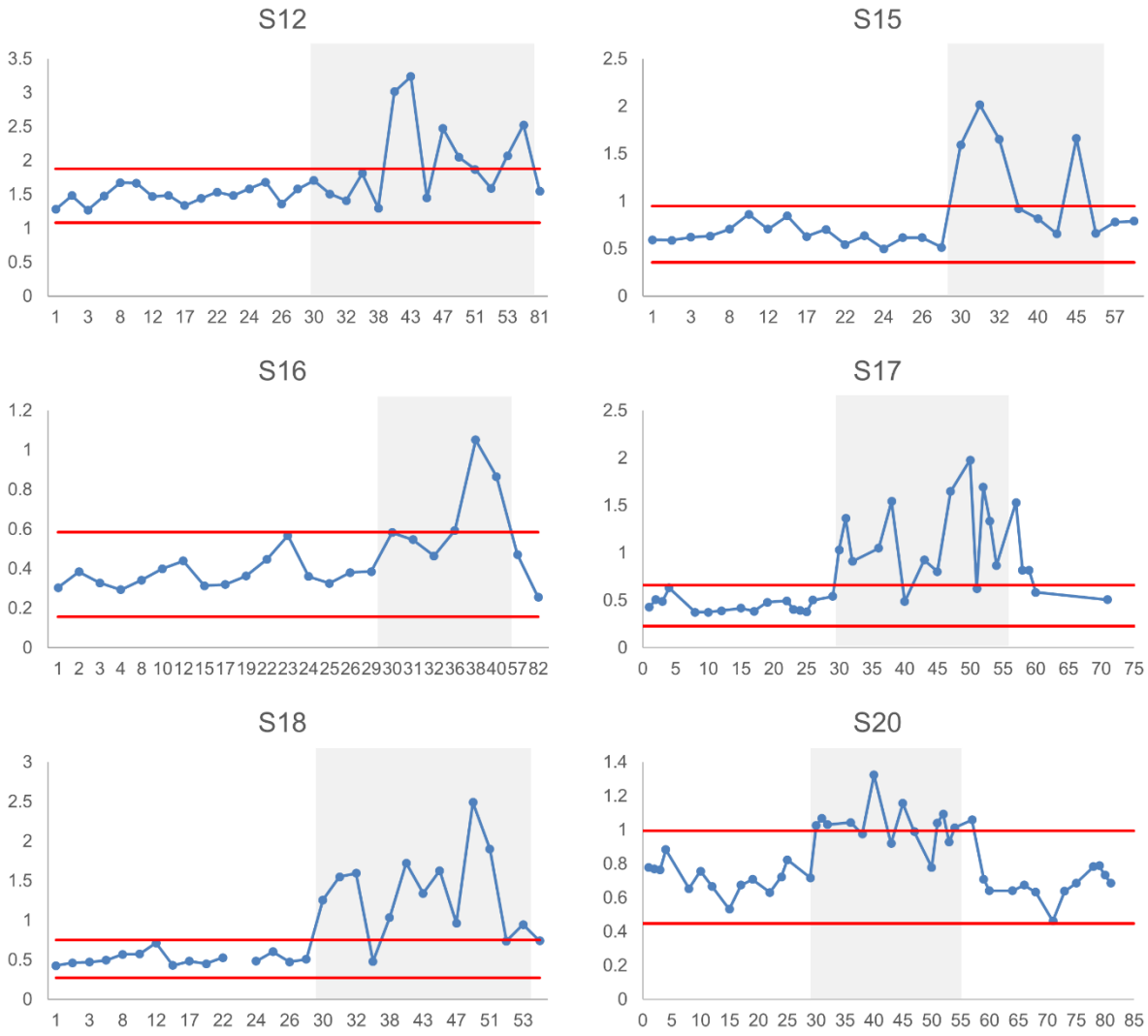


**Figure S5** (continued)

**T/A4 (serum)**

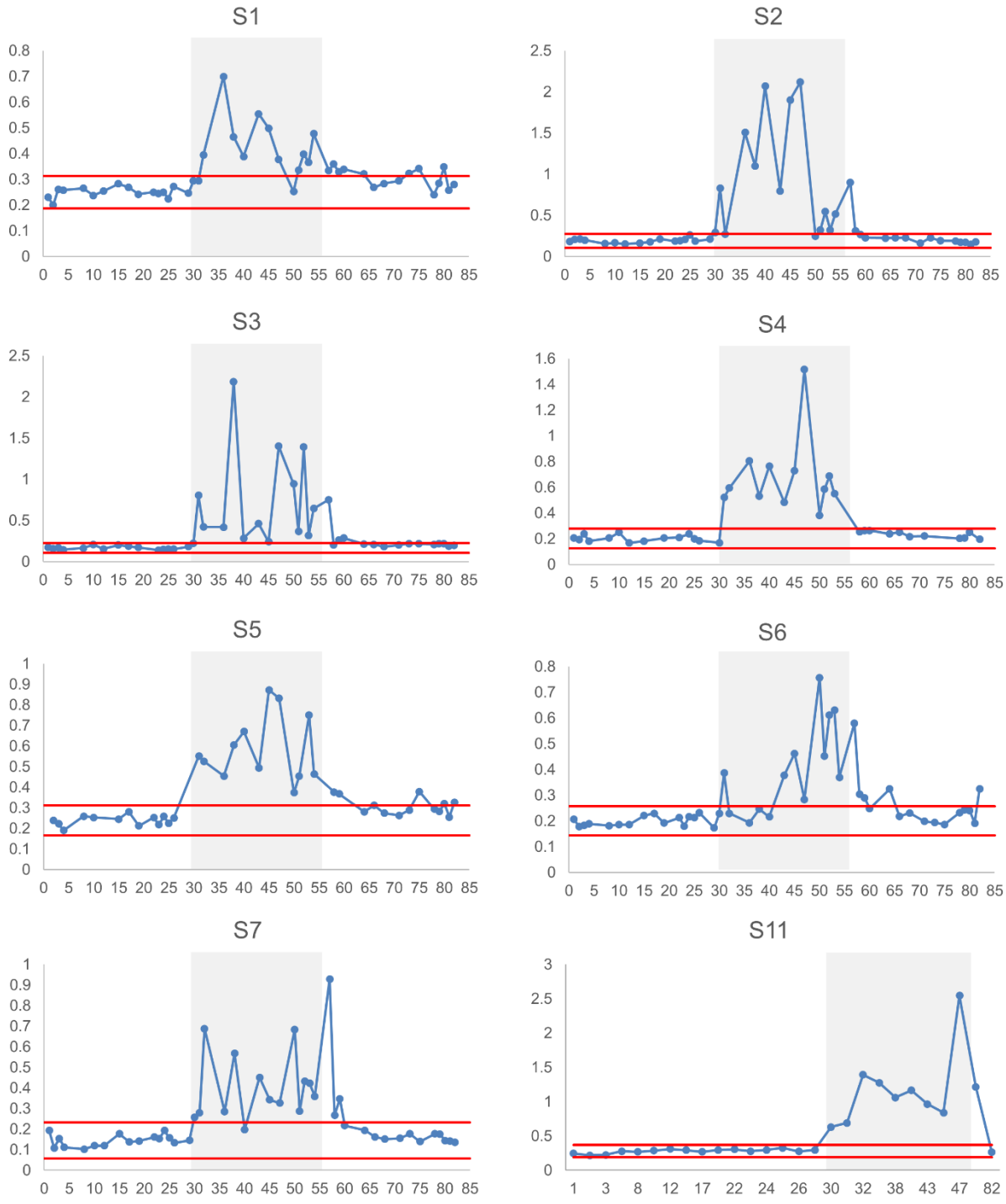


**Figure S6.** Longitudinal monitoring of T/A4 ratio. The reference individual limits (red lines) for Phase 2 (T treatment, 2nd cycle) and Phase 3 (wash-out or 3rd cycle) were extrapolated from the limits provided by mean ± 3xSD of the baseline samples collected until the end of the Phase 1 period (n=15). The blue line indicates the T/A4 for the subject and the grey area indicates T treatment period. The x-axis represents the days of the study period and y-axis T/A4 values.

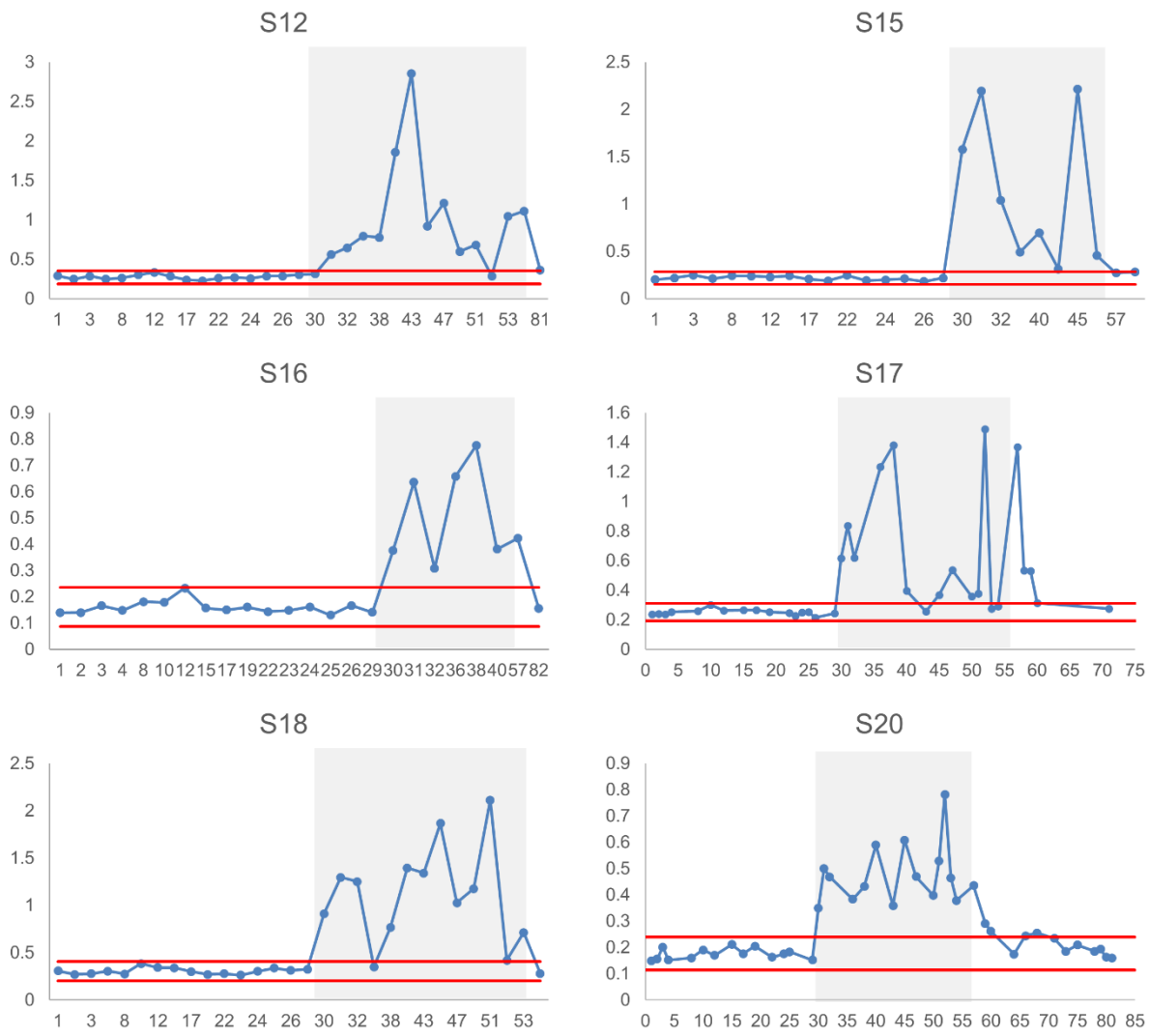


**Figure S6** (continued)

## 3.3 Article 6: Development and validation of an UHPLC-MS/MS method for extended serum steroid profiling in female populations

Besides the development of a method for the serum quantification of endogenous steroids, our group also carried out an exploratory untargeted steroidomics study to expand the panel of potential biomarkers of T administration [212]. This study highlighted that the increase of serum concentrations was more pronounced for phase II steroid metabolites than free steroids after oral T administration leading to a significant improvement in the detection of EAAS abuse. Based on these findings, a targeted metabolomic method was developed and validated for the simultaneous determination of 14 free and 14 conjugated steroids in serum, including potential T biomarkers, as described in Article 6. The inclusion of phase II metabolites are also of particular interest given that androgen glucuronides were reported as potential indicators of androgen activity in women [213].

Optimization was performed for: the chromatographic separation of isomeric compounds in addition to the peak shape; the MS/MS conditions due to the use of the polarity switching mode as most glucuro and sulfo-conjugated metabolites were detected in negative ionization mode; the sample preparation in order to find the best compromise for the simultaneous extraction of unconjugated and conjugated endogenous steroids.

The method was then applied to the analysis of serum samples originating from two different female populations (healthy and PCOS women), first to estimate which analytes were present in serum, and to assess the potential of the method for the discrimination of female populations with different steroid profiles.

This extended steroid profile supported to highlight differences between healthy and PCOS women and to link subsets of relevant biomarkers with prior biological knowledge, demonstrating its applicability in the clinical context for understanding disorders related to steroid metabolism.

Research Article

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Bioanalysis

# Development and validation of an UHPLC–MS/MS method for extended serum steroid profiling in female populations

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**Aim:** Quantitative endogenous steroid profiling in blood appears as a complementary approach to the urinary module of the World Anti-Doping Agency's Athlete Biological Passport Steroidal Module for the detection of testosterone doping. To refine this approach further, a UHPLC–MS/MS method was developed for the simultaneous determination of 14 free and 14 conjugated steroids in serum. **Results:** The method was validated for quantitative purposes with satisfactory results in terms of selectivity, linearity range, trueness, precision and combined uncertainty (<20 %). The validated method was then applied to serum samples from both healthy women and women diagnosed with mild hyperandrogenism. **Conclusion:** The UHPLC–MS/MS method showed promising capability in quantifying free and conjugated steroids in serum and determining variations of their concentration/distribution within serum samples from different populations.

First draft submitted: 25 February 2020; Accepted for publication: 5 May 2020; Published online: 1 June 2020

**Keywords:** female populations • hyperandrogenism • serum • steroid profiling • UHPLC–MS/MS

Anabolic androgenic steroids (AAS) represent the most prevalent class of substances reported in anti-doping analyses. While the majority of AAS prohibited in sport are exogenous, in other words, not naturally produced by the human body, they are detectable by qualitative methods based on chromatographic-mass spectrometric assays. On the contrary, the misuse of endogenous AAS (EAAS), such as testosterone (T), is much more challenging for antidoping laboratories. This complexity is mainly due to the necessity of developing quantitative methods and distinguishing between naturally occurring concentrations and those of exogenous origin (i.e., doping practice).

In males, the main EAAS, T, is principally secreted by testes, while in females it is produced in low quantity by the ovaries and adrenal glands, but the majority is synthesized by peripheral conversion [1]. It is responsible for the development of male secondary sexual characteristics as well as the increase of muscle mass, physical strength or aggressiveness. In addition to its anabolic action at high doses, T administered in small and repeated doses can also reduce fatigue and stimulates the erythropoietic system [2–4]. For its beneficial effect on recovery, the scenario of the misuse of testosterone is hence expanded from power/strength sports to endurance sports [5]. A recent study demonstrated that a moderate increase in testosterone level improved physical performance in young women [6].

T and EAAS doping is currently targeted by the urinary steroidal module of the Athlete Biological Passport (ABP), which was implemented in 2014 by the World Anti-Doping Agency (WADA) [7]. The steroid profile consists of an individual and longitudinal monitoring of urinary concentrations of free and glucuro-conjugated fractions of T, epitestosterone (E) androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol) and 5β-androstane-3α,17β-diol (5βAdiol) measured by GC–MS(/MS). These concentrations are adjusted with the specific gravity of urine samples and are combined into five specific ratios T/E, A/T, A/Etio, 5αAdiol/5βAdiol

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and 5αAdiol/E for evaluation of the ABP-profile. Among these, T/E is the only ratio which automatically triggers a confirmatory analysis with GC-combustion-isotope ratio MS (GC–C–IRMS) through profile evaluation via the Anti-Doping Administration and Management System (ADAMS) database [8].

While this individualized approach has certainly improved the EAAS detection capability from to the previous T/E >4 threshold based on population reference intervals, it still suffers from some drawbacks mainly related to the urinary matrix and the analytical method used (i.e., GC–MS/MS) [9]. Urinary EAAS concentrations can be influenced by several general (ethanol consumption, bacterial contamination, medication, etc.) and individual factors (sex, age and enzyme polymorphism) [10,11]. In particular, limitations in analytical sensitivity occur when analyzing urine samples of individuals with deletion polymorphism of UGT2B17 enzyme responsible for T glucuronidation [12]. These individuals (*del/del*) excrete low amounts of glucuronide-conjugated T in urine and consequently, their T/E ratio may lack sensitivity to trigger GC–C–IRMS analysis. Similar issues associated with limited GC–MS(/MS) sensitivity can be encountered with urine samples of female athletes due to lower urinary T and other related EAAS concentrations.

Recently, targeted blood steroid profiling using UHPLC–MS/MS was proposed as a complementary approach to the urinary steroid profile [13]. Furthermore, Ponzetto *et al.* demonstrated that monitoring some endogenous steroids in serum (mostly T and dihydrotestosterone [DHT]) resulted in a significant improvement of the detection of testosterone administration in men, particularly for the *del/del* genotype of the UGT2B17 enzyme, and a longer detection window after transdermal administration of testosterone [13]. This approach was also particularly effective for the detection of T in female athletes [14,15]. Indeed, although serum T concentrations are 10- to 20-times lower in women than in men, the UHPLC–MS/MS platform provided excellent performance in terms of analytical sensitivity. While serum steroid profiling has the potential to provide a valid complement to urine steroid profiling, this matrix might also be influenced by external factors such as concomitant medication and involves invasive sampling associated with stringent logistics related to its more strict conditions required for transportation

In addition to this targeted approach, an exploratory untargeted steroidomic study has been carried out to expand the panel of potential T biomarkers [9]. This study highlighted that the increase of serum concentrations was more pronounced for Phase II steroid metabolites than free steroids after oral T administration, notably with a strong response of glucuronide-conjugated androgen metabolites. Moreover, epiandrosterone sulfate was evidenced as a long-term biomarker of T administration, which corroborates previous results highlighting its utility as a long-term urinary biomarker of misuse of T, androstenedione and DHT [16]. Individual and longitudinal monitoring of these analytes improved significantly the detection of T doping, especially regarding detection time and sensitivity for individuals with the *del/del* UGT2B17 genotype.

In the clinical context, several diseases are associated with alterations of steroid metabolism due to enzyme dysfunctions or environmental factors. The main difficulty related to the diagnosing of these endocrine syndromes relies on the similarities of the symptoms and therefore, on the necessity to discriminate the nature of these disorders to benefit from the appropriate treatment [17]. Initially, most steroid analyses were performed using immunoassays. While this measurement method is simple and fast with reduced sample preparation, it does not allow for measuring multiple compounds at the same time, and may suffer from problems of cross-reactivity between steroids. On the contrary, LC–MS/MS analyses offer the possibility of multiplexed configuration to analyze a panel of steroids in a small volume of serum and therefore, quantifying multiple metabolites simultaneously to discover potential interferences in steroidogenesis. Moreover, in the case of very low concentrated steroids, there is also the possibility to further enhance sensitivity with chemical derivatization such as isotope labeling derivatization [18]. Consequently, LC–MS/MS is of particular interest for the distinction of syndromes related to steroid metabolism. A quantitative method including both free and conjugated steroids would allow for obtaining a broad overview of the interfered metabolic pathways.

With the earlier experience from the biomarkers in blood and the prevailing problematic of result interpretation for the ABP, the aim of this study was to develop a quantitative method for the simultaneous determination of free and conjugated EAAS including potential T biomarkers using UHPLC–MS/MS for a further implementation of the blood steroidal profile in the anti-doping context. The applicability of the method was then assessed by analyzing serum samples from two female populations, namely healthy women and women diagnosed with polycystic ovary syndrome (PCOS), which were supposed to have distinct steroid profiles.

## Experimental

### Chemical & reagents

Methanol (MeOH) was purchased from Macron Fine Chemicals (Deventer, The Netherlands), formic acid (FA; UHPLC/MS, >99%) and UPLC grade MeOH from Biosolve BV (Valkenswaard, The Netherlands) while ammonium hydroxide ($NH_4OH$; 28-30%) and phosphoric acid solutions were obtained from Sigma-Aldrich (Buchs, Switzerland). Charcoal Dextran Stripped Human Serum was supplied by Dunn Labortechnick GMbH (Asbach, Germany). Milli Q quality water was obtained from a Milli-Q® grade system (Millipore, MA, USA) and was used for the preparation of LC mobile phases and washing/elution solutions. Oasis Prime HLB Prime 96-well µElution plates (3 mg) were purchased from Waters Corporation (CT, USA).

Testosterone (T), androstenedione (A4), androsterone (A) were purchased from Fluka (Buchs, Switzerland), 11-deoxycortisol, 21-deoxycortisol, deoxycorticosterone, 17α-hydroxyprogesterone (17αOHProg), cortisol, epitestosterone (E), etiocholanolone (Etio) from Sigma–Aldrich (Buchs, Switzerland), DHT, corticosterone, dehydroepiandrosterone (DHEA) from Chemie Brunschwig (Basel, Switzerland), testosterone glucuronide (TG), epitestosterone glucuronide (EG), etiocholanolone glucuronide (EtioG), epiandrosterone glucuronide (EpiAG), androsterone sulfate (AS), deyhdroepiandrosterone sulfate (DHEAS), testosterone sulfate (TS), epiandrosterone sulfate (EpiAS) from Steraloids (Newport, RI, USA), androsterone glucuronide (AG), 5β-androstane-3α,17β-diol-17-O-glucuronide (5βAdiol-17-G), 5β-androstane-3α,17β-diol-3-O-glucuronide (5βAdiol-3-G), epitestosterone sulfate (ES), etiocholanolone sulfate (EtioS), noretiocholanolone sulfate (NorEtioS) from LGC Standards (Wesel, Germany) and progesterone (Prog) from Laboratoire Golaz (Lausanne, Switzerland). Internal labeled standards (IS) were provided by National Measurement Institute (Pymble, Australia).

### Analytes solutions, calibration & validation samples

A stock solution at 1 mg/ml of each compound was prepared in MeOH; intermediate solutions at appropriate concentrations were prepared through consecutive dilution of the stock solutions in MeOH. Working solutions containing mixtures of standard compounds at different concentrations were also prepared in MeOH and used for calibration and validation samples in depleted serum. A working IS solution containing IS at different appropriate concentrations was also prepared (Supplementary Table 1). Stock solutions, intermediate and working solutions were stored at -20°C in glass tubes. Calibration and validations samples were prepared by spiking 20 µl of mix solution at appropriate concentrations into 180 µl of charcoal depleted serum and extracted according to the sample preparation protocol.

### Sample preparation

Serum sample aliquots of 300 µl were first centrifuged for 20 min at 16,000 rpm to remove the lipid fraction and 200 µl of supernatant was then transferred to a 96-well collection plate. Samples were spiked with 20 µl of IS mix and then diluted with 200 µl of 4% $H_3PO_4$ aqueous solution. Solid phase extraction (SPE) on Oasis Prime HLB Prime 96-well µElution plates was used to extract steroid hormones from serum samples using an Extrahera™ automation system (Biotage, Uppsala, Sweden) as described earlier [9]. After loading of the pretreated 400 µl serum samples in each well, a positive pressure (1 bar) was applied for 150 s to facilitate sample loading. A washing step was performed by adding 400 µl of a $H_2O$/MeOH (95/5, v/v) solution containing 0.1% $NH_4OH$ and applying positive pressure (1 bar) for 150 s. Elution was finally performed with 50 µl of a MeOH/ $H_2O$ (90/10, v/v) solution and application of 1 bar positive pressure for 1 min. Extracts were collected with collection plates equipped with 1.5 ml glass inserts and were then diluted with 50 µl of $H_2O$. After 10 min of gentle shaking, 10 µl of each extract was injected into the UHPLC–MS/MS system.

### Instrumentation & analytical conditions

An Acquity UPLC I-class (Waters Corporation) equipped with a Kinetex C18 column (150 × 2.1 mm, 1.7 µm; Phenomenex, CA, USA) was used for chromatographic separation. The mobile phase A solution was $H_2O$ + 5 mM ammonium formate and mobile phase B was MeOH + 5 mM ammonium formate. Separation was performed with a flow rate at 300 µl/min and a column temperature at 55°C. The solvent gradient started from 40% B and increased linearly to 80% over 20 min, followed by 7 min at 98% B, with a 8 min re-equilibration step at initial conditions for a total run time of 35 min.

MS/MS analysis was carried out using a Xevo-TQ S triple quadrupole mass spectrometer (Waters Corporation) equipped with an ESI source operated in polarity switching (positive/negative) mode. Capillary voltage was set

at ± 2.5 kV and source temperature was maintained at 150°C. Desolvation gas temperature and flow rate were set at 500°C and 1000 l/h. The cone gas flow was set at 150 l/h and nebulizer gas flow at 7 bar. The collision gas was argon with a collision gas flow of 0.15 ml/min.

Mass spectrometric conditions (MS/MS transitions, cone voltage, collision energy) were optimized by direct infusion of methanolic standard solutions of each compound at 100 ng/ml. A multiple reaction monitoring (MRM) method using detection windows was constructed including two transitions when possible for each compound, one for quantitation and the other for identification (Table 1).

Data were acquired and handled using MassLynx software version 4.2 and TargetLynx application was used to calibrate, quantify and process data.

## Method validation

Validation protocol implied the evaluation of selectivity, matrix effects, recovery and quantitative performances (trueness, repeatability, intermediate precision, combined uncertainty, linearity range, limit of quantification) of the presented method.

Selectivity was evaluated with the extraction and analysis of three depleted serum samples (negative control), three serum samples spiked with IS mix and three depleted serum samples spiked with the analytes of interest at moderate concentration. In addition, three depleted serum spiked with a mix solution containing approximately 60 exogenous steroids at concentrations between 2 and 5 ng/ml were also prepared to evaluate possible interferences coming from structurally related compounds.

Extraction recovery and matrix effect were also evaluated using the approach of Matuszewski *et al.* [19]. Extraction recovery was calculated as the ratio of the peak areas of the analyte from the pre-extracted spiked and post-extracted spiked samples prepared in triplicate. Matrix effects were evaluated by comparing peak areas of analytes in post-extracted spiked samples to peak areas of the corresponding reference standard in methanolic aqueous solution. The calibration solution 5 was used for these analyses (Supplementary Table 2).

The calibration curves were prepared by spiking depleted serum samples at 7 (conjugated) or 8 (free) levels of concentration in concentration ranges specific for each analyte (Supplementary Table 2). Linear calibration curves were generated from the peak area ratio of each steroid to its respective IS using a $1/\times$ weighted regression. In addition to the determination coefficient ($R^2$) > 0.98, the criteria for acceptance of the calibration curves were a minimum of six calibrator levels to be ± 15% of theoretical concentrations except at LLOQ where the calibrators should be ± 20% of nominal concentrations [20].

According to ISO/IEC requirements, trueness and precision (repeatability and intermediate precision) of the described methods were assessed on three different analytical series [21]. For each series, eight calibration and eight validation samples were analyzed in simple and triplicate respectively in depleted serum at adapted concentrations for each analyte (Supplementary Table 2). Carry-over was assessed by injecting an extracted depleted serum sample after the most concentrated validation sample. Then, in accordance with the WADA technical document (TD2019DL), combined measurement uncertainty ($u_c$) was obtained by quadratic combinations of the intermediate precision and the root mean square of the bias estimates [22]. A pre-defined uncertainty acceptance criterion was set at 20% of the mean result at each concentration level of the validation samples. Uncertainty profiles were then established by plotting the $u_c$ according to the related concentration.

## Application to serum samples

Twenty-one serum sample from healthy women blood donors and thirty serum samples from women diagnosed with PCOS (approved by the Human Research Ethics Committee of the Canton of Vaud in Switzerland, Protocol 2018-01147) were analyzed using the developed method to quantify endogenous steroid hormones as a preliminary application for the distinction of two populations with different steroid blood profiles. A matrix-matched standard calibration curve with 7 and 8 levels of concentrations for free and conjugated analytes respectively was used to quantify steroid metabolites. Calculated concentrations were then used for multivariate analysis using principal component analysis (PCA) after unit variance (UV) scaling using SIMCA (Version 15.0, MKS Umetrics, Umea, Sweden). Statistical comparisons between both populations were performed using nonparametric Mann–Whitney test. Descriptive and statistical comparisons were performed using R studio software.

Table 1. Mass spectrometric conditions for each analyte.

| Compound | Ionization mode | Precursor ion (m/z) | Product ion (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| Testosterone | Pos | 289.2 | **97**; 109 | 28 | 45 |
| Testosterone-d3 | Pos | 292 | 97 | 20 | 20 |
| Epitestosterone | Pos | 289 | **97**; 109 | 30; 34 | 18; 22 |
| Epitestosterone-d3 | Pos | 292 | 97 | 42 | 20 |
| Androstenedione | Pos | 287 | **97**; 109 | 44 | 26 |
| Androstenedione-d7 | Pos | 294 | 113 | 42 | 26 |
| DHEA | Pos | **253**; 271† | **197**; 253 | 56; 32 | 20; 12 |
| DHEA-d5 | Pos | 276 | 258 | 8 | 14 |
| DHT | Pos | 291 | 159; **255** | 22 | 26; 18 |
| DHT-d3 | Pos | 294 | 258 | 36 | 14 |
| Androsterone | Pos | 255; **273**† | 159; **147** | 52; 14 | 18 |
| Androsterone-d4 | Pos | 259 | 163 | 50 | 20 |
| Etiocholanolone | Pos | 255† | **145**; 159 | 48 | 24; 20 |
| Etiocholanolone-d5 | Pos | 260† | 173 | 52 | 26 |
| Progesterone | Pos | 315 | **97**; 109 | 28 | 45 |
| Progesterone-d9 | Pos | 324 | 113 | 16 | 26 |
| 17α-hydroxyprogesterone | Pos | 331 | **97**; 109 | 22 | 22; 28 |
| 17α-OHProgesterone-d8 | Pos | 339 | 100 | 26 | 24 |
| Cortisol | Pos | 363 | **91**; 121 | 38 | 25; 55 |
| Cortisol-d4 | Pos | 367 | 121 | 38 | 26 |
| Corticosterone | Pos | 347 | **91**; 121 | 40 | 44; 45 |
| Corticosterone-d4 | Pos | 351 | 121 | 28 | 28 |
| Deoxycorticosterone | Pos | 331 | **97**; 109 | 48 | 22; 24 |
| Deoxycorticosterone-d8 | Pos | 339 | 113 | 42 | 28 |
| 11-Deoxycortisol | Pos | 347 | **97**; 109 | 26 | 22; 24 |
| 11-deoxycortisol-d2 | Pos | 349 | 97 | 26 | 22 |
| 21-Deoxycortisol | Pos | 347 | 121; **311** | 44 | 28; 16 |
| 21-deoxycortisol-d8 | Pos | 355 | 319 | 25 | 18 |
| Testosterone Glucuronide | Pos | 465 | **97**; 109 | 42; 48 | 20; 26 |
| Testosterone Glucuronide-d3 | Pos | 468 | 97 | 32 | 26 |
| Epitestosterone Glucuronide | Pos | 465; **487**‡ | 271; **311** | 14; 28 | 16; 22 |
| Epitestosterone Glucuronide-d3 | Pos | 468 | 292 | 32 | 16 |
| Androsterone Glucuronide | Neg | 465 | 75; **85** | 74 | 34 |
| Androsterone Glucuronide-d4 | Neg | 469 | 85 | 74 | 34 |
| Etiocholanolone Glucuronide | Neg | 465 | 75; **85** | 74 | 34 |
| Etiocholanolone Glucuronide-d5 | Neg | 470 | 85 | 40 | 30 |
| 5βαβ-Adiol 3-Glucuronide | Neg | 467 | **75**; 85 | 60 | 32; 30 |
| 5βαβ-Adiol 17-Glucuronide | Neg | 467 | **75**; 85 | 60 | 32; 30 |
| 5βαβAdiol-17-Glucuronide-d3§ | Neg | 470 | 85 | 60 | 32 |

Ion transitions used for quantification in bold.
† Precursors with loss of (2) $H_2O$.
‡ [M+$Na^+$] adduct.
§ Used as IS for 5βαβ-Adiol 3-Glucuronide.
¶ Used as IS for Epiandrosterone Sulf.
# Used as IS for Noretiocholanolone Sulf.
DHEA: Dehydroepiandrosterone; DHT: Dihydrotestosterone.

Table 1. Mass spectrometric conditions for each analyte (cont.).

| Compound | Ionization mode | Precursor ion (m/z) | Product ion (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| Epiandrosterone Glucuronide | Neg | 465 | **85**; 113 | 74 | 34; 28 |
| Testosterone Sulfate | Pos | 369 | **97**; 109 | 46 | 24; 28 |
| Testosterone Sulfate-d3 | Pos | 372 | 97 | 48 | 20 |
| Epitestosterone Sulfate | Pos | 369 | 109; **271** | 46; 42 | 50; 20 |
| Epitestosterone Sulfate-d3 | Pos | 372 | 97 | 48 | 20 |
| Androsterone Sulfate | Neg | 369 | **97** | 88 | 46 |
| Androsterone Sulfate-d4¶ | Neg | 373 | 98 | 88 | 34 |
| Etiocholanolone Sulfate | Neg | 369 | **97** | 88 | 46 |
| Etiocholanolone Sulfate-d5# | Neg | 374 | 98 | 88 | 34 |
| DHEA Sulfate | Neg | 367 | **97** | 66 | 60 |
| DHEA Sulfate-d6 | Neg | 373 | 98 | 66 | 50 |
| Noretiocholanolone Sulfate | Neg | 355 | **97** | 84 | 34 |
| Epiandrosterone Sulfate | Neg | 369 | **97** | 90 | 46 |

Ion transitions used for quantification in bold.
†Precursors with loss of (2) $H_2O$.
‡[M+$Na^+$] adduct.
§Used as IS for 5βαβ-Adiol 3-Glucuronide.
¶Used as IS for Epiandrosterone Sulf.
#Used as IS for Noretiocholanolone Sulf.
DHEA: Dehydroepiandrosterone; DHT: Dihydrotestosterone.

## Results & discussion

### Method development

In the present study, a single method was developed, optimized and validated for the simultaneous analysis of 28 EAAS, including free and conjugated (sulfates and glucuronides) metabolites for which reference standard material was commercially available.

First, MS conditions were optimized involving the selection of MRM transitions for each target analyte. While no major issue was encountered with free steroids, all detected in positive ionization mode, the selection of ion transitions for glucuro- and sulfoconjugated steroids was found to be much more challenging. This was mainly due to the possibility to achieve positive and negative ionization modes for the majority of conjugated target compounds. Therefore, further tests were required to compare the sensitivity and selectivity of positive and negative monitored ions. Concerning sulfoconjugated metabolites, an additional issue was due to the presence of a single predominant, characteristic ion fragment at $m/z$ 97 ($HSO_4^-$) in negative ionization mode [23]. Consequently, a proper chromatographic separation of compounds presenting identical MS/MS transitions such as AS, EtioS or EpiAS ($m/z$ 369 > 97) was mandatory. Glucuronide- and sulfo-conjugated EAAS metabolites were analyzed in negative ionization mode with the exception of TG/TS and EG/ES detected using $[M + H]^+$ as precursor ion. It was indeed highlighted that in negative mode ion transitions $m/z$ 367 > 97 for TS and ES suffer from significant endogenous interferences due to other sulfates [23]. A 13 min MS scan in negative mode (from $m/z$ 100 to 1000) was added at the end of the gradient to avoid a potential reduction of MS signal, (mainly for glucuronide and sulfate steroids) which could be caused by a hypothetical charging effect in the quadrupole [24]. Finally, a reduction of MS signal was observed in negative ionization mode with a consecutive decline of sensitivity for glucuronide and sulfate steroids. To circumvent this deterioration, the dwell time, normally calculated automatically, was increased and fixed for compounds detected in negative mode. Moreover, ion energy of the analyzer was increased to improve MS signals. The most sensitive and selective MRM transitions for each compound are presented in Table 1.

As mentioned, the presence of identical MS/MS transitions and the large occurrence of isomeric compounds required an efficient chromatographic separation for an appropriate identification of each analyte. Initially, conditions described in [9], comprising mobile phases composed of $H_2O$ 0.1% FA/ACN 0.1% FA were used with a linear gradient from 5 to 95% of organic phase in 16.8 min. However, these conditions did not enable appropriate

peak resolution for isomeric compounds and the peak shape of sulfoconjugated compounds was unsatisfactory. The organic phase was first modified using MeOH instead of ACN as organic modifier and, due to the reduced eluting strength of MeOH, the gradient was adapted starting at 40% of organic phase increasing to 80% in 20 min. Nonetheless, while the change of organic phase improved peak separation of isomers, the peak shape of sulfate analytes was still poor. To overcome this limitation and to obtain a proper peak shape, the ionic strength of the mobile phase was increased with the addition of 5 mM ammonium formate to both solvents. The improvement of peak shape was attributed to the role of ion-pairing agent of ammonium ions present in the mobile phase as previously suggested by Esquivel *et al.* [23]. An example of chromatograms using the final conditions is depicted in Figure 1.

Concerning serum extraction, SPE using HLB Prime µElution plates was found to be the best compromise for the simultaneous extraction of unconjugated and conjugated EAAS in order to develop a single method for extended blood steroid profiling. The same protocol and conditions were already used for an untargeted steroidomics study [9]. A centrifugation step, increasing slightly the initial serum sample volume, was deemed mandatory to remove lipid fraction and thereby to limit clogging of the SPE sorbent. Extractions are simple and fast, do not require postelution evaporation and can be performed in a 96 well-plate format using an automated extraction system. After serum pretreatments, the final conditions included a washing step using $H_2O$/MeOH (95/5, v/v) solution containing 0.1% $NH_4OH$ while the elution was finally performed with MeOH/$H_2O$ (90/10, v/v). Using these conditions, matrix effects were negligible (<15%) and satisfactory recoveries were obtained for free (ranging from 64% to 91.7%) and glucuroconjugated (ranging from 52.4 to 70.3%) steroids (Table 2). However, extraction recoveries for sulfate metabolites were below expectations (range 30.2–47.5%), but still considered acceptable. Indeed, the aim of this work was to find a rapid, simple and generic SPE method able to perform the simultaneous extraction of neutral steroids (free), small acidic steroids (glucuronides) and strong acidic steroids (sulfates). The recoveries were particularly reproducible for each class of substances (between 1.7 and 8.6% for free, between 2.4 and 10.7% for glucuronides and between 4.4 and 8.4% for sulfates) (Table 2). In this context, to obtain accurate quantification performances, the careful selection of a high number of deuterated IS with a similar recovery compared with the unlabeled compounds was judged essential to account for these recoveries differences. Finally, the acceptance criteria depends also on the global method sensitivity and the applicability to the concentrations encountered in the routine samples, and along with the obtained recoveries, the compromised method was fit-for-purpose to the analysis of target compounds at required sensitivity in biological matrix.

Alternative conditions were tested using this extraction plate by modifying the percentage of $NH_4OH$ present in the washing and/or elution solution. Indeed, it was previously demonstrated that a low percentage of ammonia was crucial for extracting sulfate analytes from serum [9]. However, none of the tested conditions improved significantly the extraction recovery and initial extraction conditions were preserved for the developed method.

## Method validation

Quantitative performance of the presented method and a summary of validation results (accuracy, precision, uncertainty, linearity range, LLOQ) are presented in Table 2 (complete results at each concentration level are shown in Supplementary Table 3).

For the evaluation of selectivity, three depleted serum samples, three serum samples spiked with a low concentration mix solution and three depleted serum spiked with a mix solution containing exogenous steroids at different concentrations were analyzed with the presented method. No interference was detected on the MRM chromatogram close to the retention time of each analyte.

Calibration curves were prepared by spiking charcoal-dextran stripped serum at 8 (free) and 7 (conjugated) different concentration levels for each analyte with determination coefficient above 0.98 for all analytes (data not shown). These calibration curves were used for the determination of method trueness and precision. For this, three different analytical series were conducted, including each time eight calibration and eight validation samples in simple and triplicate, respectively, in depleted serum at adapted concentrations for each analyte. In addition, the operator was changed between batches and the column was replaced.

The method demonstrated satisfactory trueness and precision (Table 2). The relative bias represented ranged between -11.7 and 12%. The precision displayed as repeatability and intermediate precision was also measured for each analyte concentration level. These values varied from 0.7 to 8.8%, for repeatability and from 1.2 to 12.8% for intermediate precision. Precision varied with analyte concentration and demonstrated the highest RSD % values at the lowest concentrations, while values were all below 10% for higher concentration (Supplementary Table 3).

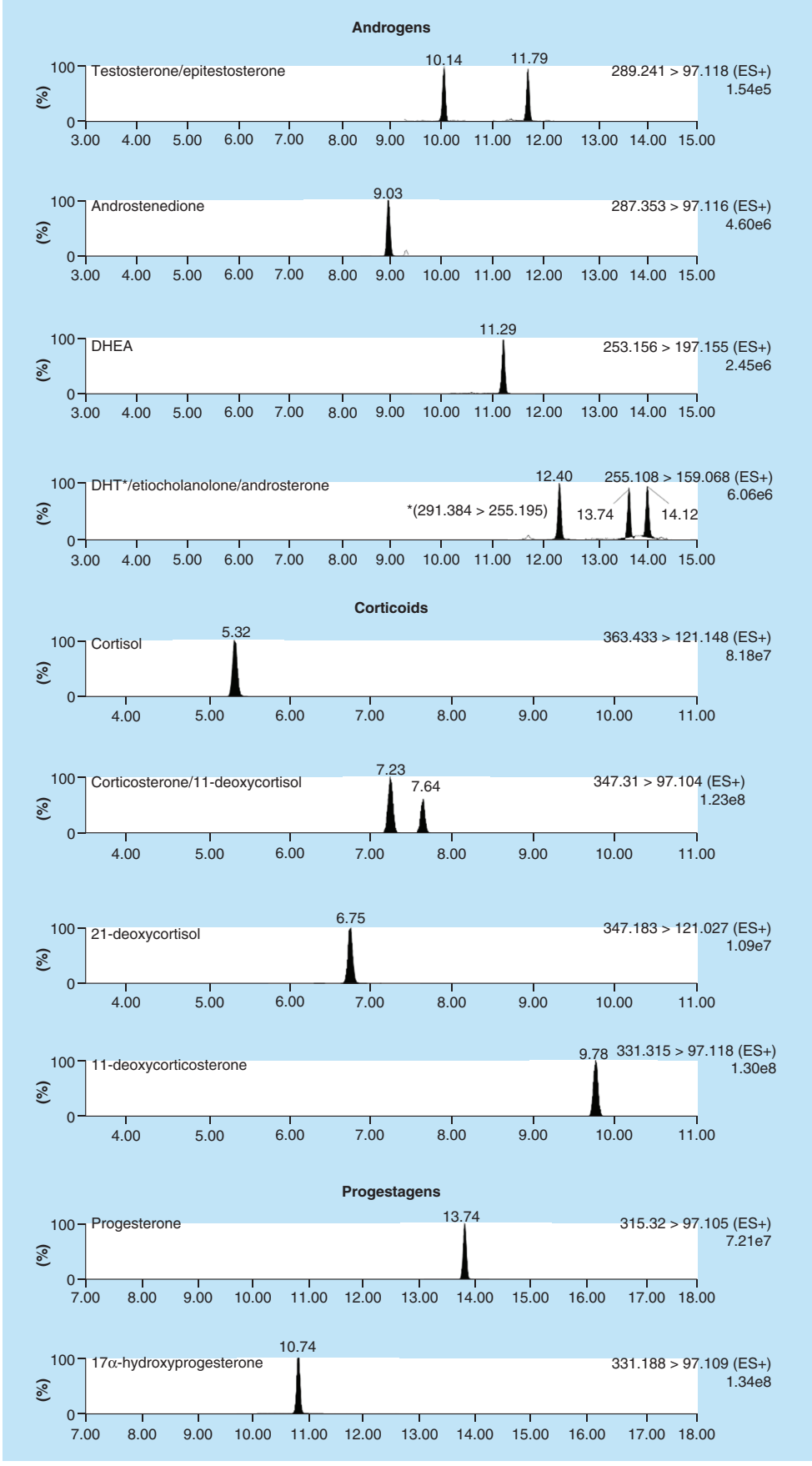


**Figure 1. Chromatograms of solutions containing target free and conjugated steroid hormones.**

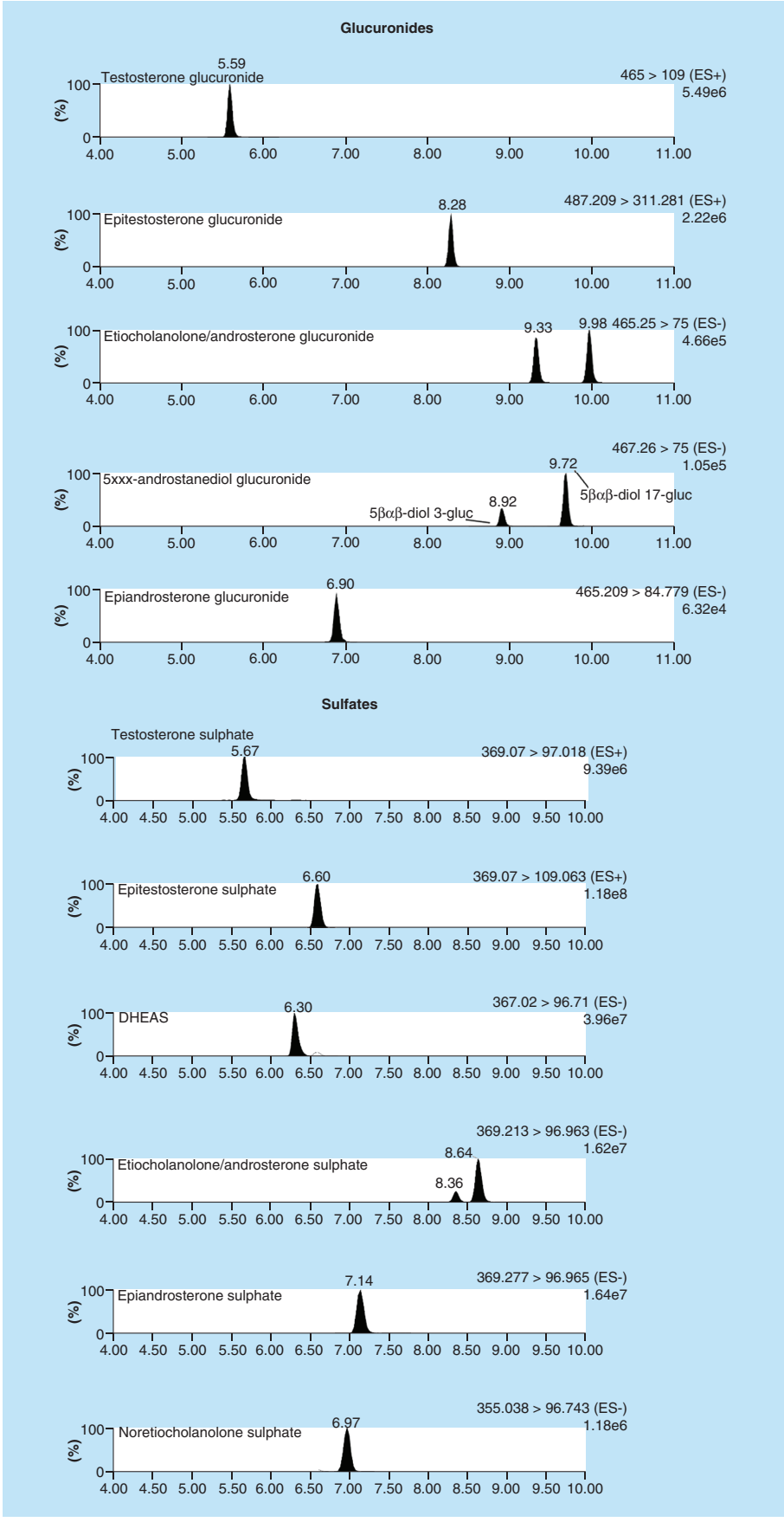


**Figure 1. Chromatograms of solutions containing target free and conjugated steroid hormones (cont.).**

Table 2. Summary of validation results.

| Analyte | RT (min) | Trueness (%) | Repeatability (%) | Intermediate precision (%) | Combined uncertainty (%) | Linearity range (ng/ml) | LLOQ (pg/ml) | Recovery, CV (%) | Matrix effect, CV (%) |
|---|---|---|---|---|---|---|---|---|---|
| Testosterone | 10.14 | 92.9–107.0 | 1.6–5.4 | 2.4–9.0 | 5.9–12.0 | 0.02–25 | 20 | 83.8 (1.7) | 101 (3.7) |
| Epitestosterone | 11.79 | 94.6–109.8 | 1.3–5.8 | 3.4–10.1 | 4.9–13.4 | 0.02–10 | 20 | 87.2 (3.2) | 97.6 (4.2) |
| Androstenedione | 9.03 | 88.3–108.7 | 2.0–4.6 | 6.0–10.1 | 8.1–15.6 | 0.05–25 | 50 | 66.0 (5.7) | 98.5 (3.5) |
| DHEA | 11.29 | 94.0–106.1 | 2.3–6.8 | 2.4–12.8 | 3.1–17.2 | 0.5–150 | 500 | 80.3 (3.2) | 97.6 (6.7) |
| DHT | 12.40 | 96.2–105.8 | 2.9–6.7 | 4.0–10.1 | 5.4–13.3 | 0.05–10 | 50 | 79.1 (4.1) | 90.7 (6.9) |
| Androsterone | 14.12 | 97.7–102.0 | 1.3–3.8 | 2.3–4.8 | 3.5–6.0 | 0.1–10 | 100 | 84.9 (2.6) | 96.7 (6.6) |
| Etiocholanolone | 13.74 | 98.6–103.0 | 1.3–3.5 | 2.6–4.6 | 3.8–6.2 | 0.1–10 | 100 | 89.5 (2.8) | 103.8 (7.8) |
| Progesterone | 13.74 | 93.7–112.0 | 2.2–8.8 | 3.1–9.9 | 4.4–15.1 | 0.015–25 | 15 | 64.0 (2.3) | 96.3 (6.6) |
| 17α-hydroxyprogesterone | 10.74 | 93.0–108.6 | 1.7–4.4 | 4.3–11.8 | 6.7–15.4 | 0.1–25 | 100 | 82.6 (5.6) | 96.6 (4.3) |
| Cortisol | 5.32 | 91.9–108.9 | 1.4–3.8 | 3.4–10.1 | 5.3–15.4 | 1-400 | 1000 | 87.3 (2.3) | 94.5 (4.3) |
| Corticosterone | 7.23 | 89.7–107.3 | 2.0–2.7 | 2.5–4.5 | 3.8–11.8 | 0.25–100 | 250 | 88.5 (5.1) | 104.8 (14.9) |
| Deoxycorticosterone | 9.78 | 91.1–110.1 | 2.0–3.2 | 5.4–12.3 | 7.4–18.2 | 0.025–10 | 25 | 79.0 (3.0) | 98.0 (4.1) |
| 11-deoxycortisol | 7.64 | 96.1–106.5 | 2.0–5.2 | 2.0–7.9 | 4.8–10.6 | 0.025–2.5 | 25 | 85.2 (8.6) | 97.5 (8.6) |
| 21-deoxycortisol | 6.75 | 91.0–109.8 | 1.9–8.8 | 3.5–10.7 | 7.9–17.0 | 0.025–10 | 25 | 91.7 (5.1) | 99.4 (0.4) |
| Testosterone glucuronide | 5.59 | 96.8–103.3 | 1.7–7.0 | 2.5–7.0 | 3.5–9.9 | 0.05–25 | 50 | 69.0 (4.9) | 100.1 (2.4) |
| Epitestosterone glucuronide | 8.28 | 99.3–102.2 | 1.1–2.6 | 1.5–4.9 | 2.1–6.8 | 0.1–10 | 100 | 67.3 (7.4) | 117.9 (4.8) |
| Androsterone glucuronide | 9.98 | 97.1–102.2 | 0.8–5.6 | 1.2–9.9 | 2.2–13.4 | 0.25–100 | 250 | 70.3 (7.5) | 105.6 (4.6) |
| Etiocholanolone glucuronide | 9.33 | 96.9–104.0 | 2.7–7.6 | 2.7–8.1 | 4.1–11.5 | 0.25–100 | 250 | 63.2 (2.4) | 105.2 (1.8) |
| 5βαβ-androstanediol-3-glucuronide | 8.92 | 97.0–100.8 | 2.5–8.1 | 3.4–8.1 | 4.5–10.5 | 0.1–10 | 100 | 52.4 (6.6) | 113.0 (1.4) |
| 5βαβ-androstanediol-17-glucuronide | 9.72 | 97.7–100.5 | 1.6–4.3 | 2.1–6.3 | 2.9–8.7 | 0.25–25 | 250 | 66.8 (3.6) | 96.9 (5.5) |
| Epiandrosterone glucuronide | 6.90 | 99.0–101.7 | 1.2–5.8 | 2.7–6.6 | 3.9–8.3 | 0.1–10 | 100 | 64.4 (10.7) | 98.4 (6.9) |
| Testosterone sulfate | 5.67 | 100.0–106.1 | 1.7–5.4 | 2.1–8.7 | 3.1–13.0 | 0.1–25 | 100 | 42.6 (4.6) | 100.8 (2.6) |
| Epitestosterone sulfate | 6.60 | 93.2–107.5 | 0.7–4.2 | 2.2–5.8 | 3.1–9.6 | 0.05–2.5 | 50 | 45.1 (7.1) | 99.7 (3.6) |
| Androsterone sulfate | 8.64 | 96.9–100.5 | 1.3–3.2 | 1.4–6.5 | 2.9–8.8 | 1-2500 | 1000 | 37.3 (6.9) | 114.1 (6.0) |
| Etiocholanolone sulfate | 8.36 | 97.5–102.8 | 1.9–4.8 | 2.1–6.1 | 3.3–8.6 | 1-2500 | 1000 | 42.8 (8.2) | 101.7 (1.2) |
| DHEA sulfate | 6.30 | 93.7–108.2 | 1.4–3.0 | 2.6–4.4 | 4.1–9.9 | 50–10,000 | 50,000 | 39.6 (8.4) | 104.7 (4.2) |
| Noretiocholanolone sulfate | 6.97 | 99.5–103.7 | 0.8–2.6 | 1.6–3.0 | 2.2–4.7 | 0.1–100 | 100 | 47.5 (7.8) | 107.2 (6.8) |
| Epiandrosterone sulfate | 7.14 | 100.4–105.8 | 1.3–2.9 | 1.8–3.8 | 3.3–7.7 | 5–2500 | 5000 | 30.2 (4.4) | 103.5 (1.4) |

Negligible carry-over was found with values below 0.1% for most analytes with the exception of 0.2% for DHEA, 0.3% for AS and 0.6% for EtioG.

Following these results, validated concentration ranges of each steroid were established as presented in Supplementary Table 2. LLOQ ranged between 15 pg/ml and 50 ng/ml depending on the analyte, highlighting the important heterogeneity of the concentration ranges covered by the quantitative method.

Finally, measurement uncertainty, which represents the statistical dispersion of the values attributed to a measured quantity, was calculated according to the WADA requirements for quantitative methods [22]. "Combined uncertainty included the sources of variations that causes both random (precision) and systematic errors (accuracy) by combining the variations in replicate observations (random error) and the variation in measurements from the true or reference value (systematic error)" [25]. This way of calculating uncertainty is mainly used in the anti-doping context for decision limits of threshold substances. In our context, the combined uncertainty was used to establish uncertainty profiles computing the relationship between the concentration and the combined uncertainty for each analyte (Figure 2). This approach is particularly interesting for assessing measurement uncertainty of analytes covering large concentration ranges such as T or DHT with important differences of concentration levels between male and female samples. For T, the concentration range corresponding to female (20–100 pg/ml) had uncertainties between

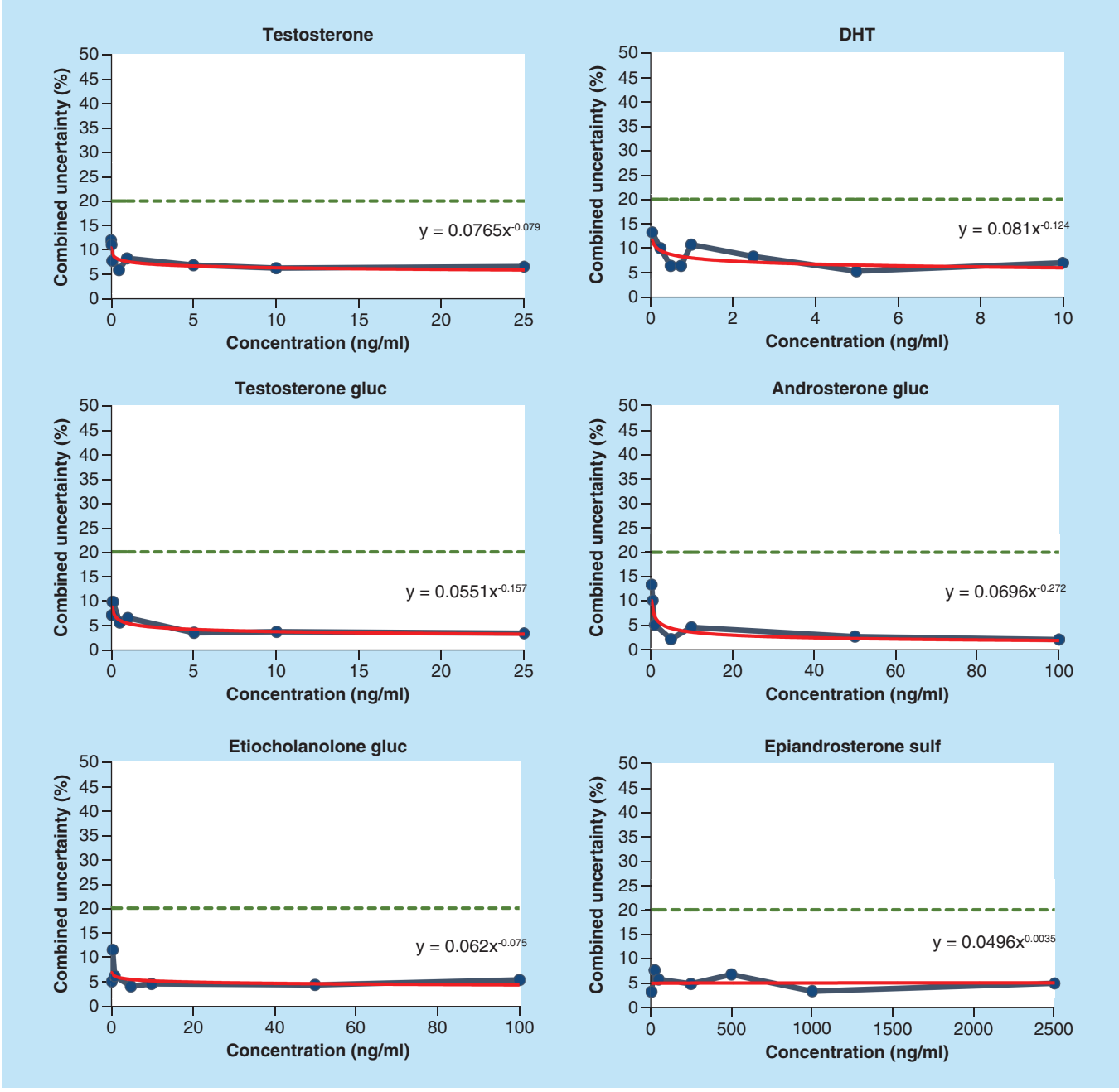


**Figure 2. Uncertainty profiles of testosterone, dihydrotestosterone, testosterone glucuronide, androsterone glucuronide, etiocholanolone glucuronide and epiandrosterone sulfate.**

7.8 and 12% while the rest of the interval was below 10% (Figure 2). The same was also true for DHT with all values below 15% in agreement with a predefined uncertainty acceptance criterion set at ± 20%. For conjugated metabolites, uncertainty was even lower especially given that potential biomarkers of T doping such as AG, EtioG or EpiAS are expected to be at high concentrations in serum. Graphs for T, DHT, TG, AG, EtioG and EpiAS are depicted in Figure 2, while the others are shown in Supplementary Figure 1.

## Application to serum samples

The developed method was applied to the quantification of serum samples from two different female populations (healthy and PCOS women) which were supposed to have different steroid profiles, allowing to determine the


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concentrations of the different steroids. PCOS is a fairly common endocrine disorder among women at reproductive age with a prevalence of approximately 6-10% [26]. This syndrome is characterized by mild hyperandrogenism and it is of particular interest in the sports context due to its over-representation among elite female athletes [6]. Hence, improved physical performance with muscle mass and power performance correlating with androgen levels has been suggested in female PCOS athletes [27]. Due to the restricted number of volunteers analyzed, it has to be noted that the presented data only provide an estimation of these population levels.

Concerning unconjugated steroids, most of them were detected and easily quantified with the exception of androsterone, etiocholanolone, epitestosterone and 21-deoxycortisol and deoxycorticosterone. Androsterone, etiocholanolone and 21-deoxycortisol were not detected or were below the LLOQ of our method in both groups, while for epitestosterone and deoxycorticosterone only a few samples were quantified above the LLOQ. For DHT, 82% of the samples were quantified and the remaining percentage was detected but below the LLOQ.

For the conjugated metabolites including sulfates and glucuronides, the majority was quantified at high concentrations. For glucuronide-conjugated metabolites, EG and epiAG were not detected while TG concentration was below the LLOQ for the majority of the samples. Concerning sulfo-conjugated analytes, TS concentrations were mostly close or below the LLOQ, and ES and NorEtioS were not detected as already demonstrated [28]. In addition to estimation of EAAS concentrations in the serum of females, the application of the present method also allowed gaining insights into the human steroid metabolism with some disparities in the ratio of free steroids and their conjugated forms. In particular, some hormones such as T or DHT were mostly detected in their free form, while analytes such as androsterone or etiocholanolone were only detected in their conjugated (glucuronide or sulfate) form. Furthermore, EpiA was quantified in its sulfated form but was not detected in its glucuronide form.

As androgen excess is a defining feature of PCOS, only metabolites from androgen origin (in addition to Prog and 17α-OHProg) were selected for PCA. Differentiation between healthy and PCOS groups was observed on the score plot PC1 versus PC2 (representing 40.8 and 14.9% of the total variability, respectively). As seen in Figure 3A, the major trend in the data summarized by PC1 is related to differences between both groups. The corresponding loading plot highlighted that most androgens were increased in the PCOS population with the exception of Prog and TS for which most of the samples were below the LLOQ (Figure 3B).

Not surprisingly, T and A4 are both increased in PCOS resulting from the increased ovary production driven by increased luteinizing hormone and increased primary androgen secretion by thecal cells [29]. Besides this origin, it has been suggested that there is more than one source of androgen hypersecretion in women with PCOS [30]. For example, adrenal androgen secretion can also be increased. Hence, it was observed that DHEA and DHEAS were both elevated in our PCOS group. Moreover, a previous study demonstrated that 50% of PCOS women have elevated circulating levels of DHEAS.

Among analytes characterized by the most marked differences, AG and EtioG concentrations were significantly higher in the PCOS population (Supplementary Figure 2). Interestingly it has been reported that AG reflects adrenal androgen secretion from hepatic 5α-reductase activity and to a lesser extent peripheral 5α-reductase activity that converts DHEAS to AG [31,32]. This metabolite accounted for 93% of the total androgen glucuronide derivatives [33]. The same was also observed for its sulfated counterpart as sulfated steroids may also serve as sources of steroid precursors [17]. It is suggested that these analytes may reflect peripheral androgen action and therefore represent reliable markers for the effects of androgens at the target tissue level especially in hirsute women with PCOS [34]. In particular, the biological significance of serum testosterone concentration without taking the large amount of androgens made in peripheral tissues into account was questioned [33]. The estimated concentrations for most androgens including T, A4, DHT, DHEA and DHEAS were similar to previously reported concentration levels in both groups while and AG concentrations were slightly lower in both groups [31,33,35].

Epiandrosterone sulfate was reported as either a urinary or serum potential long-term biomarker of exogenous T administration [9,16]. It is interesting to highlight that circulating levels of this metabolite were not found significantly different between a population with naturally elevated T concentration (PCOS) and a normal population, suggesting that an increase of EpiAS concentration might be specific to exogenous T administration (Supplementary Figure 2).

These results demonstrate the applicability of the analytical method for the quantification of a broad range of endogenous steroids including free and conjugated analytes in two female populations. This extended steroid profile allowed differences between healthy and PCOS women to be clearly underlined and subsets of relevant biomarkers to be linked with prior biological knowledge.

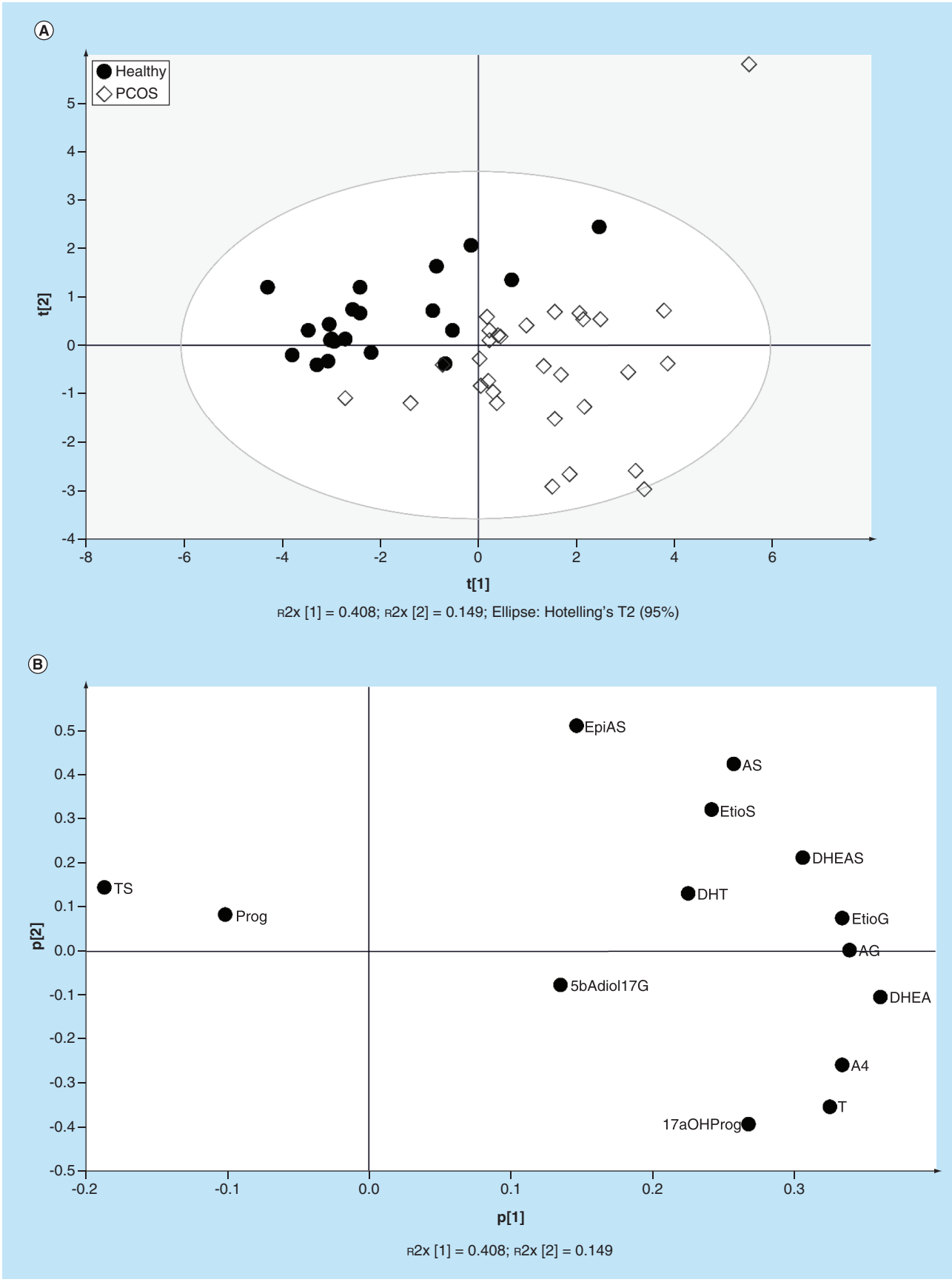


**Figure 3. Principal component analysis plots. (A)** Scores scatter plot for healthy and polycystic ovary syndrome women samples. Each point represents an individual. Healthy group is represented by black circle and polycystic ovary syndrome group is represented by white diamond. **(B)** Loadings scatter plot corresponding to the contributions of the targeted steroids.

## Conclusion

In this work, a method involving the simultaneous determination of unconjugated and conjugated steroids was developed and validated. The validation of such a method is challenging due to the difference in physicochemical properties and in concentration ranges between the large panel of analytes quantified.

The optimized chromatographic conditions allowed for adequate chromatographic resolution of isomers and acceptable peak shape of sulfated steroids. The polarity-switching mode was successfully used for mass spectrometric analyses. All free steroids were monitored in positive modes while conjugated (glucuronide and sulfate) analytes were detected in negative ionization mode with the exception of TG/TS and EG/ES. The method validation demonstrated acceptable results for trueness, precision, matrix effect, limits of detection and linear range.

The analytical method was successfully used for the analysis of serum samples from two female groups. Quantitative results allowed for distinguishing between a group with mild hyperandrogenism and a group with regular circulating androgen levels, underlining alteration of specific steroids related to mild hyperandrogenism. While the method was initially developed for anti-doping purposes, this application demonstrates that an extended steroid profiling may also be appropriate in the clinical context for the understanding of disorders related to steroid metabolism.

## Future perspective

The longitudinal monitoring of serum steroid concentrations represents a promising approach for the detection of EAAS misuse and a complement to the current urinary steroid profiling, especially in female athletes. Indeed, sequential measurement of serum T by LC–MS/MS has already been used for the profiling of athletes in cases where the urinary steroid profile did not show evidence of suspicious fluctuations [14]. To investigate this approach further, the method should be extended by additional markers of T doping (included in the presented method) and applied to the analysis of serum samples from T administration studies in female population. The results of a normal population should be then compared with the ones originating from female populations with androgen metabolism alterations, with the aim of possibly discriminating between naturally elevated androgen levels and EAAS doping.

### Executive summary

**Background**

- Quantification of serum endogenous steroid concentrations represents a complementary approach to urinary steroid profiling of the Athlete Biological Passport (ABP) for the detection of testosterone doping, especially for elite female athletes.
- To further refine this approach and expand the panel of testosterone biomarkers, a UHPLC–MS/MS method was developed for the simultaneous quantification of endogenous unconjugated and conjugated steroid in human serum.

**Experimental**

- Sample preparation, chromatographic separation and multiple reaction monitoring transitions conditions were optimized for the quantification of 14 free and 14 conjugated steroids in serum.
- The validation of the method implied the evaluation of selectivity, matrix effects, recovery and quantitative performances.
- The analytical method was used for the quantitative analysis of serum samples from two different female populations (healthy and polycystic ovary syndrome women).

**Results**

- A UHPLC–MS/MS method for the determination of an extended steroid profile in human serum was established.
- The method validation demonstrated suitable results for accuracy, precision, matrix effect and limits of detection and quantification.
- The application of the method allowed to discriminate between two female groups supposed to have different steroid profile.

**Conclusion**

- Our method is a useful analytical method for the quantification of endogenous steroids in serum for the detection of testosterone doping as well as the distinction of alterations in androgen metabolism.

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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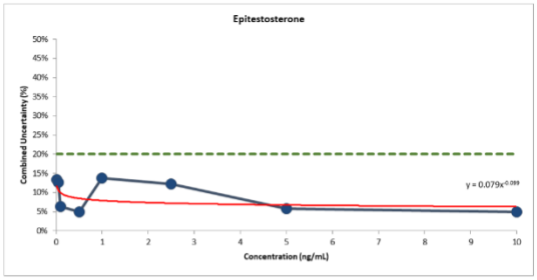
*Bioanalysis*

## Electronic Supplementary Material

# Development and validation of an UHPLC-MS/MS method for extended serum steroid profiling in female populations

### Table of contents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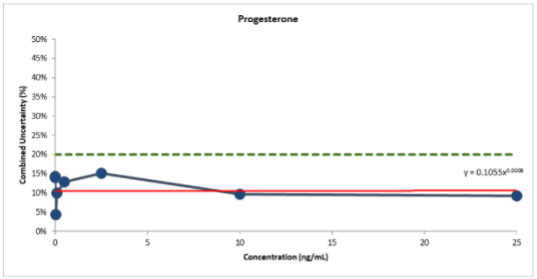
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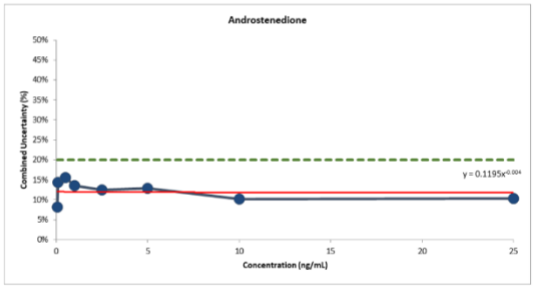
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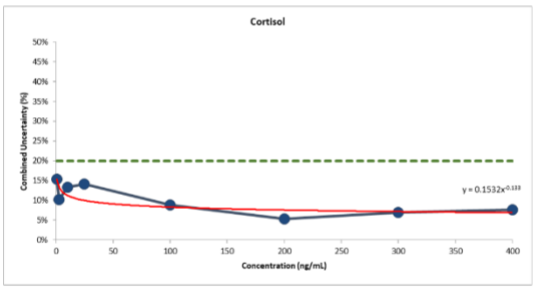
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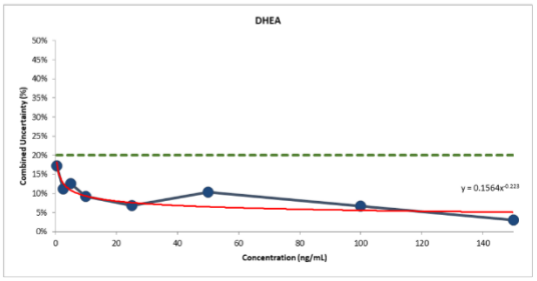
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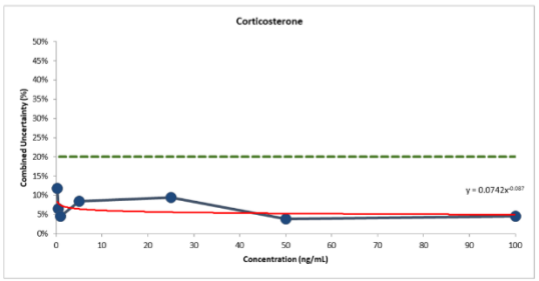
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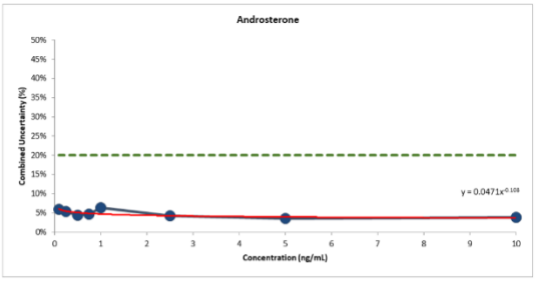
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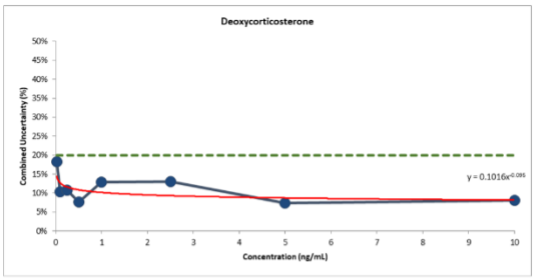
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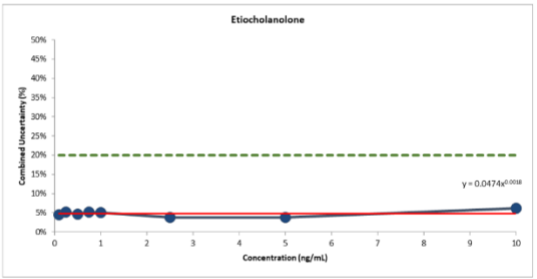
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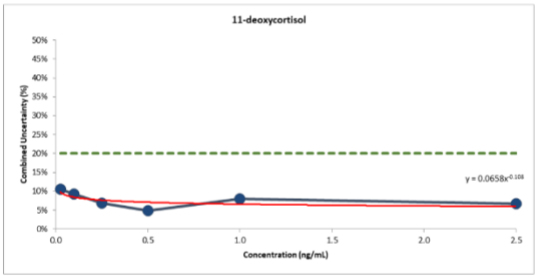
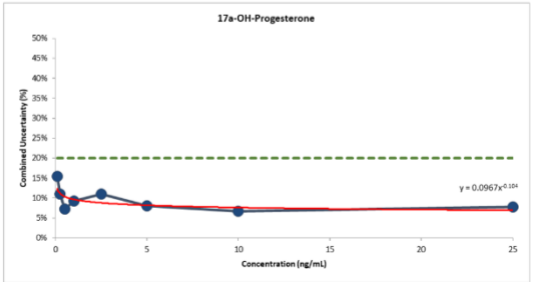
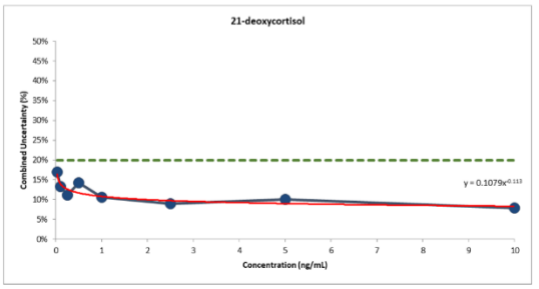
Concentration (ng/mL)
y = 0.0474x^0.0018


11-deoxycortisol
Combined Uncertainty (%)
Concentration (ng/mL)
y = 0.0658x^-0.108


17a-OH-Progesterone
Combined Uncertainty (%)
Concentration (ng/mL)
y = 0.0967x^-0.104


21-deoxycortisol
Combined Uncertainty (%)
Concentration (ng/mL)
y = 0.1079x^-0.113

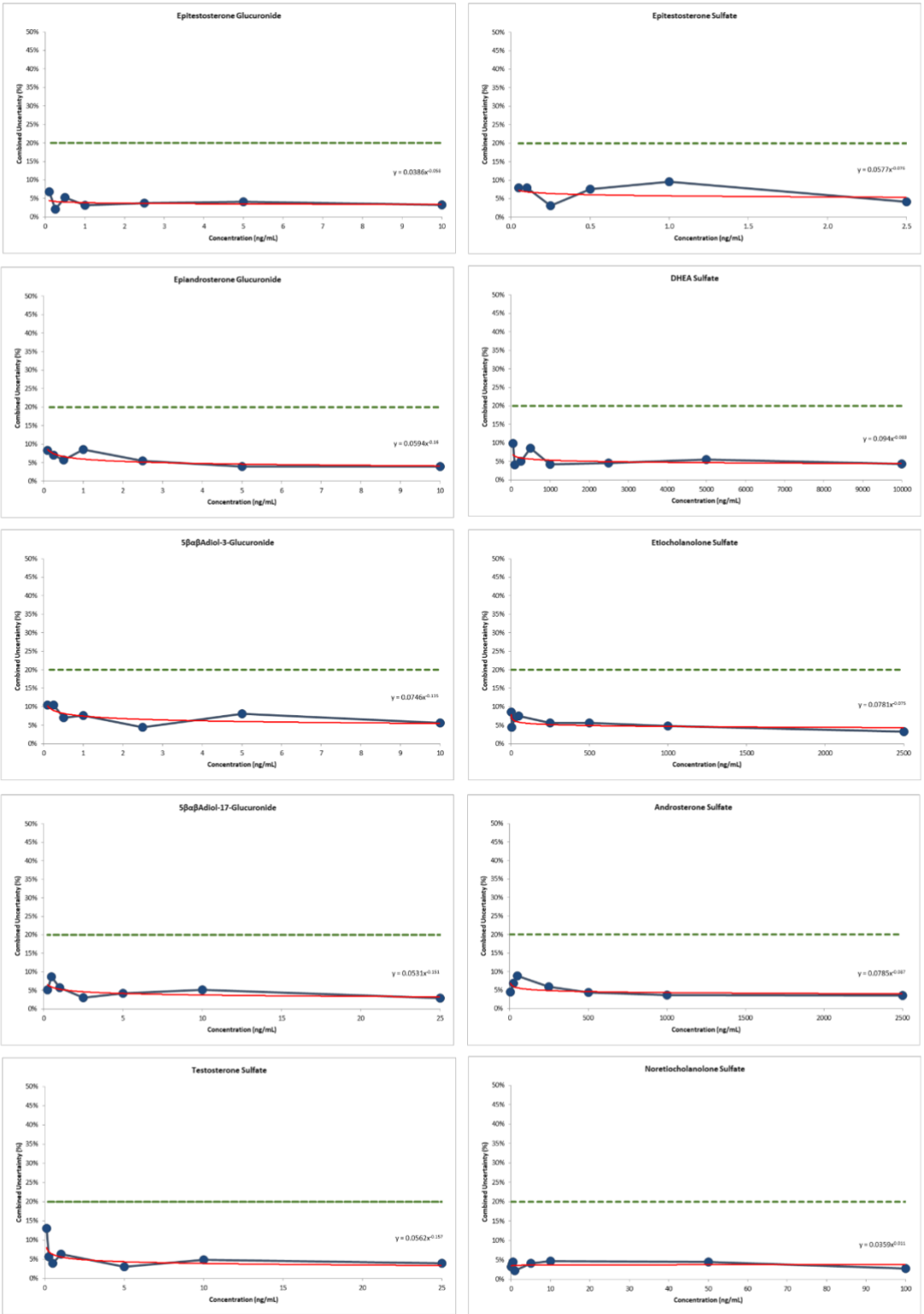



**Figure S1** - Uncertainty profiles for each analyte included into the analytical method

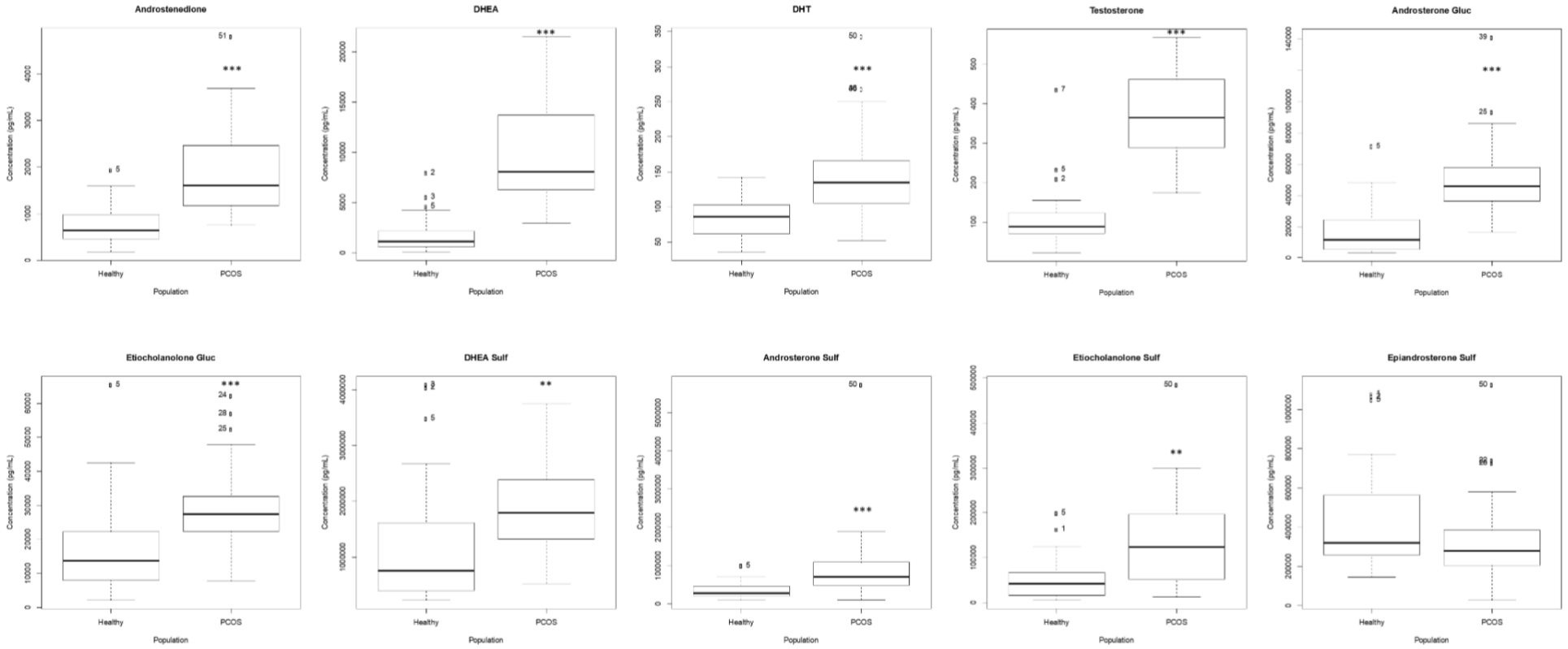



**Figure S2** - Scores of the first and second predictive components (TP1 and TP2) associated with the Time main effect for the negative ionization mode dataset related to oral administration.

Table S1 - Internal Standard Mix composition and concentration (final conc. in serum)

| Compound | Concentration (ng/mL) |
|---|---|
| **Testosterone-d3** | 0.2 |
| **Epitestosterone-d3** | 0.5 |
| **DHT-d3** | 2 |
| **DHEA-d5** | 5 |
| **Corticosterone-d4** | 5 |
| **Androstenedione-d7** | 5 |
| **17α-OHProgesterone-d8** | 10 |
| **11-deoxycortisol-d2** | 0.5 |
| **Deoxycorticosterone-d8** | 2 |
| **Progesterone-d9** | 0.5 |
| **Cortisol-d4** | 100 |
| **Androsterone-d4** | 5 |
| **Etiocholanolone-d5** | 5 |
| **21-deoxycortisol-d8** | 0.5 |
| **5βαβAdiol-17-Gluc-d3** | 10 |
| **Androsterone Gluc-d4** | 20 |
| **Etiocholanolone Gluc-d5** | 20 |
| **Epitestosterone Gluc-d3** | 5 |
| **Testosterone Gluc-d3** | 1 |
| **Androsterone Sulf-d4** | 250 |
| **Etiocholanolone Sulf-d5** | 250 |
| **DHEA Sulf-d6** | 1500 |
| **Testosterone Sulf-d3** | 2 |
| **Epitestosterone Sulf-d3** | 5 |

Table S2 - Calibration and validation samples composition and concentration (final conc. in serum)

| Analyte | Concentrations (pg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cal1/Val1 | Cal2/Val2 | Cal3/Val3 | Cal4/Val4 | Cal5/Val5 | Cal6/Val6 | Cal7/Val7 | Cal8/Val8 |
| **Testosterone** | 20 | 50 | 100 | 500 | 1000 | 5000 | 10000 | 25000 |
| **Epitestosterone** | 20 | 50 | 100 | 500 | 1000 | 2500 | 5000 | 10000 |
| **Androstenedione** | 50 | 100 | 500 | 1000 | 2500 | 5000 | 10000 | 25000 |
| **DHEA** | 500 | 2500 | 5000 | 10000 | 25000 | 50000 | 100000 | 150000 |
| **DHT** | 50 | 250 | 500 | 750 | 1000 | 2500 | 5000 | 10000 |
| **Androsterone** | 100 | 250 | 500 | 750 | 1000 | 2500 | 5000 | 10000 |
| **Etiocholanolone** | 100 | 250 | 500 | 750 | 1000 | 2500 | 5000 | 10000 |
| **Progesterone** | 15 | 25 | 50 | 100 | 500 | 2500 | 10000 | 25000 |
| **17α-hydroxyprogesterone** | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 | 25000 |
| **Cortisol** | 1000 | 2500 | 10000 | 25000 | 100000 | 200000 | 300000 | 400000 |
| **Corticosterone** | | 250 | 500 | 1000 | 5000 | 25000 | 50000 | 100000 |
| **Deoxycorticosterone** | 25 | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **11-Deoxycortisol** | 25 | 100 | 250 | 500 | 1000 | 2500 | | |
| **21-Deoxycortisol** | 25 | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **Testosterone Glucuronide** | | 50 | 100 | 500 | 1000 | 5000 | 10000 | 25000 |
| **Epitestosterone Glucuronide** | | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **Androsterone Glucuronide** | | 250 | 500 | 1000 | 5000 | 10000 | 50000 | 100000 |
| **Etiocholanolone Glucuronide** | | 250 | 500 | 1000 | 5000 | 10000 | 50000 | 100000 |
| **5βαβ-Androstanediol 3-Glucuronide** | | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **5βαβ-Androstanediol 17-Glucuronide** | | 250 | 500 | 1000 | 2500 | 5000 | 10000 | 25000 |
| **Epiandrosterone Glucuronide** | | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **Testosterone Sulfate** | | 100 | 250 | 500 | 1000 | 5000 | 10000 | 25000 |
| **Epitestosterone Sulfate** | | 100 | 250 | 500 | 1000 | 2500 | 10000 | 25000 |
| **Androsterone Sulfate** | 1000 | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |
| **Etiocholanolone Sulfate** | 1000 | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |
| **DHEA Sulfate** | 50000 | 100000 | 250000 | 500000 | 1000000 | 2500000 | 5000000 | 10000000 |
| **Noretiocholanolone Sulfate** | 100 | 250 | 500 | 1000 | 5000 | 10000 | 50000 | 100000 |
| **Epiandrosterone Sulfate** | 1000 | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |

**Table S3** - Quantitative validation results for all analytes at each concentration level

| Compound | Concentration | Trueness (%) | Intermediate Precision (%) | Repeatability (%) | Combined Uncertainty (%) |
|---|---|---|---|---|---|
| **Testosterone** | 20 | 98.9 | 9.0 | 5.4 | 12.0 |
| | 50 | 93.0 | 6.6 | 2.4 | 11.1 |
| | 100 | 92.9 | 2.4 | 2.4 | 7.8 |
| | 500 | 98.6 | 4.3 | 3.1 | 5.9 |
| | 1000 | 107.0 | 3.2 | 3.0 | 8.3 |
| | 5000 | 105.4 | 3.3 | 1.6 | 6.9 |
| | 10000 | 103.7 | 3.8 | 2.8 | 6.3 |
| | 25000 | 98.2 | 4.8 | 2.8 | 6.6 |
| **Epitestosterone** | 20 | 97.4 | 10.1 | 4.3 | 13.4 |
| | 50 | 94.6 | 8.6 | 5.8 | 12.7 |
| | 100 | 98.6 | 4.7 | 4.7 | 6.3 |
| | 500 | 98.3 | 3.4 | 3.4 | 4.9 |
| | 1000 | 109.7 | 7.4 | 4.0 | 13.8 |
| | 2500 | 109.8 | 5.6 | 1.3 | 12.2 |
| | 5000 | 101.1 | 4.3 | 2.4 | 5.8 |
| | 10000 | 98.7 | 3.6 | 2.3 | 4.9 |
| **Androstenedione** | 50 | 101.9 | 6.0 | 3.6 | 8.1 |
| | 100 | 93.2 | 9.7 | 4.6 | 14.4 |
| | 500 | 88.3 | 7.9 | 3.6 | 15.6 |
| | 1000 | 96.9 | 10.1 | 3.7 | 13.5 |
| | 2500 | 108.7 | 6.8 | 2.9 | 12.4 |
| | 5000 | 103.0 | 9.7 | 2.1 | 12.9 |
| | 10000 | 102.2 | 7.6 | 2.5 | 10.2 |

| | | | | | |
|---|---|---|---|---|---|
| | 25000 | 96.3 | 7.4 | 2.0 | 10.3 |
| **DHEA** | 500 | 96.8 | 12.8 | 6.4 | 17.2 |
| | 2500 | 98.5 | 8.3 | 6.8 | 11.2 |
| | 5000 | 94.0 | 8.5 | 4.0 | 12.6 |
| | 10000 | 94.2 | 5.4 | 5.4 | 9.3 |
| | 25000 | 106.1 | 2.4 | 2.3 | 6.9 |
| | 50000 | 105.2 | 6.9 | 3.0 | 10.4 |
| | 100000 | 99.9 | 5.0 | 4.5 | 6.8 |
| | 150000 | 100.0 | 2.4 | 2.4 | 3.1 |
| **DHT** | 50 | 98.9 | 10.1 | 4.3 | 13.3 |
| | 250 | 96.2 | 6.9 | 6.7 | 10.1 |
| | 500 | 99.9 | 4.7 | 4.6 | 6.5 |
| | 750 | 96.3 | 4.0 | 4.0 | 6.5 |
| | 1000 | 105.8 | 6.9 | 4.5 | 10.8 |
| | 2500 | 105.7 | 4.5 | 4.2 | 8.4 |
| | 5000 | 99.6 | 4.0 | 2.9 | 5.4 |
| | 10000 | 96.9 | 4.7 | 3.8 | 7.1 |
| **Androsterone** | 100 | 102.0 | 4.2 | 3.5 | 6.0 |
| | 250 | 98.5 | 3.8 | 3.8 | 5.4 |
| | 500 | 98.1 | 3.0 | 2.4 | 4.4 |
| | 750 | 98.1 | 3.1 | 2.9 | 4.7 |
| | 1000 | 101.1 | 4.8 | 2.6 | 6.4 |
| | 2500 | 102.0 | 2.7 | 2.3 | 4.2 |
| | 5000 | 100.6 | 2.6 | 1.3 | 3.5 |
| | 10000 | 97.7 | 2.3 | 2.3 | 3.8 |

| | | | | | |
|---|---|---|---|---|---|
| **Etiocholanolone** | 100 | 99.2 | 3.3 | 2.3 | 4.5 |
| | 250 | 100.3 | 3.9 | 2.4 | 5.2 |
| | 500 | 100.5 | 3.5 | 3.5 | 4.7 |
| | 750 | 98.9 | 3.9 | 2.1 | 5.2 |
| | 1000 | 103.0 | 3.0 | 1.8 | 5.0 |
| | 2500 | 101.4 | 2.6 | 2.4 | 3.8 |
| | 5000 | 99.4 | 2.9 | 1.3 | 3.8 |
| | 10000 | 98.6 | 4.6 | 1.8 | 6.2 |
| **17a-OHProgesterone** | 100 | 97.5 | 11.8 | 1.7 | 15.4 |
| | 250 | 98.2 | 8.3 | 3.4 | 11.0 |
| | 500 | 95.7 | 4.3 | 4.0 | 7.3 |
| | 1000 | 93.0 | 4.4 | 4.4 | 9.2 |
| | 2500 | 108.6 | 5.1 | 3.5 | 11.0 |
| | 5000 | 101.0 | 6.0 | 3.0 | 8.0 |
| | 10000 | 97.7 | 4.8 | 2.3 | 6.7 |
| | 25000 | 96.8 | 5.5 | 2.0 | 7.8 |
| **Progesterone** | 15 | 94.0 | 9.9 | 3.8 | 14.3 |
| | 25 | 93.7 | 9.2 | 8.8 | 14.0 |
| | 50 | 98.3 | 3.1 | 3.1 | 4.4 |
| | 100 | 96.5 | 6.8 | 6.2 | 9.9 |
| | 500 | 110.6 | 5.7 | 2.2 | 12.9 |
| | 2500 | 112.0 | 6.9 | 4.1 | 15.1 |
| | 10000 | 98.6 | 7.3 | 3.2 | 9.7 |
| | 25000 | 99.9 | 7.1 | 2.3 | 9.3 |

| | | | | | |
|---|---|---|---|---|---|
| **Cortisol** | 1000 | 108.9 | 6.7 | 1.4 | 15.4 |
| | 2500 | 99.8 | 10.1 | 3.8 | 10.2 |
| | 10000 | 93.1 | 5.9 | 3.1 | 13.3 |
| | 25000 | 91.9 | 9.0 | 3.8 | 14.2 |
| | 100000 | 98.7 | 6.6 | 3.6 | 8.8 |
| | 200000 | 97.6 | 3.4 | 3.4 | 5.3 |
| | 300000 | 101.9 | 5.0 | 3.8 | 6.9 |
| | 400000 | 99.4 | 5.8 | 1.4 | 7.6 |
| **Corticosterone** | 250 | 89.7 | 4.4 | 2.0 | 11.8 |
| | 500 | 95.6 | 3.5 | 2.7 | 6.5 |
| | 1000 | 97.0 | 2.5 | 2.2 | 4.5 |
| | 5000 | 107.2 | 3.3 | 2.0 | 8.4 |
| | 25000 | 107.3 | 4.5 | 2.5 | 9.4 |
| | 50000 | 100.1 | 2.8 | 2.7 | 3.8 |
| | 100000 | 98.4 | 3.1 | 2.3 | 4.5 |
| **Deoxycorticosterone** | 25 | 91.1 | 12.3 | 3.2 | 18.2 |
| | 100 | 95.1 | 6.9 | 2.8 | 10.3 |
| | 250 | 101.8 | 8.0 | 3.8 | 10.7 |
| | 500 | 97.1 | 5.4 | 2.7 | 7.7 |
| | 1000 | 109.9 | 6.4 | 2.9 | 12.9 |
| | 2500 | 110.1 | 6.3 | 2.3 | 13.0 |
| | 5000 | 102.3 | 5.4 | 2.4 | 7.4 |
| | 10000 | 98.0 | 6.0 | 2.0 | 8.1 |
| **11-Deoxycortisol** | 25 | 102.7 | 7.9 | 3.1 | 10.6 |

| | | | | | |
|---|---|---|---|---|---|
| | 100 | 97.9 | 6.9 | 3.3 | 9.3 |
| | 250 | 100.7 | 5.2 | 5.2 | 6.9 |
| | 500 | 96.1 | 2.0 | 2.0 | 4.8 |
| | 1000 | 106.5 | 3.5 | 3.5 | 8.0 |
| | 2500 | 96.4 | 4.1 | 3.9 | 6.7 |
| **21-Deoxycortisol** | 25 | 91.0 | 10.7 | 8.3 | 17.0 |
| | 100 | 95.6 | 9.2 | 8.8 | 13.3 |
| | 250 | 105.3 | 7.2 | 6.2 | 11.1 |
| | 500 | 104.2 | 10.0 | 8.5 | 14.2 |
| | 1000 | 109.8 | 3.0 | 1.9 | 10.6 |
| | 2500 | 107.7 | 3.5 | 1.9 | 8.9 |
| | 5000 | 108.5 | 3.9 | 3.9 | 10.0 |
| | 10000 | 104.1 | 5.1 | 3.4 | 7.9 |
| **Testosterone Gluc** | 50 | 101.1 | 5.3 | 4.4 | 7.2 |
| | 100 | 96.8 | 7.0 | 7.0 | 9.9 |
| | 500 | 97.5 | 3.9 | 2.5 | 5.7 |
| | 1000 | 103.3 | 4.3 | 2.7 | 6.6 |
| | 5000 | 100.3 | 2.6 | 2.4 | 3.6 |
| | 10000 | 99.5 | 2.8 | 2.3 | 3.8 |
| | 25000 | 100.7 | 2.5 | 1.7 | 3.5 |
| **Epitestosterone Gluc** | 100 | 102.2 | 4.9 | 2.0 | 6.8 |
| | 250 | 99.3 | 1.5 | 1.1 | 2.1 |
| | 500 | 100.0 | 4.1 | 1.2 | 5.3 |
| | 1000 | 102.2 | 1.7 | 1.7 | 3.2 |
| | 2500 | 101.5 | 2.6 | 2.6 | 3.8 |
| | 5000 | 101.6 | 2.8 | 2.2 | 4.1 |

| | | | | | |
|---|---|---|---|---|---|
| | 10000 | 101.9 | 1.9 | 1.7 | 3.3 |
| **Androsterone Gluc** | 250 | 97.1 | 9.9 | 5.6 | 13.4 |
| | 500 | 100.9 | 7.9 | 1.8 | 10.2 |
| | 1000 | 98.9 | 3.9 | 1.8 | 5.2 |
| | 5000 | 98.9 | 1.5 | 1.4 | 2.3 |
| | 10000 | 102.2 | 3.2 | 3.2 | 4.7 |
| | 50000 | 100.4 | 2.0 | 2.0 | 2.8 |
| | 100000 | 101.5 | 1.2 | 0.8 | 2.2 |
| **Etiocholanolone Gluc** | 250 | 98.8 | 3.6 | 3.6 | 5.1 |
| | 500 | 96.9 | 8.1 | 7.6 | 11.5 |
| | 1000 | 97.8 | 4.4 | 2.9 | 6.2 |
| | 5000 | 98.1 | 2.7 | 2.7 | 4.1 |
| | 10000 | 100.2 | 3.6 | 3.6 | 4.6 |
| | 50000 | 100.8 | 3.2 | 2.7 | 4.4 |
| | 100000 | 104.0 | 2.8 | 2.8 | 5.4 |
| **Epiandrosterone Gluc** | 100 | 99.9 | 6.1 | 5.8 | 8.3 |
| | 250 | 100.8 | 5.2 | 5.2 | 7 |
| | 500 | 101.2 | 4.0 | 3.9 | 5.7 |
| | 1000 | 99.0 | 6.6 | 1.2 | 8.6 |
| | 2500 | 100.2 | 4.2 | 2.3 | 5.5 |
| | 5000 | 99.4 | 2.9 | 1.8 | 3.9 |
| | 10000 | 101.7 | 2.7 | 2.0 | 4 |
| **5bab-Adiol-3 Gluc** | 100 | 100.5 | 8.1 | 8.1 | 10.5 |

| | | | | | |
|---|---|---|---|---|---|
| | 250 | 100.8 | 7.8 | 6.4 | 10.5 |
| | 500 | 100.1 | 5.2 | 5.2 | 7.1 |
| | 1000 | 99.5 | 5.8 | 2.9 | 7.6 |
| | 2500 | 101.2 | 3.4 | 3.4 | 4.5 |
| | 5000 | 97.0 | 5.7 | 2.5 | 8.1 |
| | 10000 | 100.4 | 4.2 | 3.0 | 5.6 |
| **5bab-Adiol-17 Gluc** | 250 | 99.3 | 3.9 | 3.0 | 5.2 |
| | 500 | 97.7 | 6.3 | 3.4 | 8.7 |
| | 1000 | 100.0 | 4.3 | 4.3 | 5.7 |
| | 2500 | 100.5 | 2.2 | 1.6 | 3 |
| | 5000 | 98.2 | 2.8 | 2.2 | 4.2 |
| | 10000 | 97.9 | 3.5 | 2.8 | 5.1 |
| | 25000 | 100.3 | 2.1 | 2.1 | 2.9 |
| **Testosterone Sulf** | 100 | 106.1 | 8.7 | 5.4 | 13 |
| | 250 | 102.8 | 3.7 | 3.7 | 5.7 |
| | 500 | 100.0 | 3.0 | 3.0 | 4 |
| | 1000 | 105.2 | 2.7 | 1.7 | 6.4 |
| | 5000 | 101.1 | 2.1 | 2.1 | 3.1 |
| | 10000 | 100.3 | 3.6 | 3.1 | 4.9 |
| | 25000 | 101.0 | 2.9 | 1.8 | 4 |
| **Epitestosterone Sulf** | 50 | 107.5 | 2.2 | 0.7 | 8 |
| | 100 | 93.2 | 3.2 | 1.2 | 7.9 |
| | 250 | 99.8 | 2.3 | 2.3 | 3.1 |
| | 500 | 94.3 | 3.7 | 2.2 | 7.6 |
| | 1000 | 105.7 | 5.8 | 4.2 | 9.6 |

| | | | | | |
|---|---|---|---|---|---|
| | 2500 | 97.7 | 2.6 | 1.9 | 4.2 |
| **Androsterone Sulf** | 1000 | 97.7 | 1.4 | 1.3 | 2.9 |
| | 5000 | 96.9 | 2.4 | 2.1 | 4.5 |
| | 25000 | 98.8 | 5.1 | 3.2 | 6.8 |
| | 50000 | 97.8 | 6.5 | 3.2 | 8.8 |
| | 250000 | 98.8 | 4.4 | 2.6 | 5.9 |
| | 500000 | 100.5 | 3.2 | 3.2 | 4.3 |
| | 1000000 | 99.2 | 2.7 | 2.7 | 3.6 |
| | 2500000 | 99.7 | 2.7 | 1.7 | 3.5 |
| **Etiocholanolone Sulf** | 1000 | 102.8 | 6.1 | 4.0 | 8.6 |
| | 5000 | 102.4 | 2.8 | 1.9 | 4.4 |
| | 25000 | 101.2 | 5.1 | 4.8 | 7.1 |
| | 50000 | 98.3 | 5.4 | 4.2 | 7.5 |
| | 250000 | 97.5 | 3.7 | 3.2 | 5.6 |
| | 500000 | 98.5 | 4.0 | 3.4 | 5.6 |
| | 1000000 | 98.3 | 3.2 | 3.2 | 4.8 |
| | 2500000 | 98.3 | 2.1 | 2.1 | 3.3 |
| **Noretiocholanolone Sulf** | 100 | 101.0 | 2.3 | 1.7 | 3.2 |
| | 250 | 101.9 | 2.6 | 2.0 | 3.9 |
| | 500 | 103.5 | 2.2 | 1.5 | 4.5 |
| | 1000 | 99.5 | 1.6 | 1.6 | 2.2 |
| | 5000 | 100.6 | 3.0 | 2.6 | 4.1 |
| | 10000 | 103.6 | 2.3 | 1.8 | 4.7 |
| | 50000 | 103.7 | 2.0 | 0.8 | 4.5 |
| | 100000 | 100.5 | 2.0 | 1.8 | 2.8 |

| | | | | | |
|---|---|---|---|---|---|
| **Epiandrosterone Sulf** | 5000 | 101.9 | 2.0 | 1.7 | 3.3 |
| | 25000 | 105.8 | 3.8 | 1.8 | 7.7 |
| | 50000 | 103.2 | 3.6 | 2.7 | 5.8 |
| | 250000 | 104.3 | 1.8 | 1.8 | 4.9 |
| | 500000 | 103.3 | 4.6 | 1.5 | 6.8 |
| | 1000000 | 100.9 | 2.5 | 1.3 | 3.4 |
| | 2500000 | 100.4 | 3.7 | 2.9 | 5 |
| **DHEA Sulf** | 50000 | 108.2 | 4.2 | 2.3 | 9.9 |
| | 100000 | 99.7 | 3.1 | 1.4 | 4.1 |
| | 250000 | 102.5 | 3.2 | 3.0 | 5 |
| | 500000 | 93.7 | 4.4 | 2.6 | 8.6 |
| | 1000000 | 97.9 | 2.8 | 1.6 | 4.2 |
| | 2500000 | 98.9 | 3.2 | 2.9 | 4.5 |
| | 5000000 | 98.2 | 4.0 | 1.4 | 5.5 |
| | 10000000 | 102.5 | 2.6 | 2.2 | 4.3 |

## 3.4 Article 7: A comprehensive investigation of the steroid metabolism in eumenorrheic women following testosterone gel administration (submitted to the Journal of Clinical Endocrinology and Metabolism)

To further benefit from the development of this extended steroid profiling, it was applied on the serum samples collected during the T administration study to investigate the steroid metabolism in healthy eumenorrheic women and its response to T gel administration. The response to T administration in women was further explored with the analysis of samples collected over 12 months from four transgender patients (female-to-male) under T treatment.

The quantitative analysis enabled to establish reference ranges of each detected compound during a menstrual cycle and to highlight potential additional biomarkers of T gel administration. Alternative androgen biosynthesis in women such as the 'backdoor' pathway could also be evaluated with correlation analysis between the steroids.

In addition to T and DHT, which were already reported to increase during T administration (Article 5), androsterone and epiandrosterone sulfate demonstrated higher levels the week following the end of T administration underlining an increased systemic 5α-reductase activity while androgen glucuronides did not seem impacted. Moreover, the 28-day T gel administration did not impact the menstrual status of the subjects as naturally cycling hormones such as progesterone, estradiol, or LH remained unaffected by the treatment. The responses of DHT, androsterone and epiandrosterone sulfate following T administration were confirmed with transgender patients with a dose-effect response over the 12 months of treatment.

These findings suggest that serum T and DHT as well as sulfo-conjugated androsterone and epiandrosterone may represent potential markers of short- and long-term T gel administration respectively in women.

*Article*

# A comprehensive investigation of the steroid metabolism in eumenorrheic women following testosterone gel administration

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**Keywords**: steroid metabolism; testosterone gel; women; UHPLC-MS/MS; androgen

Clinical Trial Registration number**:** ISRCTN10122130

Disclosure Statement: The authors have nothing to disclose

## Abstract

**Context:** Few studies have investigated the effect of exogenous testosterone (T) administration on the steroid hormones and their regulation in healthy women.

**Objective:** This work aimed to investigate the steroid metabolism in healthy eumenorrheic women and the female response to T gel administration by quantifying a large panel of endogenous steroids.

**Methods:** Fourteen healthy women with regular menstrual cycle and basal testosterone <2 nmol/L participated in an open-label trial divided into three phases of 4-weeks, corresponding to three consecutive menstrual cycles. The first cycle was the control phase, the second one a treatment phase during which 10-mg T gel was administered daily, followed by the third, a post-treatment phase. Serum samples were analysed with UHPLC-MS/MS for the simultaneous determination of 28 endogenous steroids including free and conjugated metabolites. To further explore the response to T administration in women, serum samples collected over 12 months from four transgender patients under T treatment were also analysed.

**Results:** Among the quantified steroids, testosterone and DHT levels increased significantly during the 28-day T treatment while androsterone and epiandrosterone sulfate demonstrated higher concentrations after T administration, referring to an increased 5α-reductase activity. SHGB level decreased during T administration phase but the treatment did not impact the menstrual status of the subjects. The responses of DHT, androsterone and epiandrosterone sulfate to T administration were confirmed with transgender patients via dose-effect response.

**Conclusion:** Serum T, DHT, androsterone and epiandrosterone sulfate concentrations exhibit potential markers of long-term T gel administration in women.

## Introduction

The diagnosis and monitoring of various endocrine conditions relies often on the quantitative analysis of steroids in blood. Due to the similarities of the symptoms between steroid-related endocrine diseases, it is imperative to discriminate the nature of alterations in the steroid metabolome for a better understanding, diagnosis, prognosis and treatment. For various endocrine pathologies, the standard test is commonly based on the immunoanalytic measurement of a single steroid (1). However, the accuracy and sensitivity of these methods suffer from compromised performance due to narrow detection range for low-molecular-weight compounds, protein binding interference and cross-reaction (2). Furthermore, as this approach does not allow for measuring multiple target compounds at the same time, the analytical scope suffers from limitations for method extension. For some time now, clinical laboratories have rather used liquid chromatography-tandem mass spectrometry (LC-MS/MS) as gold standard method to measure the levels of steroid hormones which allows quantifying multiple analytes simultaneously with an increased sensitivity and specificity (3). Therefore, the simultaneous determination of a panel of steroids has become an attractive approach in clinical and research laboratories to obtain a large coverage of pathways and hence to study potential interferences in steroidogenesis due to hormone imbalances (1).

Traditionally, the developed methods are used to quantify free steroids including precursors, hormones, and their principal phase I metabolites. However, in the context of androgens for instance, the intracrine synthesis is not often reflected by circulating androgens but rather by androgen metabolites and conjugates, especially in women in whom certain circulating androgen levels are low (4). It is suggested that conjugated analytes (phase II metabolites) may reflect peripheral androgen action, representing reliable markers for the effects of androgens at the target tissue level (5,6). For instance, hyperandrogenism diagnosis is mainly based on the quantification of testosterone or few compounds. Quantification of an extended panel of

steroids including conjugated metabolites is therefore necessary to provide further insight into the intracrine metabolism and its role in endocrine disorders (4). Our group recently developed a method for an extended serum steroid profiling involving free and conjugated steroids (Fig. 1) using UHPLC-MS/MS and applied the method for the discrimination between healthy and women with polycystic ovary syndrome (PCOS) (7).

In women, the regulation of androgens differs from men. While most androgens are produced in testes in males, androgens in women derive either from a modest ovarian production or mostly from peripheral conversion of adrenal steroid precursors. The main active androgen, testosterone (T), is responsible for the development of male secondary sex characteristics and the increase of muscle mass and physical strength. The circulating level of T in women is seven to eight times lower than in men and its production declines with age (8). Nevertheless, T is a vital element in female sexuality and is associated with greater well-being, improved energy and vitality. Indeed, several studies have demonstrated that transdermal T administered in small doses had benefic effects on sexual function, mood and well-being as well as anxiolytic effects in premenopausal and menopausal women (9–12). Several forms of transdermal T specific to women have been developed to treat principally hypoactive sexual desire dysfunction (HSDD) that affects up to 40% of women (13) and a T-based formulation (AndroFeme® 1) has just been included on the Australian Register of Therapeutic Goods for the treatment of postmenopausal women experiencing HSDD (14). In North America, despite the absence of an approved T formulation for HSDD, off-label prescription is widespread, even in the absence of low T levels (15).

The administration of exogenous T is also particularly attractive in the context of sports to benefit from an ergogenic impact. While exogenous T is particularly effective in strength sports, frequent and small T doses can compensate the lower amount of endogenous T induced by overtraining, reducing fatigue and also stimulate the erythropoietic system (16,17). In

particular, testosterone in transdermal form is preferred to oral or parenteral form because its administration results in minor increase in urinary concentrations and is therefore more complex to detect for anti-doping purposes. Given the lower basal T concentration in women, an increase in the circulating T level is supposed to generate a significant increase in performance. Hence, a study demonstrated that a moderate increase of testosterone levels consecutive to a 10-weeks treatment with 10-mg transdermal T was sufficient to significantly improve physical performance by increasing total lean mass, skeletal muscle oxidative capacity and capillary-to-fiber ratio (18,19).

Despite the number of studies involving the transdermal T administration in women, few of them have investigated the impact of exogenous T administration on a large panel of steroids including precursors, active hormones, and phase I and II metabolites in combination with the hormonal fluctuations of the menstrual cycle. In this study, the biological response to the administration of exogenous T in healthy women with regular menstrual cycles was investigated with the profiling of 28 steroids in serum. To further explore the steroid response to T administration, samples from transgender patients (female-to-male (FTM)) treated with higher T doses were also analyzed. In addition to a broader investigation of steroid metabolism in healthy women, this study allowed for evaluating possible biomarkers of exogenous T administration that could be used in the clinical context to assess the response to T treatment but also for anti-doping purposes through longitudinal monitoring for T detection.

## Material & Methods

*Study subjects*

The study involved 14 healthy women volunteers with the following characteristics: 20 to 40 years of age, a body mass index between 18 and 30 kg/m$^2$, regular menstruations (cycle length between 26 and 32 days), hemoglobin concentration (HGB) between 12 and 16 g/dL, basal testosterone concentration ≤ 2 nmol/L, negative pregnancy test at screening, acceptance to use highly efficient non-hormonal contraception during the study period. All participants were in general good health based on history and physical examination. Women planning a pregnancy within 6 months after study completion or breastfeeding were excluded. Women with acne or hirsutism considered clinically significant, cardiovascular, liver, biliary or renal disease, endocrine or metabolic disorder, uncontrolled hypertension, dyslipidemia, hyperprolactinemia, eating disorder, taking medications known to interfere with steroid metabolism (ketoconazole, ACTH, corticoids or anticoagulants) or hormonal contraception in the 2 months prior to the study and who donated blood in the 3 months prior to the study were also excluded. To comply with anti-doping rules, women subjects agreed not to participate in any sports competition during the study period. The necessity of contraception during the protocol was stressed and all participants received contraceptive counseling. Subjects were permitted to moderate consumption of alcohol beverages (≤3 units/week) and to use over the counter medication and instructed to report any at the time of the study visit.

Participants were recruited from the general population via calls posted at the university hospital. All subjects provided written informed consent prior to any study procedures and the open-label trial was approved by the local Ethical Committee of the Canton de Vaud in Switzerland (2018-02106, SNCTP000003264) and Swissmedic (2018DR1168), registered on www.isrctn.com (ISRCTN10122130) and conducted in accordance with the Declaration of Helsinki.

141 *Study design*

142 The protocol was an open-label trial, with each subject being her own control, divided into three
143 28-days phases for a total of 12 study weeks. Each phase corresponded to a menstrual cycle.
144 The protocol started on the Monday following the start of menses. The first menstrual cycle
145 corresponded to the control phase (phase 1-P1). During the first week, serum samples were
146 collected the first four days (Monday to Thursday) while in the second and third week, samples
147 were collected the Monday, Wednesday and Friday and in the fourth week, collection occurred
148 from Monday to Friday. The second phase (phase 2-P2) corresponded to the treatment stage
149 during which 0.5 g of Tostran® 20 mg/g gel (equivalent to 10 mg testosterone) was self-
150 administered every morning on the upper thigh or abdomen for 28 days. To prevent skin
151 irritations caused by the gel, the application site was altered every day. On visiting days,
152 samples were collected before the application of the treatment, corresponding approximately to
153 24 hours after gel administration, and sampling was similar to P1. In case of delay of menses,
154 subjects had to wait for one week until menstruation before starting the treatment. The
155 compliance was monitored at each study visit by weighting the medical preparation. The third
156 phase (phase 3-P3) corresponded to the post-treatment period and was similar to first phase. In
157 addition to serum, whole blood samples were collected once a week (on Wednesday) for full
158 blood count. Blood samples were collected from antecubital vein in 4 mL BD Vacutainer®
159 K2EDTA tubes for full blood count and in 8.5 mL BD Vacutainer® SST$^{TM}$ II Advance tubes
160 and serum was separated by centrifugation and stored at-20°C until analysis. The Tostran®
161 product was supplied and labelled by the pharmaceutical service of Lausanne University
162 Hospital (CHUV). Study visits involving sample collection took place at the Clinical Trial Unit
163 (CTU) of CHUV in the morning approximately at the same hour for each subject between 7
164 AM and 11 AM.

165 *Evaluation of safety*

The reported adverse events (AEs) possibly related to the administration of testosterone were monitored during the whole treatment phase at each visit. All participants were required to have a pregnancy test once a week during P2 and once a week the first two weeks of P3. Hirsutism was evaluated using the Ferriman and Gallwey score once a week during P2 and the first and last week of P3. Acne and voice changes were also assessed clinically. Any adverse events or changes in medication were recorded in the case report form. Blood pressure was recorded each week. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and C-reactive protein (CRP) serum levels were monitored to detect any potential liver damage. As regular blood sampling in addition to menstruations could cause an onset of iron deficiency, ferritin level was measured once a week. In case of ferritin decrease, the subjects were supplemented with oral iron until the end of the protocol.

*Steroid hormone measurements*

Serum samples were analyzed using a validated method for the simultaneous determination of 14 free (testosterone (T), epitestosterone (E), androstenedione (A4), progesterone (Prog), 17a-hydroxyprogesterone (17OHP), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), corticosterone, cortisol, deoxycorticosterone, 11-deoxycortisol, androsterone (A), etiocholanolone (Etio), 21-deoxycortisol) and 14 conjugated (testosterone glucuronide (TG), epitestosterone glucuronide (EG), androsterone glucuronide (AG), etiocholanolone glucuronide (EtioG), epiandrosterone glucuronide (EpiAG), 5β-androstane-3α,17β-diol-17-glucuronide (5βαβAdiol-17-G), 5β-androstane-3α,17β-diol-3-O-glucuronide (5βαβAdiol-3-G), testosterone sulfate (TS), epitestosterone sulfate (ES), DHEA sulfate (DHEAS), androsterone sulfate (AS), etiocholanolone sulfate (EtioS), epiandrosterone sulfate (EpiAS) and dehydroandrosterone sulfate (DehydroAS)) steroids in serum as previously described (7). Briefly, a solid phase extraction (SPE) with Oasis Prime HLB Prime 96-well µElution plate (Waters, Milford USA) was used to extract steroid hormones. Each serum aliquot (200 µL),

previously centrifuged to remove lipid fraction, was spiked with 20 µL of the internal standard mixture, diluted with 200 µL of 4% $H_3PO_4$ aqueous solution and 400 µL of sample was loaded on each well using positive pressure. After a washing step with 400 µL of a $H_2O$/MeOH (95/5, v/v) solution containing 0.1% $NH_4OH$, elution was carried out by adding 50 µL of a MeOH/ $H_2O$ (90/10, v/v) solution. Extracts were collected with collection plates equipped with 1.5 mL glass inserts, were then diluted with 50 µL of $H_2O$ and 10 µL of each extract was injected into UHPLC-MS/MS system.

Chromatographic separation was performed on a UPLC system (Waters, Milford, MA, USA) equipped with a Kinetex C18 column (150 x 2.1 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) set at 60°C. The mobile phases were composed of 5 mM ammonium formate in water (A) and 5 mM ammonium formate in MeOH (B) with a flow rate set at 300 µL/min. Gradient started from 40% B and increased linearly to 80% over 20 min, followed by 7 min at 98% B, with a 8 min re-equilibration step at initial conditions for a total run time of 35 min. The UPLC system was coupled to a Xevo-TQ S triple quadrupole MS/MS system (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source operated in polarity switching (positive/negative) mode. Multiple reaction monitoring (MRM) acquisition was employed and the detailed instrumental conditions (MRM transitions, ESI conditions, cone voltages and collision energies) are described in (7). For each batch of analyses, calibration curves were performed using a 8-point linear calibration model in spiked depleted serum for each compound, prepared freshly within each batch. Linear calibration models were generated with the peak area of each steroid to its respective internal standard using a 1/x weighted least-squared regression and were used to obtain quantitative values of each sample.

Free and bioavailable testosterone was calculated using Vermeulen method, with a standard average albumin concentration of 4.3 g/dL (20). Free androgen index (FAI) was calculated as total testosterone (nmol/L) divided by SHBG (nmol/L) x 100.

For estrogen quantification (estradiol, estrone, estriol), a second serum aliquot was analysed using the method described in (21).

*Immunological analyses*

Alanine transaminase (ALT), Aspartate transaminase (AST), and C-reactive protein (CRP) serum levels were monitored using a Dimension EXL200 automated system (Siemens Healthcare Diagnostic SA, Zurich, Switzerland). Serum luteinizing hormone (LH), follicular stimulating hormone (FSH), sex-hormone binding globulin (SHBG), and ferritin were measured by direct chemiluminescence using a Siemens ADVIA® Centaur™ Immunoassay System. LH peak was taken to denote mid-cycle, while the mid-luteal phase was marked by a peak in systemic P. Other phases of the cycle were extrapolated from these two time points.

Erythropoietin (EPO) and anti-mullerian hormone (AMH) levels were quantified on serum samples for each study week using enzyme-linked immunosorbent assay (ELISA) microplates according to manufacturer recommendations (StemCell Technologies for EPO; Sigma-Aldrich for AMH).

*Full blood count*

Full blood count was determined on whole blood collected in EDTA tubes using an automated hematology analyzer Sysmex XN-1000 (Sysmex, Japan).

*Evaluation of self-esteem and quality of life*

Self-esteem was assessed before and after testosterone administration using the Rosenberg Self Esteem scale. The health-related quality of life was similarly evaluated using the 36-item short form health survey questionnaire (SF-36) measuring eight scales: physical functioning (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH) (22).

*Body composition measurement*

Body composition assessment was done using the Lunar iDXA® System (GE Medical Systems, Madison, WI, USA), at baseline (during P1) and the week following the end of the treatment (P2), in accordance with published guidelines by the International Society for Clinical Densitometry. This analysis could only be performed for eight subjects. All participants wore paper gowns, and removed jewellery and other personal items capable of interfering with the DXA exam. Participants were placed in a supine position with palms down and arms at sides, slightly separated from the trunk, and correctly centred on the scanning field. Regions of interest (ROIs) were defined by the analytical program and included total body, trunk, head, pelvis, upper limbs, lower limbs, android and gynoid regions. For each region, DXA scanned weight of total mass, bone mineral content, bone areal size and weight (in grams) of bone, fat and lean body mass. Finally, the intravisceral or visceral fat mass (VAT) was assessed using a special algorithm provided by the manufacturer.

*Transgender patients population*

Serum samples from four transgender female-to-male (FTM) patients treated with intra-muscular exogenous testosterone at therapeutic dosage were collected before and one, three, six and twelve months after the start of the treatment. These samples come from biological material collected by health professionals as part of daily clinical activity and stored in the biobank of the Endocrinology, Diabetology and Metabolism Department of the CHUV. These patients gave their consent for the storage of these samples and for their use in research on endocrine diseases.

*Statistical analysis*

Unless otherwise specified, data are expressed as the mean (± standard deviation (SD)). Normality of data was determined with the Shapiro test. In cases of non-normality, non-

263 parametric test was used. One-way repeated ANOVAs followed by post hoc pairwise
264 comparisons (Tukey's honestly significant difference) or Kruskal-Wallis' tests followed by
265 Dunn's multiple comparison test (if normality non respected) were used to test differences
266 between phases and weeks of the study. Correlations were calculated using Spearman's rank
267 order method. A p-value $< 0.05$ was considered statistically significant. Statistical analyses
268 were performed with R software.

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## Results

On 20 screened possible volunteers, 14 subjects were included in the study. The screening failure were mainly due to irregular menstruation, hyperprolactinemia or basal T >2nmol/L. Overall, eight subjects completed the whole study. The six others have incomplete data collection due to COVID-19 lockdown during which study visits were not possible. These missing data corresponded for the majority to P3 of the clinical trial. Nevertheless, 513 samples were collected corresponding to more than 80% of the total expected samples. Clinical characteristics of the 14 women are reported in Table 1.

### *DEXA scan*

The weight of the subjects differed significantly pre- and post-testosterone administration (Table 2). This change was mainly explained by an increase of total tissue mass, corresponding to the association of lean and fat mass. Both lean and fat mass demonstrated a slight increase after T administration although non-significant. In both situations, this was principally caused by an increase of the trunk area.

### *Questionnaires*

The score of the self-esteem scale was in the average range and did not demonstrate significant change after the administration of testosterone (Table 3). The eight scales of the SF-36 Quality of Life did not different before and after testosterone administration.

### *Safety*

A slight increase of acne was reported by four of the 14 women during the treatment phase, which was reversed after treatment cessation. Novel appearances of several hairs were reported by four volunteers, but the hirsutism score did not change significantly. Application of the transdermal gel was well tolerated with no women reporting any skin reaction. No serious AEs

were reported. Since the protocol involved many visits with blood collection procedures in addition to blood loss cause by menses, oral ferric complementation was given to eight subjects to limit the risk of iron deficiency due to low ferritin (<30µg/mL), which could lead to anaemia in the long term. Blood pressure, markers of hepatic function (AST/ALT) and inflammation (CRP) did not exhibit clinically significant deviation and remained constant during the treatment phase and throughout the study period (data not shown).

*Hematology and AMH levels*

Mean AMH level was in the low normal range and was not significantly different between the study phases although exhibiting slightly higher values in the treatment phase (Table 4). The same was observed for serum EPO. With the exception of the reticulocytes percentage (RET%), the main parameters of the full blood count were not influenced by the treatment. RET% was significantly higher in the treatment phase (P2) compared to P3 ($p<0.029$) and was close to significance between P1 and P2 ($p=0.05$). None of the parameters was neither influenced by the phases of the menstrual cycle.

*Menstrual cycle status*

As the subjects recruited for this study were not using hormonal contraception and with regular menstruations, the effect of the administration of testosterone on the menstrual status could be investigated. The main female sex hormones, namely progesterone, estrone and estradiol, demonstrates consistent fluctuations across the three study with no apparent effect of T gel treatment on the menstrual status of the subjects (Figure 2). Moreover, a LH peak during (P2) and after (P3) T gel administration was observed for each subject suggesting ovulation despite exposure to exogenous T (data not shown).

The influence of the menstrual phases on the serum concentration of the quantified steroids was also investigated using data from the control phase (P1). No fluctuation of the phase II steroid

metabolites in function of the menstrual cycle was observed. A significant difference between the menstrual phases was highlighted for testosterone and androstenedione with concentrations significantly higher in the ovulatory phase compared to the follicular phase. However, free T and by extension bioavailable T were not influenced by the menstrual cycle. Both analytes significantly correlated with LH level during phase 1 ($r=0.53$; $p<0.001$ for testosterone and $r=0.5$; $p<0.001$ for androstenedione). The association of testosterone and androstenedione into a T/A4 ratio, as previously proposed doping biomarker of T administration (23), was not influenced by the menstrual phases.

*Serum hormone levels and effect of T administration*

Among the 28 target steroids included in the UHPLC-MS/MS method, signal was not detected or below the LLOQ for etiocholanolone, 21-deoxycortisol, epiandrosterone glucuronide, epitestosterone glucuronide and sulfate and noretiocholanolone sulfate. For epitestosterone, deoxycorticosterone, testosterone glucuronide and sulfate, 57%, 32%, 41% and 21% respectively of the samples were below LLOQ. The median concentration and 95% reference interval of each analyte are presented in Table 5 for each phase of the clinical trial. These reference intervals were determined by combining data from the 15 serum samples per subject collected during each study phase (Table 5).

Testosterone, DHT, free and bioavailable T and FAI all increased significantly in P2 compared to the control phase (P1) and the post-treatment phase (P3), which can be attributed to the administration of exogenous T. By opposition, androstenedione and SHBG had significantly lower values during the treatment phase (P2) compared to P1 and P3. Cortisol and 11-deoxycortisol demonstrated higher concentrations in P3, but the increase could hardly be related to the administration of testosterone. Finally, among the conjugated metabolites, androsterone sulfate and epiandrosterone sulfate were the only ones to have significantly different values

between the three study phases with higher values post-treatment (P3) compared to the control phase (P1).

The fluctuations of affected steroids were then investigated by evaluating serum concentration of these analytes for each week (combination of data from samples collected each week). While androstenedione concentration was significantly affected by the study phase with lower values during testosterone administration (P2), there was no significant difference between the 12 study weeks. On the contrary, SHBG concentration demonstrated significantly lower values during week 6 and 7 in comparison to baseline weeks (week 2 and 3) (Fig. 3A). For testosterone, DHT, androsterone sulfate and epiandrosterone sulfate concentration, significant differences between the study weeks were discovered (Fig. 3). For testosterone and DHT (Fig. 3B and 3C), serum concentrations started to increase directly during the first week of treatment, reached a peak at week 7 corresponding to the third week of treatment, declined slightly during week 8 (last week of treatment) and returned rapidly to baseline level at week 9 and 10. The median testosterone concentration at week 7 was more than 3-fold that of the control phase and more than 2-fold for DHT. All treatment weeks (week 5 to 8) differed significantly from weeks of control phase (week 1 to 4) and post-treatment phase (week 10 to 12) with the exception of week 9 which includes samples collected just after the end of the treatment. Free T (Fig. 3D) and bioavailable T followed the same trend as testosterone concentration.

Concerning both sulfated steroids (Fig. 3E and 3F), while their concentration remained stable before and during the three first weeks of treatment, it started to increase during the fourth week of treatment (week 8), corroborating with the decrease of testosterone concentration, reached a peak at week 9 before gradually returning to baseline levels at week 12. The concentration of androsterone sulfate and epiandrosterone sulfate during week 9 was significantly higher than the control weeks (phase 1) with a 1.7- and 2.23-fold change increase respectively.

*Correlation between androgen hormone levels*

Three different correlation analyses were performed using data from P1 only and from the whole study period (Table 6). A significant correlation was considered to be $P < 0.05$ combined with a $r > 0.45$.

*Correlation between unconjugated androgens*

Testosterone was significantly correlated to androstenedione during P1 (r=0.65) but the correlation was suppressed when the whole study period was investigated due to the large increase of testosterone during the treatment. By opposition, testosterone demonstrated a poor correlation with DHT during P1 but when the whole study period was considered the hormone and its direct metabolite were well correlated (r=0.6). As both compounds mainly originate from adrenal production, DHEA and A4 demonstrated general satisfactory correlation (r=0.6 during P1 and r=0.64 during the whole study). Finally, DHT and androsterone which are consecutive metabolites in the 5α-reductase pathway significantly correlated during the clinical trial (r=0.6).

*Correlation between steroid glucuronides and unconjugated*

Testosterone was moderately correlated with the glucuronide metabolites during the P1, but it was no longer the case when data from the whole study were considered. Androstenedione and DHEA significantly correlated with most of the steroid glucuronides, especially with androsterone and etiocholanolone glucuronide either during P1 or over the whole study period.

*Correlation between steroid sulfates and unconjugated*

Similar to association with glucuronides, testosterone was well correlated with androsterone, epiandrosterone and DHEA sulfates during P1 but the correlation was dissipated when the whole study period was examined. Androstenedione showed acceptable correlation with most of the sulfates during the study while androsterone concentration was closely associated with its sulfated form (r=0.65).

*Correlation among conjugated steroids*

Androsterone and its 5β-epimer eticholanolone highly correlated either in their glucuronidated or sulfated forms (r=0.8) but also demonstrated acceptable correlation between the glucuronide form and the sulfate form ($r > 0.45$). The four conjugated metabolites were also significantly correlated with DHEA sulfate ($r \geq 0.64$). The most robust correlation was observed between androsterone sulfate and its 3β-isomer epiandrosterone sulfate with data of the clinical study (r=0.95).

*Comparison with FTM patients treated with transdermal T*

To further exploit the UHPLC-MS/MS analytical method used in the study and investigate the impact of T administration on the androgen metabolism of women, serum samples from four FTM patients treated with exogenous T over 1 to 12 months were analyzed. Testosterone drastically increased from one to 12 months after the start of the treatment with a ten-fold increase at 12 months (Fig. 4A). Large inter-individual variability was observed at 12 months compared to the previous months. DHT concentration also increased but to a lesser extent with less than a two-fold increase after 12 months (Fig. 4B). Similar to what was observed in the clinical study, a gradual increase in androsterone sulfate and epiandrosterone sulfate concentration was observed over the 12 months of treatment (Fig. 4D and 4E). On the contrary, although a slight increase after three months, androsterone glucuronide concentration remained unchanged after 12 months of testosterone treatment (Fig. 4C). Testosterone concentration changes were significantly correlated with those of DHT (r=0.83; $p<0.001$), androsterone sulfate (r=0.82; $p<0.001$) and epiandrosterone sulfate (r=0.82; $p<0.001$) (Fig. 4F, 4G and 4H). DHT also correlated with androsterone and epiandrosterone sulfate but to a lesser extent (r=0.73; $p<0.001$).

## Discussion

This study allowed investigating the steroidogenic pathways involved in androgen biosynthesis in eumenorrheic women and the impact of transdermal testosterone administration on the regulation of this biosynthesis. The application of a method capable of quantifying a large panel of steroids over three consecutive menstrual cycles further enabled to determine which compounds were detected in serum with the actual analytical performance, to establish the dynamic range and the reference intervals of the detected steroids, and to finally evaluate which hormones were influenced by the fluctuations of the menstrual cycle. Moreover, the influence of testosterone increase induced by exogenous administration on the hormonal profile could be compared to that induced naturally in patients with PCOS.

On the 28 compounds included in the UHPLC-MS/MS method (Fig. 1), a majority (22 analytes) was detected and quantified above LLOQ in serum samples of healthy women subjects highlighting the analytical sensitivity of the method used but also the large spectrum of circulating steroids in women. Using data from the control phase of the clinical study (P1), insights of the human steroid metabolism, particularly in women, could be obtained. We could observe that among the quantified androgen metabolites, some of them circulate predominantly in their unconjugated form such as T or DHT while their metabolites androsterone, etiocholanolone or epiandrosterone are mostly conjugated as already reported in previous studies (24). The preferences for sulfation or glucuronidation could also be highlighted. Epiandrosterone or DHEA were also exclusively present in the sulfated form while androsterone or etiocholanolone were present in high concentration in both conjugated forms. While concentrations of androsterone and etiocholanolone glucuronide were comparable, androsterone sulfate concentration was much higher than its glucuronide form and etiocholanolone sulfate underlining a potential preferred 5α-reductase pathway for the

metabolism of androgen. This finding supports the results of previous studies showing that androsterone sulfate is the most abundant of the 5α-reduced androgen conjugates (25).

The administration of testosterone gel induced a significant increase of serum testosterone over the course of the 4-week treatment period. It progressively increased over the course of the treatment until reaching a peak during the third week of treatment (week 7, median: 3.57 nmol/L) with similar values to that reported in postmenopausal women after 2 weeks of T gel administration at the same dosage (26). It slightly decreased during the last week of treatment (week 8) before returning to baseline after stopping treatment. FAI, free and bioavailable testosterone followed the same trend suggesting that the exogenous testosterone was effective at the tissue level. The administration of exogenous T induced a decreased of SHBG as expected after androgen administration (27). The level of SHBG were logically negatively correlated with free T ($r= -0.53$; $p<0.001$) but not with total testosterone. The median T concentration and FAI during the treatment period crossed the upper limit of normal female range and were close to those of PCOS (28–30). Values were similar to reported in Goldstat *et al.* after 12 weeks of treatment with 10 mg of testosterone 1% cream but lower that those reported by Hirschberg *et al.* after a 10-weeks 10 mg testosterone treatment (9,19). However, the mean T values did not overlap with the normal male range (7.7-29.4 nmol/L) (29,30). FAI demonstrated excellent relation with free T underlining its utility for women not using oral contraceptives with normal SHGB concentration (31). At the basal level during the first menstrual cycle, T and its precursor androstenedione correlated positively ($r=0.65$) but this this relation was dissipated after four weeks of T treatment, because most circulating T was exogenously derived. On the contrary, the two precursors DHEA and androstenedione correlated significantly before and after the administration of T as they remained unaffected. It suggest that exogenous T do not suppress adrenal androgen production and highlights the potential utility of these compounds for the

462 detection of T in the anti-doping context when combined in a ratio with T to increase the
463 specificity for T detection as already proposed (23).

464 The most potent androgen, DHT, also increased following the administration of transdermal T
465 although not to the same extent as T. Due to high levels of 5α-reductase present in the skin, a
466 more extensive increase was expected for DHT compared to T as reported in men administered
467 with transdermal T (21,32). This difference may be explained by a potential dimorphism of the
468 5α-reductase activity in the skin or by a rapid absorption of the gel due to its hydro-alcoholic
469 origin. Furthermore, circulating DHT levels do not necessarily reflect the high amount of DHT
470 produced in tissue with elevated expression of 5α-reductase. While T concentration was rather
471 homogenous among the subjects, DHT concentration was on the contrary variable among
472 subjects with a few outliers highlighting the difference of 5α-reductase activity between the
473 subjects. While DHT and T level exhibited poor relation ($r=0.3$) during the control phase (P1),
474 the correlation was improved when T administration period was taken into account ($r=0.6$,
475 $p<0.001$) due to the direct metabolic connection. The lack of correlation between DHT and T
476 during P1 highlighted possible non-conventional backdoor pathway to DHT in healthy cycling
477 women (33). To further emphasize this hypothesis, DHT strongly correlated with its direct
478 metabolite androsterone before ($r=0.61$, $p<0.001$) and after T administration ($r=0.57$, $p<0.001$).

479 While the inter-individual variability in serum T concentration during the control phase was
480 rather homogenous, large discrepancies between the subjects were observed during the
481 treatment period. Although there is no clear explanation for this high variability, similar
482 findings have already been reported in previous study with postmenopausal women treated with
483 transdermal testosterone (26,34). The factors contributing to this variability have been
484 investigated in hypogonadal men treated with testosterone gel with baseline characteristics
485 accounting for only a small fraction of the variance (35). The authors rather suggested that
486 SHBG and AKR3C1 polymorphisms as possible contributors to variations. The

pharmacokinetics and the bioavailability of T after transdermal T administration may also depend of several factors among which application practice, skin permeation, absorption rate and clearance capacity (36,37). In this study, while the compliance was controlled at each visit by weighing the T vial, variations could occur in case of incomplete piston push generating a slightly lower dose from the vial.

Androgen conjugates have been suggested as markers of androgen activity in women (6) but also as potential biomarkers, in addition to T and DHT, of testosterone administration in the anti-doping context (38). In this study, the principal phase II metabolites of testosterone were also quantified. Among the eleven conjugates quantified, levels of only two compounds differed between the three study phases. The concentration of androsterone and epiandrosterone sulfate started to increase during week 8 and reached a peak at week 9, corresponding to the week following the end of the treatment. This increase occurred in synchronization with the reduction of testosterone level in week 8 and 9. It suggests that the testosterone overload in week 7 stimulates the sulfotransferase activity for the production of sulfated metabolites through the 5α-reductase pathway. Indeed, androsterone and epiandrosterone sulfate have been proposed as alternative to DHT for the assessment of 5α-reductase activity (39). Both metabolites were highly correlated over the three menstrual cycles. Interestingly, epiandrosterone sulfate was also reported as a long-term marker of exogenous testosterone administration in urine (40,41). Here, although the increase is quite modest, it seems that a prolonged administration of transdermal T is necessary to increase both conjugates levels. Besides being the most abundant 5α-reduced androgen metabolite in serum, androsterone sulfate has been proposed as marker of systemic 5α-reduced activity in women (5,25). It was suggested that this metabolite is principally from adrenal origin in hirsute women (25). In our study, androsterone and epiandrosterone sulfate correlated strongly with DHEA sulfate throughout the study corroborating with this hypothesis. Although DHEA sulfate is an androgen precursor and

androsterone sulfate an androgen metabolic product, the correlation between both conjugated products is probably due to similar sulfotransferase activity or similar metabolic clearance(25). The strong correlation between androgen sulfates or between androgen glucuronides but the lower correlation between androgen sulfates and glucuronides support this hypothesis. Moreover, androstenedione, the principal substrate for 5α-reductase in women also correlated with androsterone and DHEA sulfate (42). Finally, androsterone glucuronide, which has been proposed as marker of androgenic activity and peripheral androgen action in women as well as promising biomarker of oral T administration, remained unaffected by exogenous T, such as metabolites from the 5β-reductase pathway. This finding is relatively surprising when considering that AG is the metabolite that account for 93% of the obligatory metabolites of androgen elimination (6). The low dose and the relatively short treatment period were probably not sufficient to cause significant modifications in androgen metabolism.

The inclusion of cycling women in this study allowed studying the fluctuations of steroids during a menstrual cycle but also the influence of exogenous T on the sex female hormones. Except the female sex hormones, testosterone and androstenedione were the only compounds significantly affected by the phases of the menstrual cycle with higher values in the ovulatory phase. This increase is well recognized and results from the LH surge stimulating theca cells to produce androstenedione (43). Accordingly, LH, androstenedione and testosterone were positively correlated during the control phase. The administration of exogenous T did not disturb the menstrual status of the subjects as they continued to ovulate during and after the treatment, and the female sex hormones fluctuations appeared not affected by the increased serum T in the treatment phase. This study also demonstrated that the conversion of exogenous T in E2 through a direct aromatization did not occur as no increase in E2 nor E1 was observed.

In an exploratory approach to further investigate the metabolic response to T administration in women, samples from four transgender patients treated with exogenous T were analyzed. Due

to the higher dose administered, T concentration increase was more important after one month of treatment with values just below the normal male range. The concentration continued to raise gradually over 12 months with values in the male range after one year of treatment although large inter-individual variability was observed. For DHT, while the doses were higher, a similar response to the clinical study with a moderate increase was observed one to twelve months after the start of T treatment. Following an initial increase after one month of treatment, DHT concentration remained relatively constant over 12 months. The other unconjugated remained unchanged with the exception of a decrease of progesterone level. Among the conjugated metabolites, the analysis of these transgender patients confirm the results of the clinical study with an increase observed in androsterone and epiandrosterone sulfates concentrations. Both metabolites demonstrated a duration-dependent effect response with a gradual increase over the first six months of treatment. The response of both sulfated metabolites and of DHT highly correlated with T highlighting the increased 5α-reductase pathway activity. It further confirms that androsterone and epiandrosterone sulfates may be interesting candidate biomarkers of long-term T administration. The analysis of samples from these patients also confirmed the absence of androsterone glucuronide response following exogenous T administration even at higher doses. Androsterone glucuronide was proposed as a marker of peripheral androgenic production, but in this case, most androgens are derived from exogenous T, which did not induce an increase of peripheral androgen production. Furthermore, as opposed to sulfation, glucuronidation is irreversible, ultimately inactivates the androgen, and initiates its renal excretion by increasing polarity and water solubility, which may explain the lack of correlation between androsterone glucuronide and sulfate response.

In addition to the investigation of the steroid metabolism, several additional outcomes were also evaluated for the effect of exogenous T administration. The effect of the treatment on the self-esteem and the quality of life was assessed based on both questionnaires but no influence was

observed. This could be explained either by the low dose of testosterone administered but also by the basal high scores for both items. Furthermore, the absence of a placebo group is a limitation for these outcomes.

The effect of exogenous testosterone administration on body composition was also assessed. In men, the increase of muscle mass and strength was demonstrated to be dose-dependent following T administration (44). In female subjects, the effect of testosterone increase on body composition was investigated in postmenopausal and healthy young women. The studies demonstrated that the increase of total lean mass was related to the change of serum testosterone (19,45). Huang *et al.* estimated a lean body mass increase of 0.6 kg for each 100-ng/dL change in total testosterone while Hirschberg *et al.* obtained a significant change of total muscle mass of 2.0% with a dose resulting in a moderate increase in testosterone level (19,45). In our study, the total tissue mass corresponding to the association of total lean and fat mass increased significantly after the T administration. Although a slight increase was observed, the lean body mass did not change significantly. The mean testosterone concentration during the treatment (median 2.72 nmol/L) was lower than the study of Hirschberg *et al.* (mean 4.3 nmol/L) and the administration was for a shorter period, which could explain the absence of similar effects. For instance, the administration of T in postmenopausal only induced a change in lean body mass at the highest dose associated with supraphysiological levels of circulating testosterone (45). Furthermore, the physical activity was not an inclusion criteria and the absence of training associated with T administration may explain these results. Finally, the administration of testosterone did not significantly alter erythropoiesis nor AMH levels.

The strength of the present study is the robust sampling over three consecutive menstrual cycles corresponding to three distinct study phases. The similar sample collection design between the three phases allowed discriminating between natural within-subject variability (effect of menstrual cycle) and treatment-induced variability of the evaluated biomarkers. As the design

of the study was initially set up to assess the response of selected biomarkers longitudinally and at the individual and not population level, the absence of a placebo-controlled group is a limitation of the present study. Furthermore, the dose and duration of the treatment were low resulting in a moderate testosterone increase that was just in the upper range of the normal female range. Moreover, due to the limited number of FTM patients, the comparison constitutes only preliminary results.

In summary, this study provided additional insights of the steroid metabolism of healthy eumenorrheic women as well as the effect of exogenous T gel administration on their regulation. In addition to testosterone and DHT, androsterone and epiandrosterone sulfate concentration were shown to be influenced by the exogenous T gel administration representing potential supplementary biomarkers. These altered biomarkers could be either used in the clinical context to evaluate the response to exogenous T administration (hypogonadism or FTM patients) or in the anti-doping field for a longitudinal monitoring in the so-called Athlete Biological Passport for the detection of T doping. As sulfo-conjugated androsterone and epiandrosterone are present at high circulating levels, they could also be quantified in dried blood spots for a less invasive and simplified sample collection.

**Acknowledgements**

The study was carried out with financial support from the World Anti-Doping Agency (grant 17D18MS). The authors would like to thank all subjects for participating in the study and are grateful to the research nurses at the Clinical Trial Unit of the CHUV Hospital for sample collection and logistical support.

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## Table Legends

**Table 1.** Baseline characteristics of participants.

**Table 2.** Body composition measurements before and after testosterone administration.

**Table 3.** Scores of the eight items quality of life questionnaire and self-esteem scale measured before and after testosterone administration.

**Table 4.** Mean (SD) of serum AMH and EPO levels and of haematological parameters for each study phase.

**Table 5.** Reference ranges of the quantified steroids according to the study phase. [a]: $p<0.05$ Phase 1 vs Phase 2 ; [b]: $p<0.05$ Phase 2 vs Phase 3 ; [c]: $p<0.05$ Phase 1 vs Phase 3

**Table 6.** Spearman correlation coefficients (*r*) for steroid levels during phase 1 and over the whole study.

## Figure Legends

**Figure 1.** Steroids biosynthesis including compounds quantified with the UHPLC-MS/MS method described in (7)**.**

**Figure 2.** Mean (SD) serum concentration of estradiol, estrone and progesterone during the whole study period. The grey area corresponds to the treatment phase and divides the three study phases.

**Figure 3**. Boxplots of (A) SHGB, (B) testosterone, (C) DHT, (D) free testosterone, (E) androsterone sulfate and (F) epiandrosterone sulfate concentrations according to the study week. The grey area corresponds to the treatment phase and divides the three study phases.*

778 indicates $p<0.05$ compared to week 1, 2, 3, 4, 10, 11 and 12. $^{\#}$ indicates $p<0.05$ compared to
779 week 1, 2 and 4.

780 **Figure 4.** Mean (SD) serum (A) testosterone, (B) DHT, (C) androsterone glucuronide, (D)
781 androsterone sulfate and (E) epiandrosterone sulfate levels over 12 months of testosterone
782 treatment in transgender patients. Correlation between testosterone and (F) DHT, (G)
783 androsterone sulfate and (H) epiandrosterone sulfate.

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**Table 1**

| Subject | Age (years) | Weight (kg) | Height (cm) | BMI (kg/cm$^2$) | Basal T (nmol/L) | Cycle length P1 (days) | Cycle length P2 (days) | Cycle length P3 (days) |
|---|---|---|---|---|---|---|---|---|
| 1 | 28 | 62 | 172 | 20.96 | 1.84 | 27 | 25 | 28 |
| 2 | 22 | 62 | 167 | 22.23 | 1.08 | 36 | 36 | 38 |
| 3 | 28 | 50 | 156 | 20.55 | 0.96 | 33 | 30 | 27 |
| 4 | 34 | 54 | 162 | 20.58 | 1.24 | 27 | 27 | 27 |
| 5 | 25 | 65 | 170 | 22.49 | 1.8 | 32 | 28 | 31 |
| 6 | 31 | 57 | 167 | 20.44 | 0.89 | 26 | 26 | 28 |
| 7 | 25 | 57 | 167 | 20.44 | 0.75 | 27 | 30 | 30 |
| 11 | 27 | 62 | 158 | 24.84 | 1.23 | 38 | 36 | N/A |
| 12 | 30 | 68 | 168 | 24.09 | 0.77 | 26 | 25 | 27 |
| 15 | 22 | 71 | 175 | 23.18 | 0.93 | 29 | 27 | 27 |
| 16 | 24 | 60 | 176 | 19.37 | 0.9 | 32 | 32 | 32 |
| 17 | 26 | 52 | 163 | 19.57 | 1.02 | 24 | 28 | 30 |
| 18 | 37 | 66 | 177 | 21.07 | 1.03 | 28 | 28 | 29 |
| 20 | 33 | 59 | 172 | 19.94 | 1.38 | 28 | 26 | 29 |
| Mean | 28.00 | 60.36 | 167.86 | 21.41 | 1.13 | 29.50 | 28.86 | 29.46 |
| SD | 4.52 | 6.05 | 6.43 | 1.69 | 0.34 | 4.09 | 3.61 | 2.93 |

**Table 2**

| | **Pre-T** | **Post-T** | **Delta** | ***P value*** |
|---|---|---|---|---|
| Weight (kg) | 59.08 (5.05) | 61.16 (5.81) | +2.09 | 0.0087 |
| Total lean mass (g) | 39571.7 (3807.2) | 39796.3 (3838.1) | +224.5 | 0.197 |
| Total fat mass (g) | 17265.2 (5318.2) | 17832.5 (5456.3) | +567.3 | 0.08 |
| Total tissue mass (g) | 56836.9 (4847.6) | 57628.8 (5154.3) | +791.9 | 0.01 |

798 **Table 3**

| **Quality of life (SF36)** | **Pre-T** | **Post-T** | **Delta** |
|---|---|---|---|
| Physical functioning (PF) | 29.71 (0.47) | 29.86 (0.53) | 0.14 |
| Role physical (RP) | 7.57 (1.09) | 7.43 (1.28) | -0.14 |
| Bodily pain (BP) | 3.50 (1.29) | 4.14 (1.46) | 0.64 |
| General health (GH) | 13.57 (1.16) | 14.36 (1.69) | 0.79 |
| Vitality (VT) | 14.0 (1.92) | 14.0 (1.80) | 0.00 |
| Social Functioning (SF) | 6.07 (1.0) | 6.43 (0.76) | 0.36 |
| Role emotional (RE) | 5.50 (1.09) | 5.86 (0.36) | 0.36 |
| Mental health (MH) | 20.21 (1.48) | 20.21 (1.37) | 0.00 |
| Reported health transition (HT) | 2.86 (0.53) | 2.79 (0.43) | -0.07 |
| **Total** | 103.0 (4.91) | 105.07 (3.02) | 2.07 |
| **Self-esteem scale** | 32.93 (4.34) | 33.07 (4.48) | 0.14 |

799

800 **Table 4**

| **Parameter** | **Phase 1** | **Phase 2** | **Phase 3** | ***P value*** |
|---|---|---|---|---|
| AMH (ng/mL) | 0.899 (1.44) | 0.911 (1.48) | 0.71 (0.72) | 0.94 |
| EPO (IU/L) | 6.61 (3.0) | 7.45 (3.80) | 6.71 (2.75) | 0.50 |
| HGB (g/dL) | 13.32 (0.81) | 13.22 (0.75) | 13.53 (0.90) | 0.28 |
| HCT (%) | 40.45 (2.24) | 40.18 (2.10) | 40.95 (2.57) | 0.27 |
| RBC (10^6/µL) | 4.48 (0.25) | 4.44 (0.19) | 4.52 (0.21) | 0.23 |
| RET (%) | 1.60 (0.50) | 1.74 (0.51) | 1.54 (0.45) | **0.03** |
| WBC (10^3/µL) | 6.83 (1.64) | 6.60 (1.69) | 7.25 (1.72) | 0.17 |

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802

803 **Table 5**

| | | | 95% Reference interval | | |
|---|---|---|---|---|---|
| **Compound** | **Study Phase** | **Median** | **2.5th Percentile** | **97.5th Percentile** | ***P* value** |
| Testosterone (nmol/L) | P1 | 1.03 | 0.64 | 1.93 | <0.001[a,b] |
| | P2 | 2.73 | 0.74 | 8.59 | |
| | P3 | 1.16 | 0.56 | 3.29 | |
| Epitestosterone (nmol/L) | P1 | 0.10 | 0.07 | 0.13 | 0.26 |
| | P2 | 0.09 | 0.07 | 0.16 | |
| | P3 | 0.10 | 0.07 | 0.15 | |
| Androstenedione (nmol/L) | P1 | 5.11 | 2.62 | 7.91 | 0.004 [a,b] |
| | P2 | 4.59 | 2.37 | 8.81 | |
| | P3 | 4.97 | 2.72 | 8.44 | |
| Androsterone (nmol/L) | P1 | 1.03 | 0.38 | 3.29 | 0.15 |
| | P2 | 1.12 | 0.46 | 3.31 | |
| | P3 | 1.06 | 0.46 | 3.50 | |
| DHEA (nmol/L) | P1 | 23.53 | 8.97 | 54.63 | 0.093 |
| | P2 | 19.34 | 8.96 | 54.31 | |
| | P3 | 22.01 | 10.37 | 54.39 | |
| DHT (nmol/L) | P1 | 0.45 | 0.20 | 1.58 | <0.001[a,b] |
| | P2 | 0.78 | 0.23 | 2.27 | |
| | P3 | 0.39 | 0.15 | 1.25 | |
| Progesterone (nmol/L) | P1 | 0.94 | 0.12 | 41.84 | 0.54 |
| | P2 | 0.71 | 0.11 | 40.94 | |
| | P3 | 0.94 | 0.13 | 39.95 | |
| 17aOHProgesterone (nmol/L) | P1 | 2.07 | 0.78 | 9.86 | 0.03 |
| | P2 | 1.65 | 0.63 | 7.46 | |
| | P3 | 2.22 | 0.69 | 11.46 | |
| Corticosterone (nmol/L) | P1 | 10.63 | 3.14 | 42.87 | 0.07 |
| | P2 | 9.03 | 2.16 | 36.24 | |
| | P3 | 10.84 | 2.03 | 35.85 | |
| Cortisol (nmol/L) | P1 | 350.57 | 183.18 | 540.85 | 0.006[c] |
| | P2 | 324.32 | 163.84 | 534.40 | |
| | P3 | 351.64 | 166.99 | 530.60 | |
| Deoxycorticosterone (nmol/L) | P1 | 0.15 | 0.08 | 0.34 | 0.22 |
| | P2 | 0.13 | 0.08 | 0.29 | |

| | | | | | |
|---|---|---|---|---|---|
| | P3 | 0.15 | 0.07 | 0.34 | |
| 11-Deoxycortisol (nmol/L) | P1 | 0.73 | 0.18 | 2.07 | 0.002[b] |
| | P2 | 0.61 | 0.12 | 1.85 | |
| | P3 | 0.85 | 0.20 | 2.09 | |
| Testosterone Gluc (nmol/L) | P1 | 0.17 | 0.02 | 0.66 | 0.004[a,b] |
| | P2 | 0.21 | 0.04 | 0.72 | |
| | P3 | 0.17 | 0.02 | 0.55 | |
| Androsterone Gluc (nmol/L) | P1 | 67.26 | 25.03 | 166.21 | 0.087 |
| | P2 | 69.86 | 30.16 | 204.99 | |
| | P3 | 75.96 | 31.13 | 189.74 | |
| Etiocholanolone Gluc (nmol/L) | P1 | 45.71 | 21.49 | 153.25 | 0.75 |
| | P2 | 45.86 | 21.71 | 166.87 | |
| | P3 | 47.57 | 22.28 | 155.26 | |
| 5βαβAdiol-3-Gluc (nmol/L) | P1 | 1.27 | 0.38 | 3.96 | 0.11 |
| | P2 | 1.39 | 0.35 | 3.95 | |
| | P3 | 1.34 | 0.46 | 4.44 | |
| 5βαβAdiol-17-Gluc (nmol/L) | P1 | 3.27 | 0.16 | 12.37 | 0.09 |
| | P2 | 4.06 | 0.23 | 14.88 | |
| | P3 | 2.9 | 0.19 | 15.17 | |
| Testosterone Sulf (nmol/L) | P1 | 0.28 | 0.02 | 1.47 | 0.67 |
| | P2 | 0.30 | 0.02 | 1.36 | |
| | P3 | 0.28 | 0.03 | 0.92 | |
| DHEA Sulf (nmol/L) | P1 | 4381.16 | 1636.89 | 10817.45 | 0.196 |
| | P2 | 4513.44 | 1580.18 | 9997.04 | |
| | P3 | 4549.02 | 1718.29 | 10665.53 | |
| Androsterone Sulf (nmol/L) | P1 | 1248.47 | 260.06 | 3701.85 | 0.019[c] |
| | P2 | 1370.88 | 376.35 | 4780.85 | |
| | P3 | 1756.10 | 316.98 | 5384.13 | |
| Etiocholanolone Sulf (nmol/L) | P1 | 280.67 | 47.18 | 1639.83 | 0.03 |
| | P2 | 262.47 | 42.52 | 1596.07 | |
| | P3 | 358.54 | 81.11 | 1511.74 | |
| EpiAndrosterone Sulf (nmol/L) | P1 | 596.71 | 133.07 | 1848.67 | <0.001[a,c] |
| | P2 | 675.35 | 133.10 | 2041.14 | |
| | P3 | 876.53 | 180.48 | 2385.80 | |
| DehydroAndrosterone Sulf (nmol/L) | P1 | 11.34 | 3.42 | 54.81 | 0.055 |
| | P2 | 13.34 | 3.02 | 53.77 | |
| | P3 | 14.21 | 4.64 | 55.89 | |

| | | | | | |
|---|---|---|---|---|---|
| LH (IU/L) | P1 | 5.20 | 1.75 | 25.11 | 0.11 |
| | P2 | 4.76 | 1.76 | 26.24 | |
| | P3 | 5.14 | 1.76 | 41.10 | |
| FSH (IU/L) | P1 | 5.32 | 1.49 | 10.77 | 0.48 |
| | P2 | 5.18 | 1.74 | 9.74 | |
| | P3 | 4.97 | 1.76 | 10.15 | |
| Estrone (pg/mL) | P1 | 254.84 | 120.43 | 607.66 | 0.22 |
| | P2 | 234.50 | 113.55 | 584.69 | |
| | P3 | 265.57 | 100.53 | 680.78 | |
| Estradiol (pg/mL) | P1 | 288.87 | 92.72 | 1098.11 | 0.17 |
| | P2 | 245.93 | 73.74 | 966.45 | |
| | P3 | 327.41 | 74.66 | 1141.21 | |
| SHBG (nmol/L) | P1 | 55.47 | 17.57 | 104.34 | <0.001[a] |
| | P2 | 48.18 | 13.73 | 83.24 | |
| | P3 | 51.98 | 17.45 | 92.23 | |
| FAI | P1 | 2.02 | 0.82 | 6.13 | <0.001[a,b,c] |
| | P2 | 5.74 | 1.27 | 36.85 | |
| | P3 | 2.45 | 0.87 | 11.15 | |
| Free T (nmol/L) | P1 | 0.014 | 0.007 | 0.034 | <0.001[a,b,c] |
| | P2 | 0.039 | 0.009 | 0.163 | |
| | P3 | 0.016 | 0.007 | 0.064 | |
| BioT (nmol/L) | P1 | 0.32 | 0.16 | 0.80 | <0.001[a,b,c] |
| | P2 | 0.92 | 0.22 | 3.81 | |
| | P3 | 0.38 | 0.17 | 1.50 | |
| T/A4 | P1 | 0.22 | 0.12 | 0.31 | <0.001[a,b] |
| | P2 | 0.52 | 0.19 | 2.02 | |
| | P3 | 0.22 | 0.14 | 0.83 | |

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805 **Table 6**

| Steroids | Testo | A4 | DHT | DHEA | AST | AG | EtioGluc | 5βαβAdiol3Gluc | 5βαβAdiol17Gluc | DHEAS | AS | EtioS | EpiAS | DehydroAS | SHBG | FAI | FreeT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phase 1** | | | | | | | | | | | | | | | | | |
| **Testo** | 1 | 0.65 | 0.3 | 0.27 | 0.53 | 0.45 | 0.52 | 0.51 | 0.2 | 0.52 | 0.53 | 0.38 | 0.54 | 0.38 | 0.12 | 0.5 | 0.68 |
| **A4** | 0.65 | 1 | 0.1 | 0.65 | 0.33 | 0.54 | 0.69 | 0.69 | 0.31 | 0.53 | 0.5 | 0.51 | 0.58 | 0.54 | -0.3 | 0.67 | 0.73 |
| **DHT** | 0.3 | 0.1 | 1 | 0.49 | 0.61 | 0.65 | 0.42 | 0.2 | 0.26 | 0.07 | 0.21 | -0.15 | 0.08 | -0.14 | 0.44 | -0.19 | -0.08 |
| **DHEA** | 0.27 | 0.6 | 0.49 | 1 | 0.5 | 0.65 | 0.63 | 0.42 | 0.25 | 0.25 | 0.37 | 0.27 | 0.33 | 0.27 | -0.1 | 0.26 | 0.29 |
| **AST** | 0.53 | 0.33 | 0.61 | 0.5 | 1 | 0.69 | 0.49 | 0.37 | 0.27 | 0.39 | 0.62 | 0.3 | 0.5 | 0.26 | 0.04 | 0.31 | 0.4 |
| **AG** | 0.45 | 0.54 | 0.65 | 0.65 | 0.69 | 1 | 0.85 | 0.66 | 0.37 | 0.55 | 0.69 | 0.4 | 0.62 | 0.48 | -0.09 | 0.34 | 0.4 |
| **EtioGluc** | 0.52 | 0.69 | 0.42 | 0.63 | 0.49 | 0.85 | 1 | 0.83 | 0.44 | 0.71 | 0.63 | 0.45 | 0.66 | 0.6 | -0.14 | 0.42 | 0.49 |
| **5βαβAdiol3Gluc** | 0.51 | 0.69 | 0.2 | 0.42 | 0.37 | 0.66 | 0.83 | 1 | 0.56 | 0.66 | 0.6 | 0.55 | 0.69 | 0.6 | -0.19 | 0.48 | 0.54 |
| **5βαβAdiol17Gluc** | 0.2 | 0.31 | 0.26 | 0.25 | 0.27 | 0.37 | 0.44 | 0.56 | 1 | 0.11 | 0.1 | -0.04 | 0.12 | 0.05 | -0.08 | 0.16 | 0.19 |
| **DHEAS** | 0.52 | 0.53 | 0.07 | 0.25 | 0.39 | 0.55 | 0.71 | 0.66 | 0.11 | 1 | 0.73 | 0.66 | 0.8 | 0.77 | -0.32 | 0.59 | 0.64 |
| **AS** | 0.53 | 0.5 | 0.21 | 0.37 | 0.62 | 0.69 | 0.63 | 0.6 | 0.1 | 0.73 | 1 | 0.81 | 0.96 | 0.85 | -0.24 | 0.57 | 0.62 |
| **EtioS** | 0.38 | 0.51 | -0.15 | 0.27 | 0.3 | 0.4 | 0.45 | 0.55 | -0.04 | 0.66 | 0.81 | 1 | 0.88 | 0.83 | -0.32 | 0.54 | 0.56 |
| **EpiAS** | 0.54 | 0.58 | 0.08 | 0.33 | 0.5 | 0.62 | 0.66 | 0.69 | 0.12 | 0.8 | 0.96 | 0.88 | 1 | 0.9 | -0.29 | 0.59 | 0.65 |
| **DehydroAS** | 0.38 | 0.54 | -0.14 | 0.27 | 0.26 | 0.48 | 0.6 | 0.6 | 0.05 | 0.77 | 0.85 | 0.83 | 0.9 | 1 | -0.39 | 0.57 | 0.59 |
| **SHBG** | 0.12 | -0.3 | 0.44 | -0.1 | 0.04 | -0.09 | -0.14 | -0.19 | -0.08 | -0.32 | -0.24 | -0.32 | -0.29 | -0.39 | 1 | -0.76 | -0.61 |
| **FAI** | 0.5 | 0.67 | -0.19 | 0.26 | 0.31 | 0.34 | 0.42 | 0.48 | 0.16 | 0.59 | 0.57 | 0.54 | 0.59 | 0.57 | -0.76 | 1 | 0.97 |
| **FreeT** | 0.68 | 0.73 | -0.08 | 0.29 | 0.4 | 0.4 | 0.49 | 0.54 | 0.19 | 0.64 | 0.62 | 0.56 | 0.65 | 0.59 | -0.61 | 0.97 | 1 |
| | | | | | | | | | | | | | | | | | |
| **Whole Study period** | | | | | | | | | | | | | | | | | |
| **Testo** | 1 | 0.31 | 0.6 | 0.16 | 0.46 | 0.4 | 0.38 | 0.34 | 0.19 | 0.37 | 0.37 | 0.19 | 0.4 | 0.31 | -0.09 | 0.76 | 0.87 |
| **A4** | 0.31 | 1 | 0.03 | 0.64 | 0.38 | 0.57 | 0.67 | 0.6 | 0.2 | 0.58 | 0.43 | 0.4 | 0.49 | 0.51 | -0.2 | 0.31 | 0.35 |
| **DHT** | 0.6 | 0.03 | 1 | 0.34 | 0.57 | 0.52 | 0.37 | 0.22 | 0.35 | 0.12 | 0.2 | -0.09 | 0.13 | 0 | 0.17 | 0.32 | 0.42 |
| **DHEA** | 0.16 | 0.64 | 0.34 | 1 | 0.53 | 0.67 | 0.65 | 0.46 | 0.25 | 0.4 | 0.36 | 0.24 | 0.34 | 0.33 | -0.09 | 0.13 | 0.16 |
| **AST** | 0.46 | 0.38 | 0.57 | 0.53 | 1 | 0.71 | 0.58 | 0.47 | 0.37 | 0.48 | 0.65 | 0.39 | 0.54 | 0.39 | -0.09 | 0.38 | 0.43 |
| **AG** | 0.4 | 0.57 | 0.52 | 0.67 | 0.71 | 1 | 0.87 | 0.7 | 0.36 | 0.67 | 0.67 | 0.43 | 0.63 | 0.54 | -0.16 | 0.35 | 0.4 |
| **EtioGluc** | 0.38 | 0.67 | 0.37 | 0.65 | 0.58 | 0.87 | 1 | 0.84 | 0.45 | 0.76 | 0.61 | 0.47 | 0.64 | 0.41 | -0.14 | 0.31 | 0.37 |
| **5βαβAdiol3Gluc** | 0.34 | 0.6 | 0.22 | 0.46 | 0.47 | 0.7 | 0.84 | 1 | 0.53 | 0.69 | 0.57 | 0.53 | 0.64 | 0.56 | -0.09 | 0.28 | 0.33 |
| **5βαβAdiol17Gluc** | 0.19 | 0.2 | 0.35 | 0.25 | 0.37 | 0.36 | 0.45 | 0.53 | 1 | 0.15 | 0.15 | -0.03 | 0.09 | 0.02 | -0.09 | 0.2 | 0.22 |
| **DHEAS** | 0.37 | 0.58 | 0.12 | 0.4 | 0.48 | 0.67 | 0.76 | 0.69 | 0.15 | 1 | 0.72 | 0.64 | 0.78 | 0.79 | -0.27 | 0.39 | 0.42 |
| **AS** | 0.37 | 0.43 | 0.2 | 0.36 | 0.65 | 0.67 | 0.61 | 0.57 | 0.15 | 0.72 | 1 | 0.84 | 0.96 | 0.86 | -0.29 | 0.44 | 0.45 |
| **EtioS** | 0.19 | 0.4 | -0.09 | 0.24 | 0.39 | 0.43 | 0.47 | 0.53 | -0.03 | 0.64 | 0.84 | 1 | 0.87 | 0.8 | -0.2 | 0.25 | 0.26 |
| **EpiAS** | 0.4 | 0.49 | 0.13 | 0.34 | 0.54 | 0.63 | 0.64 | 0.64 | 0.09 | 0.78 | 0.96 | 0.87 | 1 | 0.9 | -0.24 | 0.42 | 0.45 |
| **DehydroAS** | 0.31 | 0.51 | 0 | 0.33 | 0.39 | 0.54 | 0.41 | 0.56 | 0.02 | 0.79 | 0.86 | 0.8 | 0.9 | 1 | -0.37 | 0.42 | 0.42 |
| **SHBG** | -0.09 | -0.2 | 0.17 | -0.09 | -0.09 | -0.16 | -0.14 | -0.09 | -0.09 | -0.27 | -0.29 | -0.2 | -0.24 | -0.37 | 1 | -0.67 | -0.53 |
| **FAI** | 0.76 | 0.31 | 0.32 | 0.13 | 0.38 | 0.35 | 0.31 | 0.28 | 0.2 | 0.39 | 0.44 | 0.25 | 0.42 | 0.42 | -0.67 | 1 | 0.98 |
| **FreeT** | 0.87 | 0.35 | 0.42 | 0.16 | 0.43 | 0.4 | 0.37 | 0.33 | 0.22 | 0.42 | 0.45 | 0.26 | 0.45 | 0.42 | -0.53 | 0.98 | 1 |

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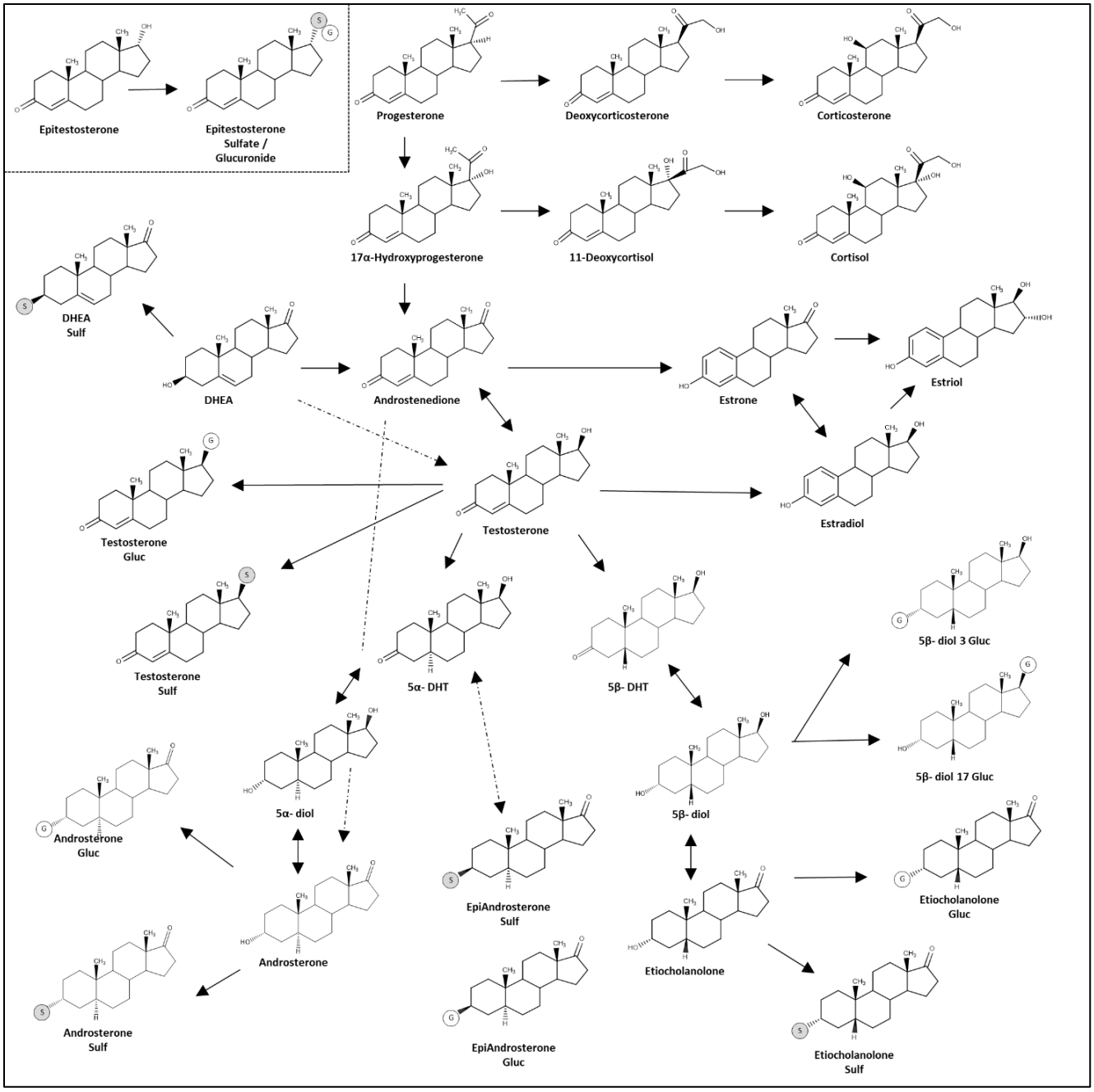
807 **Figure 1**

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809 **Figure 2**

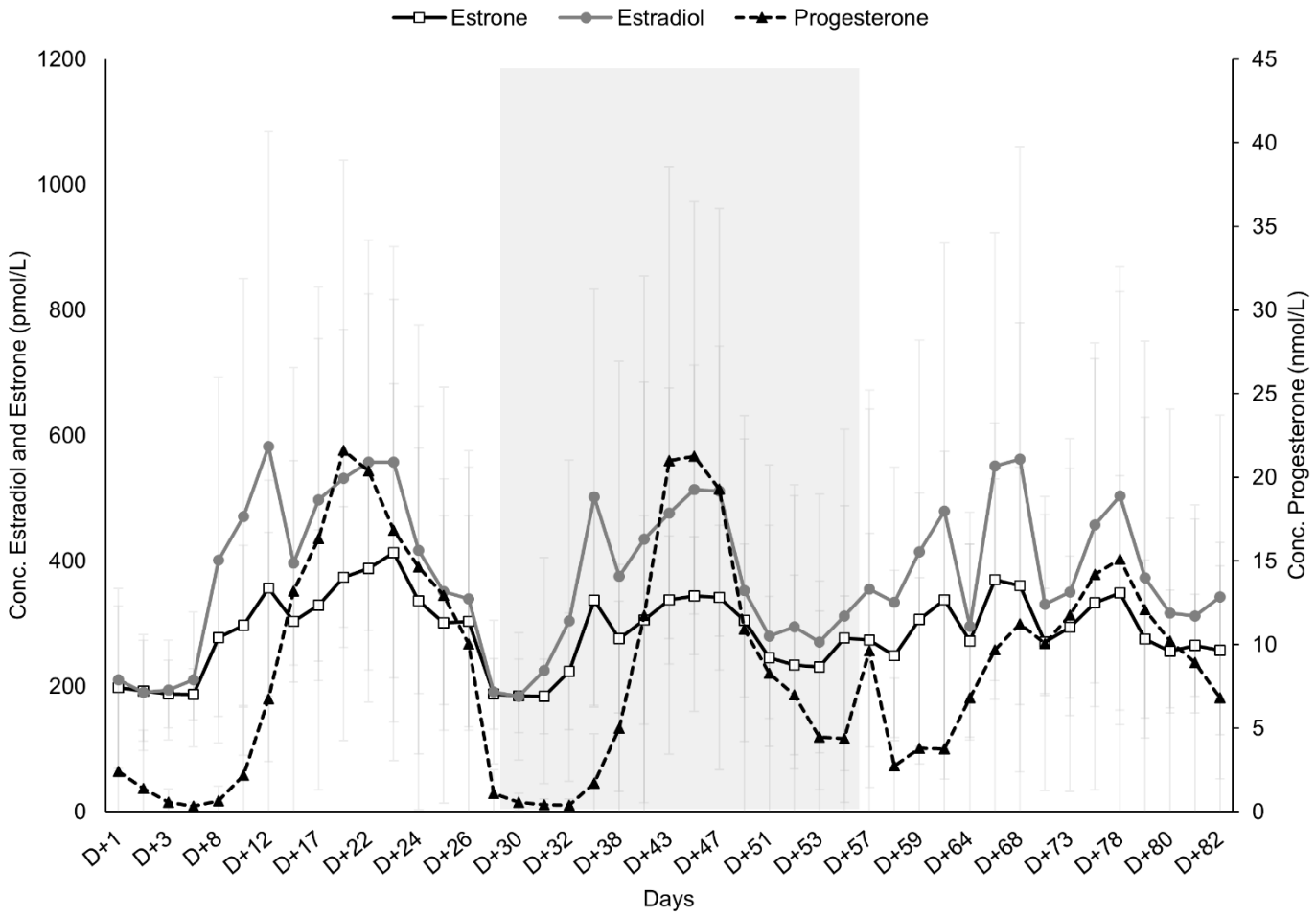


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**Figure 3**

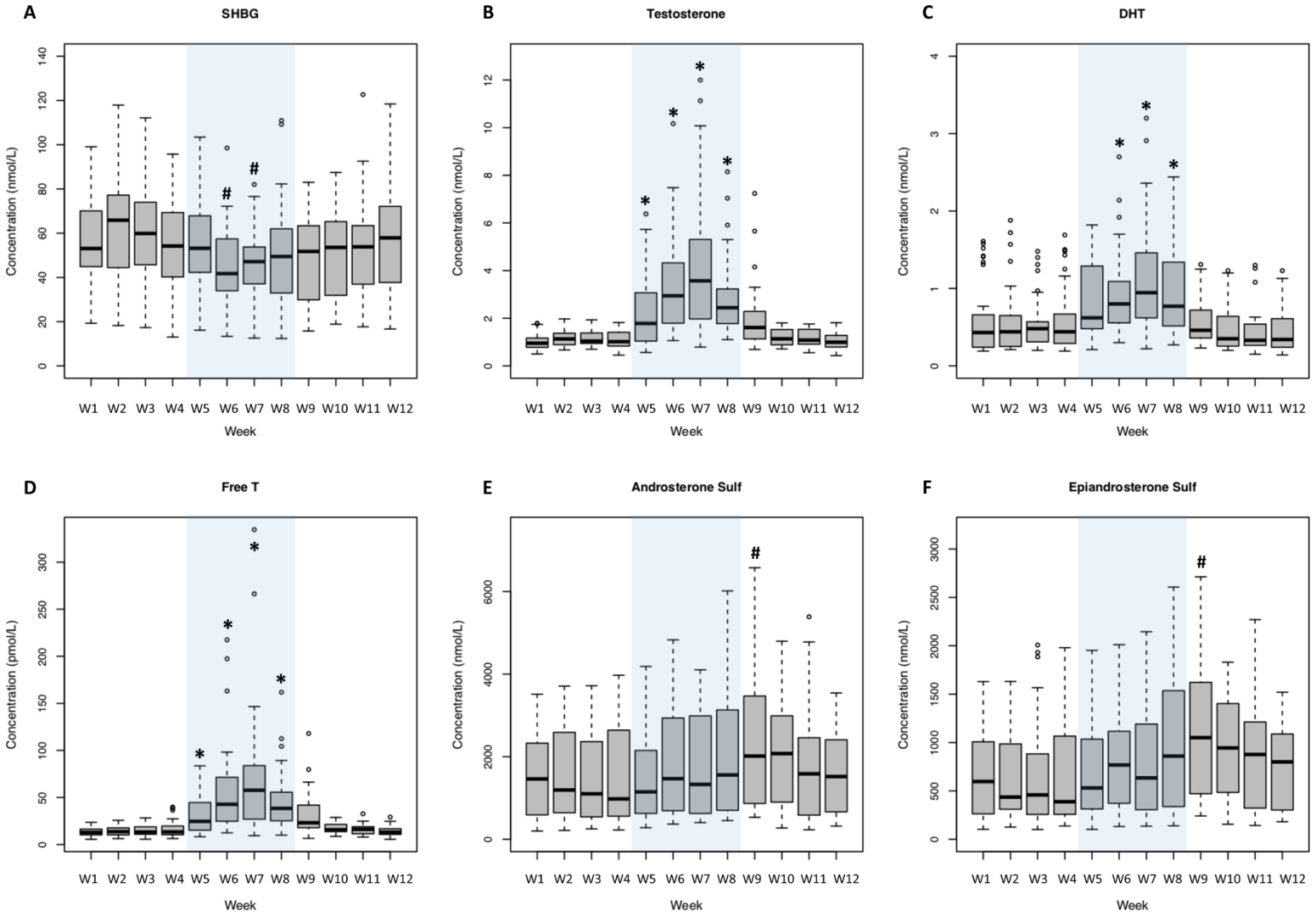
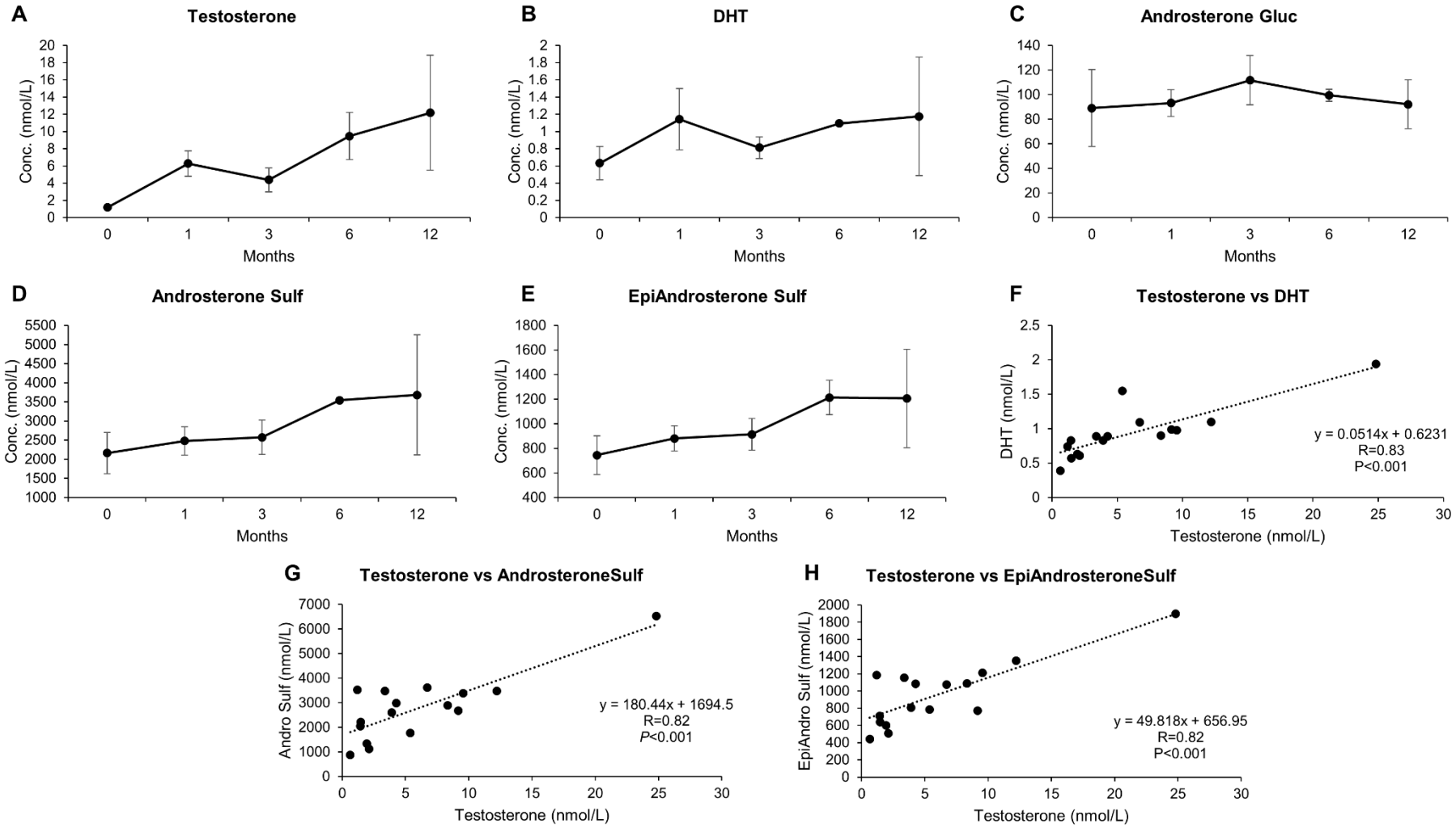

A
SHBG
Concentration (nmol/L)
B
Testosterone
Concentration (nmol/L)
C
DHT
Concentration (nmol/L)
D
Free T
Concentration (pmol/L)
E
Androsterone Sulf
Concentration (nmol/L)
F
Epiandrosterone Sulf
Concentration (nmol/L)
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12
Week

**Figure 4**


A
Testosterone
Conc. (nmol/L)
Months
B
DHT
Conc. (nmol/L)
Months
C
Androsterone Gluc
Conc. (nmol/L)
Months
D
Androsterone Sulf
Conc. (nmol/L)
Months
E
EpiAndrosterone Sulf
Conc. (nmol/L)
Months
F
Testosterone vs DHT
DHT (nmol/L)
y = 0.0514x + 0.6231
R=0.83
P<0.001
Testosterone (nmol/L)
G
Testosterone vs AndrosteroneSulf
Andro Sulf (nmol/L)
y = 180.44x + 1694.5
R=0.82
P<0.001
Testosterone (nmol/L)
H
Testosterone vs EpiAndrosteroneSulf
EpiAndro Sulf (nmol/L)
y = 49.818x + 656.95
R=0.82
P<0.001
Testosterone (nmol/L)

## 3.5 Article 8: Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration (under review in the Journal of Pharmaceutical and Biomedical Analysis)

Similar to the transcriptomic approach, serum steroid profiling requires invasive venous blood collections. Thus, a method for the analysis of endogenous steroids in DBS was developed. The methodology was mainly axed on the sample preparation as the UHPLC-MS/MS conditions were the same as developed and used in Articles 6 and 7.

The number of quantified compounds was reduced using the previous knowledge of detected circulating analytes and the method was validated for quantitative analysis of eleven free and eight conjugated steroids. Following validation, it was applied for the analysis of DBS samples collected weekly during the 'TestoFem' clinical trial and results were compared with those obtained from serum matrix.

To validate the use of DBS in clinical setting and physiological condition, the concentration measured in DBS during the control phase of the clinical study was compared with those measured in serum for each steroid and demonstrated general high agreement for most compounds. To further evaluate the use of DBS in the doping context for the detection of T doping, the samples collected during the treatment period (phase 2) and after (phase 3) were also analyzed. To avoid any potential interference from T residue on the fingers used for gel application, DBS samples generated with EDTA whole blood were also included. Testosterone concentration in capillary DBS exhibited unsatisfactory values in comparison to serum with extremely high values measured for most samples during and after T gel administration, while testosterone concentration measured in DBS generated using whole blood collected in EDTA tubes highly correlated with testosterone concentration in serum. These findings confirmed the hypothesis that residual testosterone remained on the finger skin after application, and that the local remaining testosterone could interfere and generate high concentrations in capillary blood when collected with finger prick. To overcome this potential issue, alternative device (Tasso-M20) was assessed for capillary blood and DBS collection and demonstrated high correlation with finger-prick DBS.

This study therefore highlighted the potential of DBS for monitoring endogenous steroid concentrations and their fluctuation in clinical context of steroid-related disorders, or for the detection of testosterone abuse in anti-doping.

Journal of Pharmaceutical and Biomedical Analysis 204 (2021) 114280

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

# Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration

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ARTICLE INFO

*Article history:*
Received 30 April 2021
Received in revised form 24 June 2021
Accepted 21 July 2021
Available online 26 July 2021

*Keywords:*
Dried blood spots
Steroid profiling
Testosterone gel

ABSTRACT

The quantification of a large panel of endogenous steroids in serum by LC–MS/MS represents a powerful clinical tool for the screening or diagnosis of diverse endocrine disorders. This approach has also demonstrated excellent sensitivity for the detection of testosterone misuse in the anti-doping field, especially in female athlete population. In both situations, the use of dried blood spots (DBS) could provide a viable alternative to invasive venous blood collection. Here, the evaluation of DBS sampling for the quantification of a panel of endogenous steroids using UHPLC-MS/MS is described.

The UHPLC-MS/MS method was validated for quantitative analysis of eleven free and eight conjugated steroids and was then used for the analysis of DBS samples collected in 14 healthy women during a normal menstrual cycle (control phase) followed by a 28-days testosterone gel treatment (treatment phase). Results were compared with those obtained from serum matrix. Satisfactory performance was obtained for all compounds in terms of selectivity, linearity, accuracy, precision, combined uncertainty, stability as well as extraction recovery and matrix effects. In control phase, high correlation was observed between DBS and serum concentrations for most compounds. In treatment phase, higher testosterone concentrations were observed in capillary than in venous DBS, suggesting a possible interference resulting from testosterone contamination on finger(s) used for gel application.

Steroid profiling in capillary DBS represents a simple and efficient strategy for monitoring endogenous steroid concentrations and their fluctuation in clinical context of steroid-related disorders, or for the detection of testosterone abuse in anti-doping.



## 1. Introduction

The diagnosis and the monitoring of various endocrine conditions frequently depend on the quantitative analysis of endogenous steroids in blood matrix. For many years, this analysis was performed using immunoassays allowing high-throughput. However, this technique is subject to cross-reactivity and often limited to the measurement of a single or few compounds instead of a flexible panel of substances. Actually, clinical laboratories rather use liquid chromatography-tandem mass spectrometry (LC–MS/MS) as gold standard method to measure steroid hormones, because it offers the possibility of quantifying multiple analytes at the same time with higher specificity and selectivity [1]. Along further extensions of the panel, e.g. phase II metabolites, LC–MS/MS is indeed a valuable approach for the study of potential alterations in steroidogenesis due to hormonal imbalances, especially in women for whom androgenic activity is not necessarily reflected by serum testosterone level [2,3].

In the anti-doping context, testosterone misuse is currently targeted using an individual and longitudinal monitoring of urinary biomarkers of testosterone in the so-called Athlete Biological Passport (ABP) [4]. In case of abnormal values for one or several of these markers, a time consuming and expensive analysis based on gas chromatography-combustion- isotope ratio MS (GC/C/IRMS) is performed to confirm the potential exogenous origin of testos-

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https://doi.org/10.1016/j.jpba.2021.114280
0731-7085/

terone and its metabolites. While the implementation of this tool improved the testosterone detection capability, various confounding factors may influence the urinary steroid profile complicating its interpretation and decreasing its sensitivity [5–7]. Furthermore, athletes rather resort to low doses of topical testosterone, which significantly reduces peaks of urinary concentrations that are difficult to discriminate from natural variability [7–9].

To overcome these limitations, endogenous steroid profiling in serum has been proposed as a potential complementary approach to the urinary steroid profile for the detection of testosterone misuse by athletes [9,10]. Particularly, the blood matrix is more informative than urine for the correlation between hormone concentration and the physiological responses. The longitudinal monitoring in serum has been proven particularly useful for testosterone detection in female subjects in whom menstrual fluctuations may lead to a great source of variation for urinary biomarkers disrupting their sensitivity [7,9,11].

A primary drawback of the application of serum steroid profiling is that it requires invasive venous blood sampling and sample collection by a trained phlebotomist. Moreover, these biological specimens have to be transported under cooled temperature conditions within a short timeframe, which all increase the total costs of sample collection. Dried blood spots (DBS), which are based on the transfer of a limited volume of capillary blood onto a filter paper or similar matrix, could tackle these obstacles offering a convenient and more affordable alternative. This process benefits from minimal invasiveness, simplicity of sample collection, facilitated transport and storage conditions, and reduced costs that could allow for more frequent sampling for anti-doping programs. The advent of volumetric microsampling technologies has further improved the collection of DBS for quantitative purposes. While DBS have been used for neonatal screening for decades, its applicability has been recently evaluated for SARS–CoV–2 serology assays [12], therapeutic drug monitoring [13] or alcohol abstinence [14]. In the anti-doping context, DBS have been considered as complementary matrix for many years [15–18] and have been investigated for either direct detection of prohibited substances [19–21] or indirect detection through potential biomarkers [22–24]. In particular, a method using volumetric microsampling and GC–MS/MS was recently developed for the quantification of testosterone and eight synthetic anabolic androgenic steroids (AAS) [25]. However, this method was limited to the quantification of only one endogenous AAS (EAAS) and could therefore be hardly applied in the clinical context for the monitoring of steroidogenesis disorders such as polycystic ovary syndrome or congenital adrenal hyperplasia.

In this study, we developed a UHPLC-MS/MS method for the simultaneous determination of eleven free (testosterone, epitestosterone, androstenedione, dehydroepiandrosterone (DHEA), 5α-dihydrotestosterone (DHT), progesterone, 17α-hydroxyprogesterone, cortisol, corticosterone, deoxycorticosterone and 11-deoxycortisol) and eight conjugated (glucuro-conjugated androsterone and etiocholanolone, sulfo-conjugated testosterone, DHEA, androsterone, etiocholanolone, epiandrosterone and dehydroandrosterone) steroids in DBS matrix. Following validation according to World-Anti Doping Agency (WADA) requirements, the method was applied to the analysis of DBS samples collected from healthy eumenorrheic women during a normal menstrual cycle followed by a 28-days T gel treatment and results were compared with those of serum.

## 2. Materials and methods

### 2.1. Chemical & reagents

Methanol (MeOH) was purchased from Macron Fine Chemicals (Deventer, The Netherlands), formic acid (FA; UHPLC/MS, >99 %) and UPLC grade MeOH from Biosolve BV (Valkenswaard, The Netherlands) while ammonium hydroxide ($NH_4OH$; 28–30 %) solution was obtained from Sigma-Aldrich (Buchs, Switzerland). Charcoal Dextran Stripped Human Serum was supplied by Dunn Labortechnick GMbH (Asbach, Germany). Milli Q quality water was obtained from a Milli-Q® grade system (Millipore, MA, USA) and was used for the preparation of LC mobile phases and extraction/reconstitution solutions.

Testosterone (T), androstenedione were purchased from Fluka (Buchs, Switzerland), 11-deoxycortisol, deoxycorticosterone, 17α-hydroxyprogesterone, cortisol, epitestosterone from Sigma-Aldrich (Buchs, Switzerland), dihydrotestosterone, corticosterone, dehydroepiandrosterone (DHEA) from Chemie Brunschwig (Basel, Switzerland), etiocholanolone glucuronide, androsterone sulfate, deyhdroepiandrosterone sulfate (DHEAS), testosterone sulfate, epiandrosterone sulfate from Steraloids (Newport, RI, USA), androsterone glucuronide, etiocholanolone sulfate from LGC Standards (Wesel, Germany) and progesterone from Laboratoire Golaz (Lausanne, Switzerland). Internal labelled standards (IS) were provided by National Measurement Institute (Pymble, Australia).

### 2.2. Sample preparation

#### 2.2.1. Calibration curves and quality control samples

Artificial steroid stripped blood was prepared from whole blood of a healthy volunteer using a modified method reported by Higashi et al. [26]. The whole blood sample was first centrifuged at 1500 × g for 15 min and the separated plasma was discarded. The red blood cells (RBCs) were washed with saline solution, centrifuged and the supernatant was discarded. This procedure was repeated three times and the washed RBCs were then combined with charcoal stripped serum to obtain a hematocrit of 50 %.

Calibration and quality control (QC) samples were then prepared in artificial blood by spiking with reference material of each analyte (Table S1) and 20 μL of the spiked blood was spotted onto Whatman 903™ protein saver cards (GE Healthcare). The cards were dried for a minimum of 1 h at room temperature (RT) and stored at 4 °C unless used for stability study.

#### 2.2.2. DBS samples extraction

For each sample, the whole spot of 20-μL was excised from the DBS card and transferred into a clean 1.5 mL conical polypropylene microcentrifuge tube. One milliliter of methanol/water 95:5 (v/v) containing the internal standard (IS) mixture (Table S2) was added to each tube which was then briefly vortexed and subjected to sonication for 15 min. The supernatant was transferred into a 96-well collection plate equipped with glass inserts and evaporated to dryness under nitrogen stream. The extracts were finally reconstituted in 100 μL of a MeOH/$H_2O$ (50:50, v/v) solution, and after 10 min of gentle shaking, 10 μL of each extract was injected into UHPLC-MS/MS system.

### 2.3. Instrumentation and analytical conditions

An Acquity UPLC I-class (Waters, Milford, USA) equipped with a Kinetex C18 column (150 × 2.1 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) was used for chromatographic separation. The mobile phase A solution was $H_2O$ + 5 mM ammonium formate and mobile phase B was MeOH + 5 mM ammonium formate. Column temperature was set at 60 °C and separation was performed with a flow rate at 300 μL/min, applying the same gradient as described in [27]. MS/MS analysis was carried out using a Xevo-TQ S triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) source operating in polarity switching (positive/negative) mode. Multiple reaction monitoring (MRM) mode was employed using the con-

ditions described in Table S3. MassLynx software version 4.2 was used for data acquisition and TargetLynx for data processing.

### 2.4. Method validation

#### 2.4.1. Selectivity

Selectivity was assessed by the analysis of steroid stripped blood specimens in ten replicates for the presence of interfering signals at the expected retention times of the target analytes.

#### 2.4.2. Calibration curve and linearity

Target analytes were spiked at various concentration levels (Table S1) and calibration curves were established with at least five concentration levels and prepared in three separate analytical series. Linear calibration models were generated for each compound (peak area ratio of each steroid to its respective IS) using a 1/x weighted least-squared regression. The calibration curve was accepted if the accuracy of back-calculated concentration values at each level was less than ±15 % (±20 % at the lower limit of quantification (LLOQ)). The range was considered to be linear if the determination coefficient was greater than 0.99 and in the absence of systematic pattern in the residuals.

#### 2.4.3. Bias, precision, uncertainty and LLOQ

To assess the precision and accuracy of the assay, QC fortified at four concentration levels (LLOQ, Low, Mid, High) of the target analytes were analyzed in six replicates and on three separate analytical series. In accordance with the WADA technical document TD2019DL [28], combined measurement uncertainty ($u_c$) was also assessed by quadratic combinations of the intermediate precision and the root mean square of the bias estimates. A pre-defined uncertainty acceptance criterion was set at 20 % of the mean result at each concentration level of the QCs. LLOQ was determined as the concentration at which uncertainty was below 20 %.

#### 2.4.4. Extraction recovery and matrix effects

Extraction recovery was calculated as the ratio of the peak areas of the analyte from the pre-extracted spiked and post-extracted spiked DBS samples prepared in triplicate. Matrix effects were investigated by comparing the peak areas of the analytes in post-extracted spiked samples to peak areas of the corresponding reference standard. For each analyte, the concentration corresponding to the calibration solution 6 was used for these analyses (Supplemental Table 1).

#### 2.4.5. Stability

DBS samples from a volunteer were collected from finger prick (20 μL onto Whatman 903™ protein saver cards), loaded into a foil pouch with a silica gel desiccant, and stored at three different storage conditions (RT, 4 °C and −20 °C) for 1 and 3 weeks. For each condition, three spots were used and extracted. The post-extraction stability of extracts was also investigated after one week of storage in the autosampler at approximately 10 °C.

### 2.5. Clinical samples description

#### 2.5.1. Testosterone gel study

Fourteen healthy female volunteers participated to an open-label trial. All subjects provided written informed consent prior to any study procedures and the trial was approved by the local Ethical Committee of the Canton de Vaud in Switzerland (2018−02106, SNCTP000003264) and Swissmedic (2018DR1168), registered on www.isrctn.com (ISRCTN10122130) and was conducted in accordance with the Declaration of Helsinki as described previously [7]. Briefly, the study was divided into three 4-weeks phases corresponding to three consecutive menstrual cycles for a total of 12 weeks. Phase 1 corresponded to the control phase during which samples were collected once a week. Phase 2 corresponded to the treatment during which 0.5 g testosterone gel (Tostran® 20 mg/g) was self-administered every morning on the upper thigh and/or abdomen for 28 days. Samples were collected before the application of testosterone gel, which corresponded approximately to 24 h post-application, and with the sampling scheme similar to phase 1. Phase 3 was similar to phase 1 and corresponded to the post-treatment phase.

Serum samples were collected from antecubital vein in 8.5 mL BD Vacutainer® SST™ II Advance tubes and whole blood in 4 mL BD Vacutainer® K2EDTA tubes. DBS samples were generated either using volumetric 10-μL microsampling HemaXis DB10 kits (DBS System SA, Gland, Switzerland) from finger-prick and by depositing 20 μL of EDTA whole blood onto cards (Whatman 903™ protein saver cards), which were left to dry for a minimum of 1 h at RT and stored at 4 °C with a desiccant. Hematological variables including hematocrit (HCT) were measured in whole blood samples using a fully automated hematology analyzer (Sysmex XN-1000, Sysmex).

For the analysis of DBS generated with HemaXis DB10, two 10-μL spots were used for the quantification of steroids with the method described above. Serum samples were extracted and analyzed using a method reported earlier [27] and results were compared with those measured in the DBS and corrected with HCT level using the following equation: Corrected concentration = (DBS concentration)/(1-HCT). Comparison of the steroid concentrations obtained with the two methods was performed using the Passing-Bablok regression and correlations were calculated using Spearman's correlation method with R Studio software.

#### 2.5.2. Comparison between finger prick and Tasso-M20 DBS

To assess the efficiency of the Tasso-M20 push-button blood collection device (Tasso Inc, WA, USA) for the measurement of the steroid profile, capillary blood was collected from fourteen healthy volunteers (seven males and seven females) using both finger-pricks and Tasso-M20 devices positioned on the upper arm. Blood samples from finger-pricks (20 μL) were deposited onto filter paper (Whatman 903™ protein saver cards), left to dry for at least 1 h, and then stored at room temperature with desiccant. The dried Tasso volumetric 20-μL tips samples were stored under ambient conditions with desiccant until analysis. The tips were analyzed as described previously for the DBS. The correlation between finger-prick DBS and Tasso-M20 DBS was calculated using Spearman's correlation method with R Studio software.

## 3. Results

### 3.1. Method validation

Quantitative performance of the method and the summary of validation results are described in Table 1(complete set of data for each QC level are shown in Table S4). Regarding selectivity, no interfering signals were observed for the ten replicates of negative blood samples at the expected retention times of the analytes, owing to the adequate chromatographic separation in conjunction with the two structure specific MRM transitions.

The linearity range was determined for each compound and the accuracies of the back-calculated concentrations at each calibration level met the predefined criteria (less than ±15 % and less than ±20 % bias at LLOQ). The method demonstrated satisfactory accuracy, expressed in terms of bias, and precision at each QC level (Table S4 at each QC concentration). The relative bias did not exceed 10 % for any QCs. The precision, represented by repeatability and intermediate precision, was below 10 % with the exception of the lowest QC for glucuro-conjugated androsterone and eti-

**Table 1**
Summary of validation results. RT: retention time; LLOQ: lower limit of quantification.

| Analyte | RT (min) | Bias (%) | Repeatability (%) | Intermediate Precision (%) | Combined Uncertainty (%) | Linearity range (ng/mL) | LLOQ (pg/mL) | Recovery (%) | Matrix effect (%) |
|---|---|---|---|---|---|---|---|---|---|
| Testosterone | 9.81 | 2.6–3.9 | 2.1–3.3 | 2.1–4.6 | 3.6–6.5 | 0.02–25 | 20 | 110 | 95 |
| Epitestosterone | 11.5 | 2.4–3.8 | 1.5–3.2 | 2.6–3.2 | 4.1–5.2 | 0.05–10 | 50 | 110 | 99 |
| Androstenedione | 8.74 | 2.5–6.2 | 1.3–2.3 | 1.6–3.6 | 3.5–7.3 | 0.1–25 | 100 | 108 | 93 |
| DHEA | 10.61 | 2.1–3.6 | 2.3–2.9 | 2.6–3.9 | 3.7–5.7 | 2.5–150 | 2500 | 111 | 92 |
| DHT | 12.04 | 1.9–2.6 | 1.8–2.8 | 1.9–2.8 | 3.4–4.1 | 0.25–10 | 250 | 115 | 91 |
| Progesterone | 13.42 | 2.1–4.7 | 2.1–2.8 | 2.1–3.2 | 3.1–6.0 | 0.025–25 | 25 | 112 | 93 |
| 17α-hydroxyprogesterone | 10.42 | 2.2–3.7 | 1.7–3.1 | 2.2–3.9 | 3.4–5.4 | 0.1–25 | 100 | 112 | 97 |
| Cortisol | 5.07 | 3.5–5.2 | 1.5–5.0 | 1.6–5.5 | 4.7–9.0 | 1–400 | 1000 | 110 | 97 |
| Corticosterone | 6.97 | 3.5–4.6 | 1.5–4.9 | 1.8–4.9 | 4.7–7.5 | 0.25–100 | 250 | 112 | 92 |
| Deoxycorticosterone | 9.45 | 2.6–5.9 | 1.9–2.9 | 2.4–4.1 | 4.4–6.6 | 0.1–10 | 100 | 112 | 92 |
| 11-Deoxycortisol | 7.36 | 2.2–3.6 | 2.2–3.0 | 2.2–4.5 | 3.6–6.1 | 0.1–5 | 100 | 110 | 90 |
| Androsterone Glucuronide | 9.61 | 3.7–8.6 | 3.6–7.2 | 4.8–11.8 | 6.5–16.0 | 1–100 | 1000 | 67 | 100 |
| Etiocholanolone Glucuronide | 8.99 | 3.4–9.6 | 4.8–13.3 | 4.8–14.1 | 6.7–20.4 | 1–100 | 1000 | 74 | 105 |
| Testosterone Sulfate | 5.29 | 2.8–3.4 | 2.6–3.6 | 3.5–3.9 | 5.0–5.8 | 0.25–25 | 250 | 84 | 96 |
| Androsterone Sulfate | 8.18 | 2.4–5.6 | 2.1–3.0 | 2.8–3.4 | 4.2–6.9 | 5–2500 | 5000 | 82 | 95 |
| Etiocholanolone Sulfate | 7.89 | 2.7–4.8 | 1.9–3.7 | 1.9–5.8 | 4.5–7.8 | 5–2500 | 5000 | 84 | 90 |
| DHEA Sulfate | 5.95 | 2.7–6.3 | 2.3–5.0 | 2.9–5.8 | 4.4–8.3 | 50–10000 | 50000 | 82 | 96 |
| Dehydroandrosterone Sulfate | 6.92 | 2.4–7.3 | 2.5–6.1 | 3.0–6.4 | 4.4–11.0 | 0.25–100 | 250 | 80 | 95 |
| Epiandrosterone Sulfate | 6.7 | 4.4–9.4 | 3.9–8.2 | 5.0–8.2 | 7.6–13.9 | 0.5–2500 | 5000 | 80 | 96 |

ocholanolone. Nevertheless, at this concentration (1 ng/mL), the precision was considered acceptable as the results were still below 15 %. Combined uncertainty, representing statistical dispersion of the values attributed to a measured quantity, was also assessed in agreement with WADA regulations (TD2019DL), and the estimates were all below 20 % as a predefined uncertainty acceptance criterion except for the lowest QC of etiocholanolone glucuronide with an uncertainty estimate of 20.4 %.

Recovery was excellent for all unconjugated steroids (108–115 %), while it demonstrated lower but still acceptable results for sulfate (80–84 %) and glucuronide (67–74 %) conjugated metabolites. No significant matrix effect was observed for any analyte. The carryover was negligible with all traces below 0.2 %.

Concerning the stability of the analytes in DBS, no significant difference was observed between the storage conditions (RT, 4 °C and −20 °C) for any steroid, except for progesterone. After 3 weeks of storage, progesterone level was significantly lower when stored at −20 °C compared to RT (data not shown). Extracts stored in the autosampler for one week demonstrated similar results to fresh extracts.

### 3.2. Analysis of DBS from clinical study

To validate the use of DBS in clinical setting and physiological condition, the hematocrit-adjusted concentration measured in DBS during the control phase (phase 1) of the clinical study was compared with those measured in serum for each steroid. Epitestosterone, DHT, deoxycorticosterone, 11-deoxycortisol and testosterone sulfate were below LLOQ or could not be detected and were thus not included for the further comparison. Where the reference could be made to serum concentrations, Passing-Bablok regression was assessed for each compound and the plots are presented in Fig. 1. The slope, intercept and their confidence intervals of 95 % (95 % CI) as well as the correlation coefficient were evaluated. All compounds demonstrated a correlation coefficient higher than 0.8 and a slope between 0.69 and 1.13. Exempting testosterone, androstenedione, cortisol, androsterone sulfate and etiocholanolone glucuronide, a positive intercept was obtained for steroid concentrations, suggesting a slight overestimation in DBS.

To further evaluate the use of DBS in the doping context for the detection of testosterone doping, the samples collected during the treatment period (phase 2) and after (phase 3) were also analyzed. To avoid any potential interference from testosterone residue on the fingers used for gel application, DBS samples generated with EDTA whole blood were also included and analyzed. Testosterone concentration in capillary DBS exhibited unsatisfactory values in comparison to serum with extremely high values measured for most samples during and after T gel administration (Fig. 2A), while testosterone concentration measured in DBS generated using whole blood collected in EDTA tubes highly correlated with testosterone concentration in serum (Fig. 2B). Notably, both matrices demonstrated a similar trend when mean concentrations were plotted longitudinally over the three study phases (Fig. 2C). The other compounds demonstrated similar results to phase 1 alone (Fig. S1).

### 3.3. Comparison between finger prick and Tasso device DBS

Fourteen individuals were recruited for the comparison between regular finger-prick or Tasso-M20 push-button device for the analysis of steroid concentrations in DBS. All quantified steroids demonstrated strong correlation between both collection methods. The results are presented for six free steroids (Fig. 3) and the comparison for the other compounds are available in Fig. S2.

## 4. Discussion

The method described in this study demonstrated reliable quantitative performance for the profiling of a panel of steroids in DBS. As the chromatographic separation and MS/MS conditions used in this study were already optimized for the analysis of serum samples [27], the emphasis was put on the processing of DBS samples. Based on validation data, combined uncertainty was assessed for each analyte in compliance with the WADA requirements for quantitative methods, consisting of the intermediate precision and bias components. For most compounds, uncertainty was estimated below the predefined acceptance criterion of ±20 %. In the case of etiocholanolone glucuronide, the combined uncertainty was 20.4 %

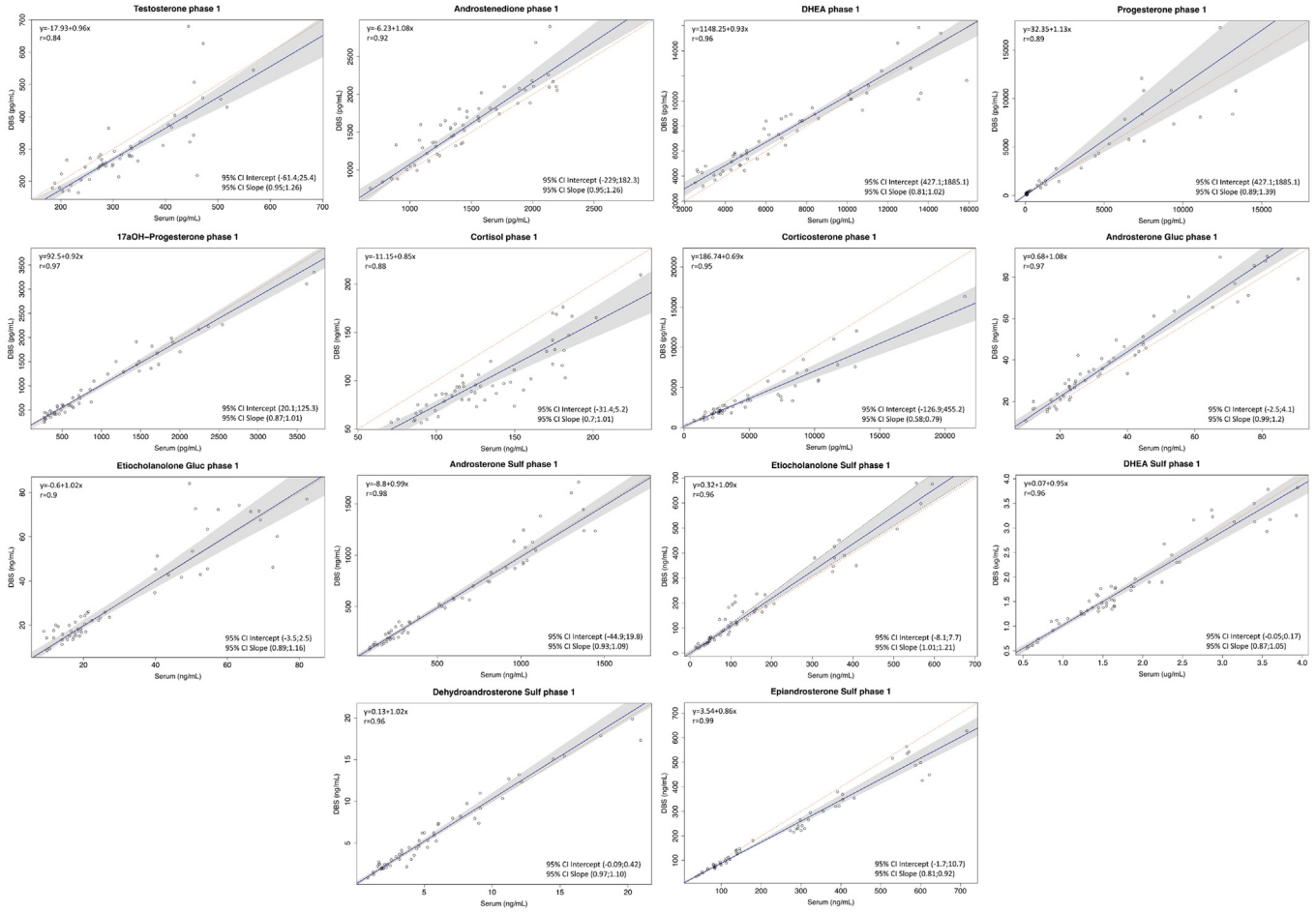


**Fig. 1.** Passing-Bablok regression plots for steroids quantification in serum vs DBS collected during phase 1. Blue line indicates regression line, red dashed line indicates identity line, and the confidence bands for regression are delimitated in grey. R is the correlation coefficient and 95 % CI corresponds to the 95 % confidence interval.

at the lowest QC level, which could be related to the low ionization efficiency of glucuronide-conjugated species in the negative mode. Nevertheless, as the result was still very close to the predefined acceptance criterion, it was considered fit for purpose.

The major challenge when developing a DBS method for steroid analysis is to reach a sufficient sensitivity for the target compounds that may be present in low circulating levels, especially in women, and to cover large and various ranges of concentration. The sensitivity of this developed method was satisfactory for the majority of the critical compounds, such as testosterone or progesterone (Fig. S3). DHT, a potential biomarker of testosterone administration, was the only compound for which the current sensitivity (LLOQ at 250 pg/mL) may be inadequate for the analysis of DBS samples of female athletes, which is in part due to its mass spectrometric properties. Furthermore, slightly higher uncertainty estimations were obtained at low concentration for the glucuro-conjugated species, but as they generally circulate at high concentrations in blood, no true sensitivity issues were encountered [29]. Finally, the concentration of the steroids panel remained generally stable when stored for 1–3 weeks either at RT, 4 °C or −20 °C. It highlights that, contrary to serum samples usually used for the steroid profiling, DBS cards can be transported and/or stored at RT with a minimal risk of degradation.

To first evaluate the applicability of the developed method in physiological condition, capillary DBS collected over 4 weeks during a clinical study involving healthy eumenorrheic women were analyzed and concentration values were compared to corresponding serum samples. For most compounds, satisfactory correlation ($r>0.84$) was discovered between DBS and serum. Passing-Bablok regression based on robust, non-parametric model was used to evaluate analytical methods agreement. The model is based on the hypothesis that if the 95 % CI for intercept includes value zero and 95 % CI for slope includes value 1, there is no constant (intercept) nor proportional (slope) difference between the two methods. These criteria were met for testosterone, androstenedione, cortisol and all conjugated metabolites (with the exception of epiandrosterone sulfate). For DHEA, 17α−OH-progesterone and progesterone, 95 % CI for intercepts did not include 0, indicating a constant difference between the two methods. It is mainly due to higher values measured in DBS at low concentration for these compounds, which could be partly explained by better recovery from DBS. For epiandrosterone sulfate, although upper limit of 95 % CI for slope was close to 1 (0.92), the two methods had a slight proportional difference with higher concentrations measured in serum. Corticosterone also demonstrated a proportional between both methods with consistent higher values in serum suggesting that both methods should not be used simultaneously. Overall, we can conclude that results obtained from both methods were in high agreement and that measured concentrations in DBS can be compared with those of serum for most compounds. These results also emphasize the necessity to adjust concentration measured in DBS with HCT [30]. Furthermore, these samples were stored for more than one year with desiccant before their analysis underlining the analytes' stability in DBS.

Longitudinal monitoring of testosterone in serum has been proposed as powerful approach for the detection of testosterone

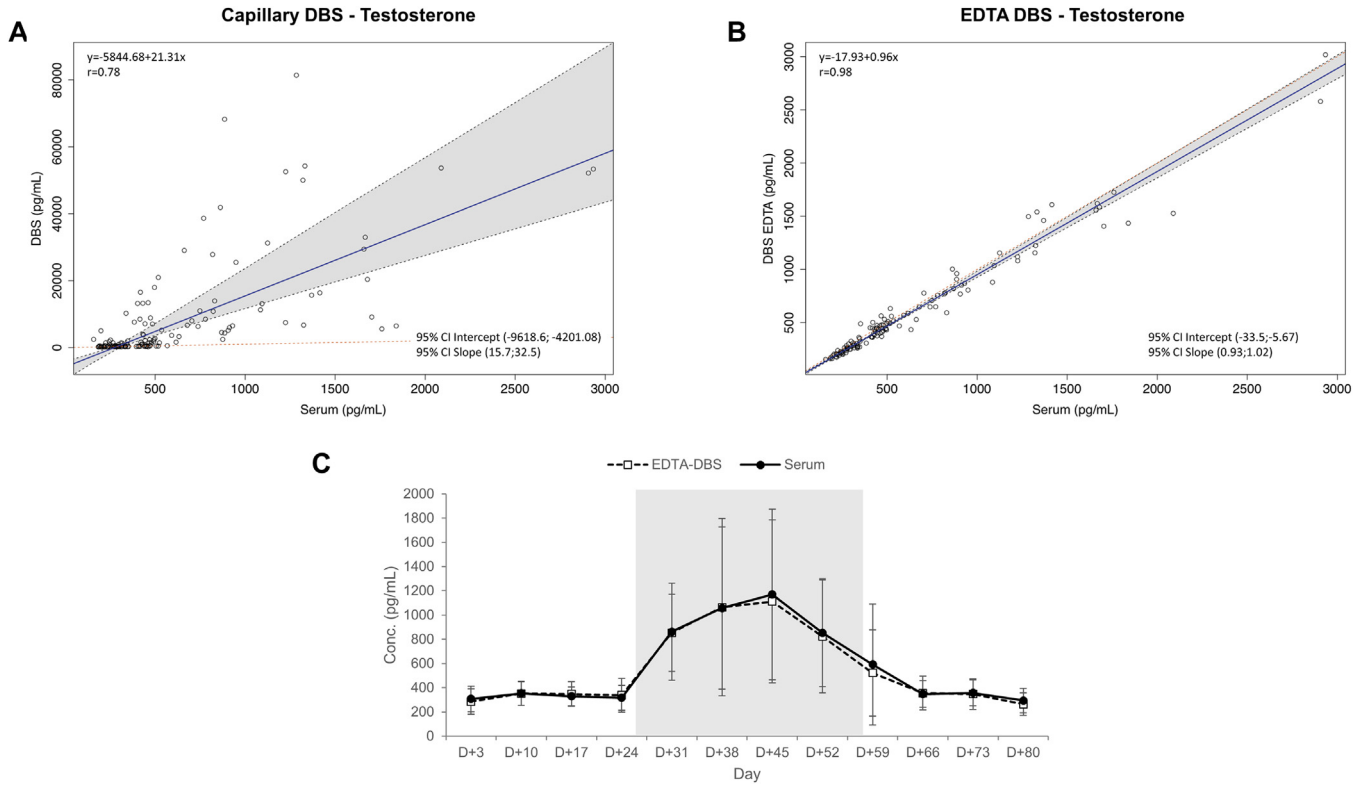


**Fig. 2.** Passing-Bablok regression plots of testosterone concentration values between serum and (A) capillary DBS and (B) EDTA-DBS during the whole study. Blue line indicates regression line, red dashed line indicate identity line, and the confidence bands for regression are delimitated in grey. R is the correlation coefficient and 95 % CI corresponds to the 95 % confidence interval. (C) Mean plot of testosterone concentration measured in EDTA-DBS and serum throughout the study. The grey area corresponds to the treatment phase and divides the three study phases.

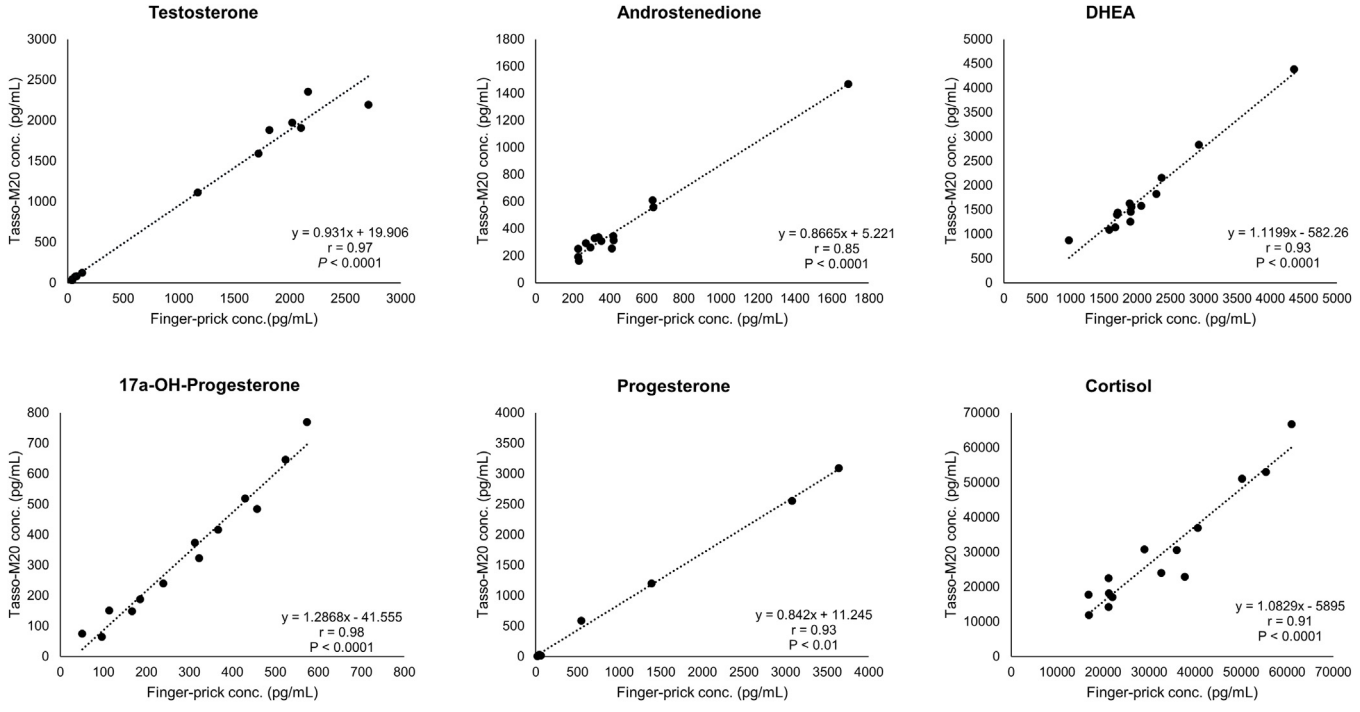


**Fig. 3.** Correlations between concentration levels of six free steroids in finger-prick vs Tasso-M20 DBS samples.

administration in women [7]. Therefore, to evaluate the applicability of DBS in the context of anti-doping, DBS samples from the two following phases (testosterone gel administration – phase 2 and wash-out – phase 3) of the clinical study were also processed. Related to the use of topical testosterone formulation, a previous study showed that considerable amounts of testosterone (60 % after 8 h) remained on the intact skin for several hours after testosterone gel application and evaporation of the alcohol

vehicle [31]. Thus, to avoid any potential interferences from testosterone residue on fingers used for gel application, DBS samples generated with EDTA tubes collected at the same time and representing rather systemic testosterone concentration were also analyzed with the developed method. While testosterone levels demonstrated high concordance between EDTA-DBS and serum, capillary DBS displayed some extreme values during and after testosterone administration. It seemed that the testosterone values increased as the treatment progressed suggesting a potential accumulation. Indeed, following its application, the gel dried and the steroid is absorbed into the stratum corneum, which acts as a reservoir before slowly releasing testosterone into the circulation [32]. On the contrary, mean testosterone concentration exhibited similar results between EDTA-DBS and serum when data were longitudinally plotted. Furthermore, the other analytes concentrations demonstrated satisfactory correlation with the serum matrix during and after the treatment period. These findings confirm the hypothesis that testosterone is indeed persisting in the finger skin, and that the local residual testosterone could interfere and generate high concentrations in capillary blood when collected with finger prick. This observation could also partly explain the low bioavailability of testosterone gel formulation. Although this hypothesis is highly likely, we emphasize that the risk of interpersonal contamination is very low as reported in [31], especially if the site of application is covered or washed [33]. We point out that the alcoholic testosterone gel, which is in contact with the application hand, remains in the finger skin and generates only locally high capillary testosterone concentration. Therefore, a high capillary testosterone concentration in DBS is highly likely to reflect a direct contact with exogenous testosterone gel. Nevertheless, further studies should be carried out to confirm this hypothesis by combining testosterone administration and multiple capillary blood collection sites.

To overcome this potential pitfall, the Tasso-M20 push button device was also evaluated as alternative collection method. This device allows collecting capillary blood on the upper arm using volumetric absorptive microsampling (Fig. S4). We demonstrated that steroid concentrations were comparable between finger-prick DBS and Tasso DBS collected from the fingers and arms of fourteen individuals, thereby demonstrating the independence from spot support and sample collection site. While this approach might be beneficial in this context, it could also generate similar issues if testosterone gel is administered on the upper arm, at the same location as the Tasso-M20.

## 5. Conclusion

In summary, a fit-for-purpose UHPLC-MS/MS method was developed and validated for the quantification of a panel of steroids in DBS. This method could be applied to anti-doping as a complementary approach for the longitudinal monitoring of steroid profile and detection of testosterone administration in the ABP allowing for more frequent sampling and for targeting blood (serum) and urine sample collection that would be used for a full steroid profile and for confirmatory GC/C/IRMS analysis. The increased sampling frequency would provide a better estimation of natural baseline variability of a given athlete and would provide a better resolution of a possible doping picture [34]. This approach could also be employed for the monitoring of steroid-related pathologies in the clinical context. Indeed, for patients requiring regular medical visits or for whom venipuncture is complicated (neonates, elderly patients), DBS collected with Tasso-M20 or finger prick with HemaXis DB10 at home or on-site could be a valuable alternative to classical serum collection.

## CRediT authorship contribution statement

**Olivier Salamin:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Raul Nicoli:** Methodology, Validation, Writing - review & editing. **Cheng Xu:** Investigation, Writing - review & editing. **Julien Boccard:** Validation, Writing - review & editing. **Serge Rudaz:** Validation, Writing - review & editing. **Nelly Pitteloud:** Resources, Supervision, Project administration. **Martial Saugy:** Supervision, Project administration, Funding acquisition. **Tiia Kuuranne:** Resources, Supervision, Funding acquisition, Writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The study was carried out with financial support from the World Anti-Doping Agency. The authors would like to thank all subjects for participating in the study and are grateful to the research nurses at the Clinical Trial Unit of the CHUV Hospital for sample collection and logistical support.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114280.

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# Supplementary data

# Steroid profiling by UHPLC-MS/MS in dried blood spots collected from healthy women with and without testosterone gel administration

## Table of contents

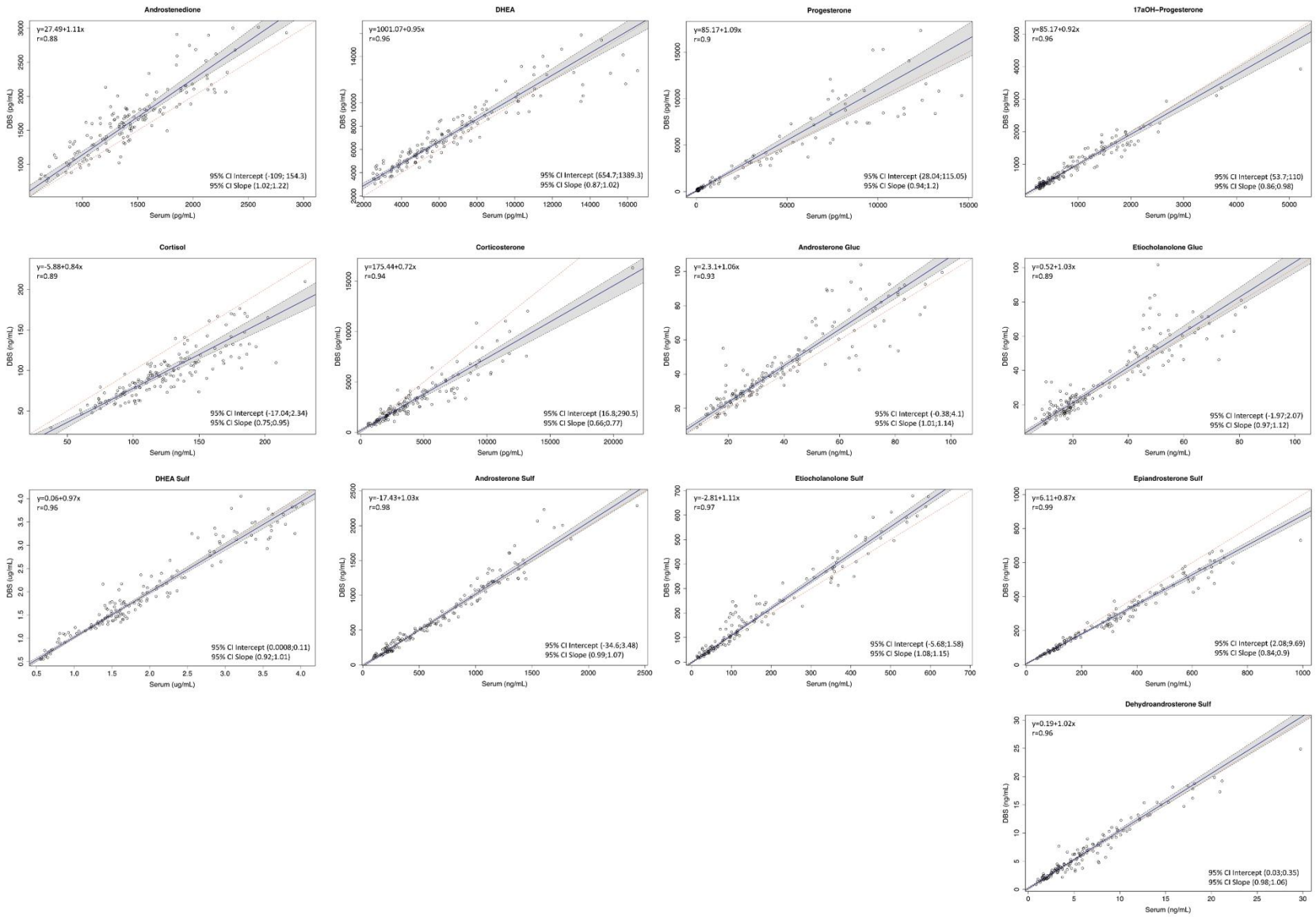



**Figure S1**. Passing-Bablok regression plots for steroids quantification in serum vs DBS collected during whole study.

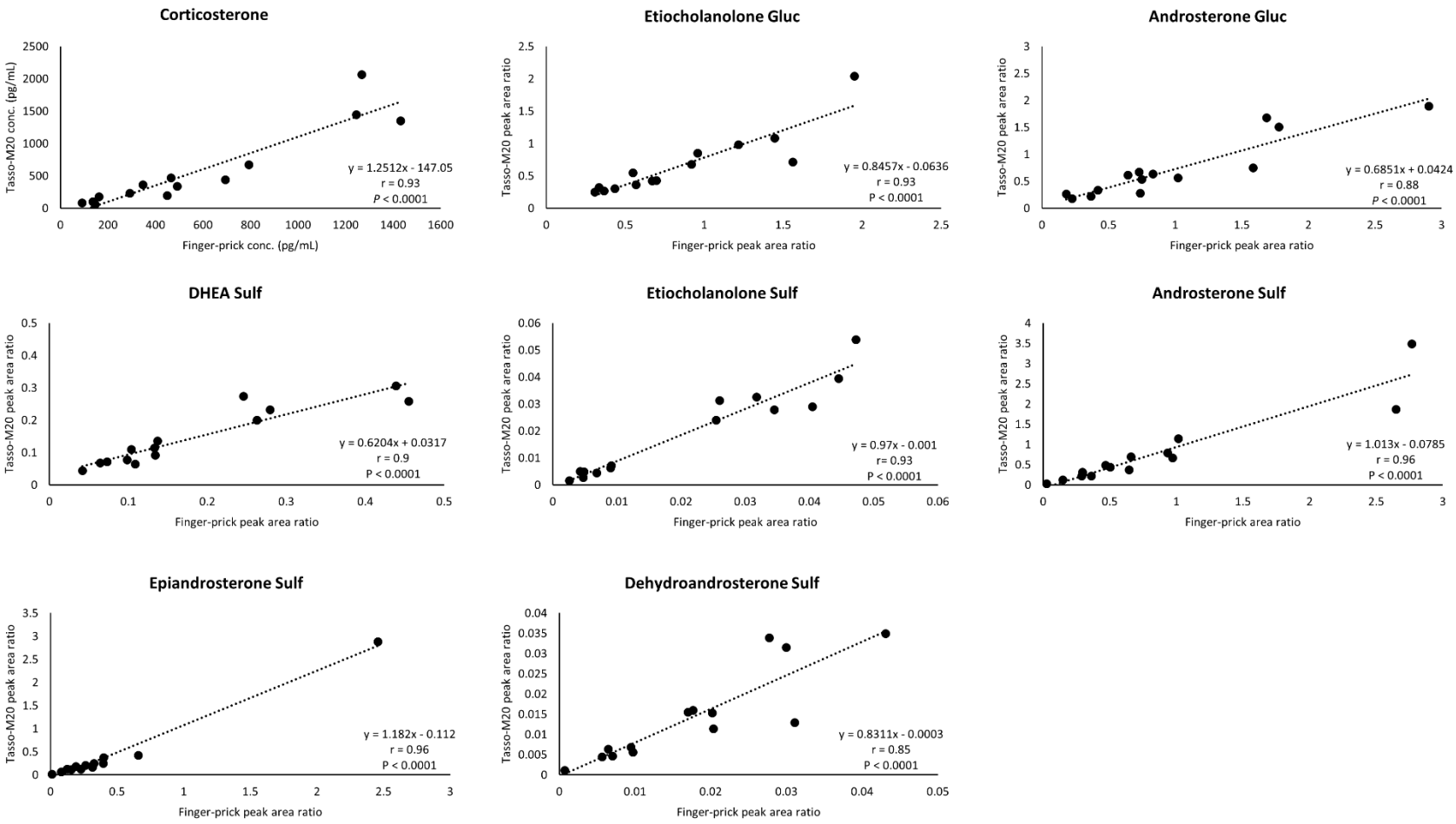
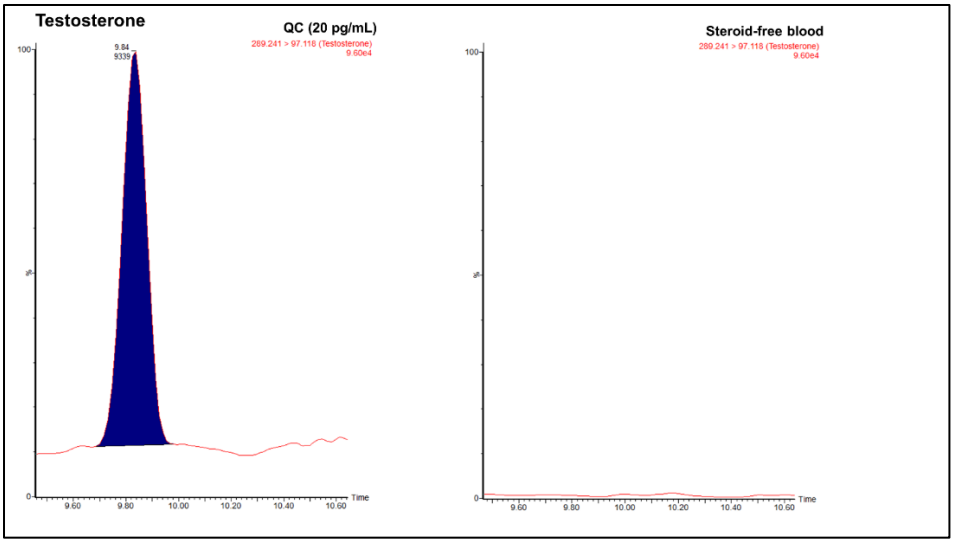
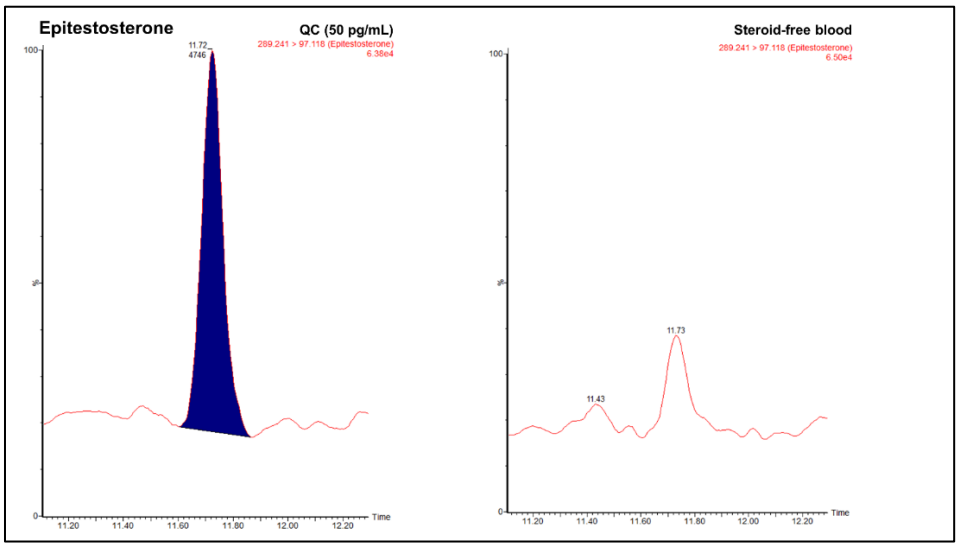
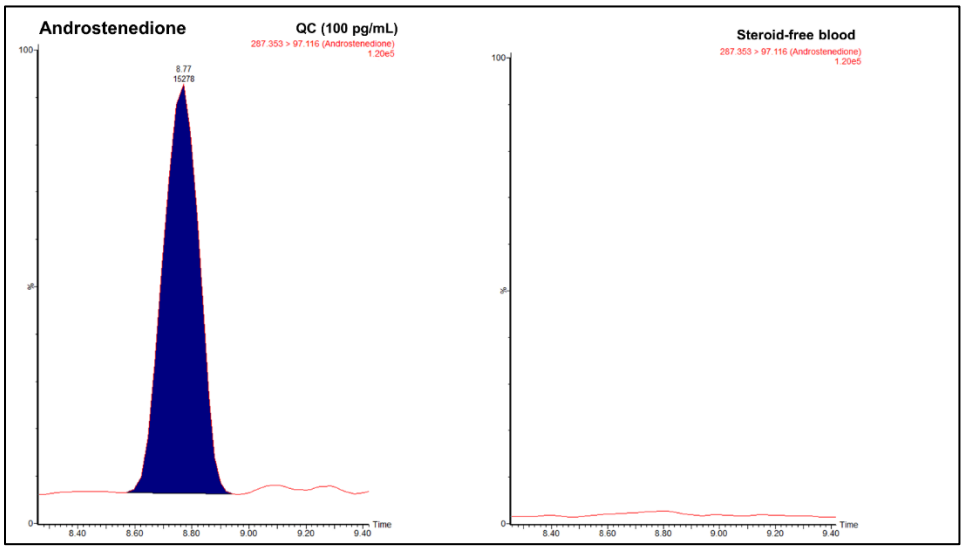
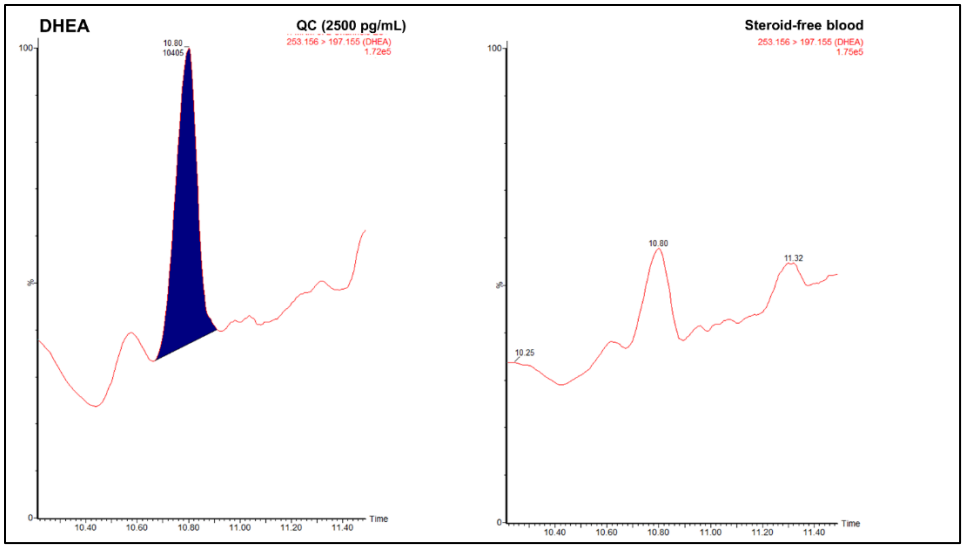


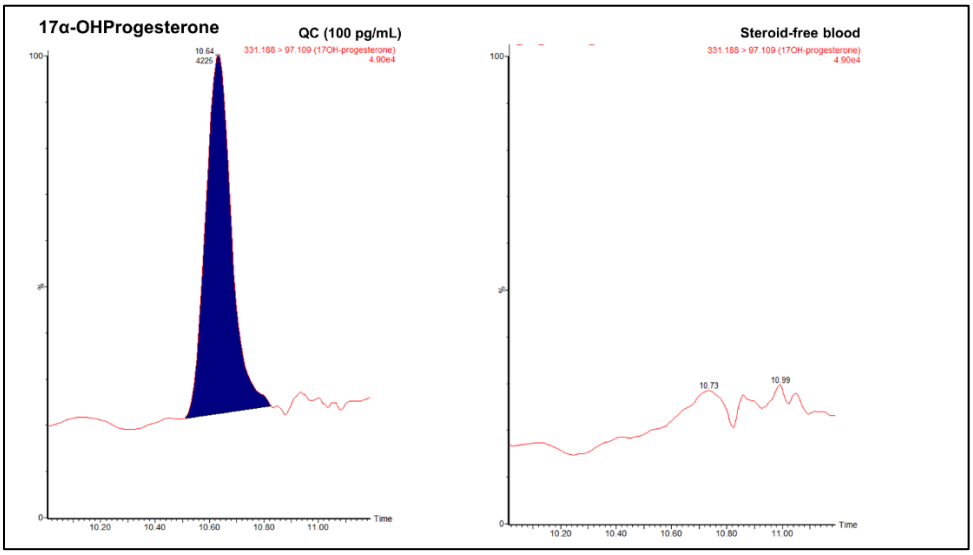

**Figure S2** – Correlation between finger-prick and Tasso-M20 DBS

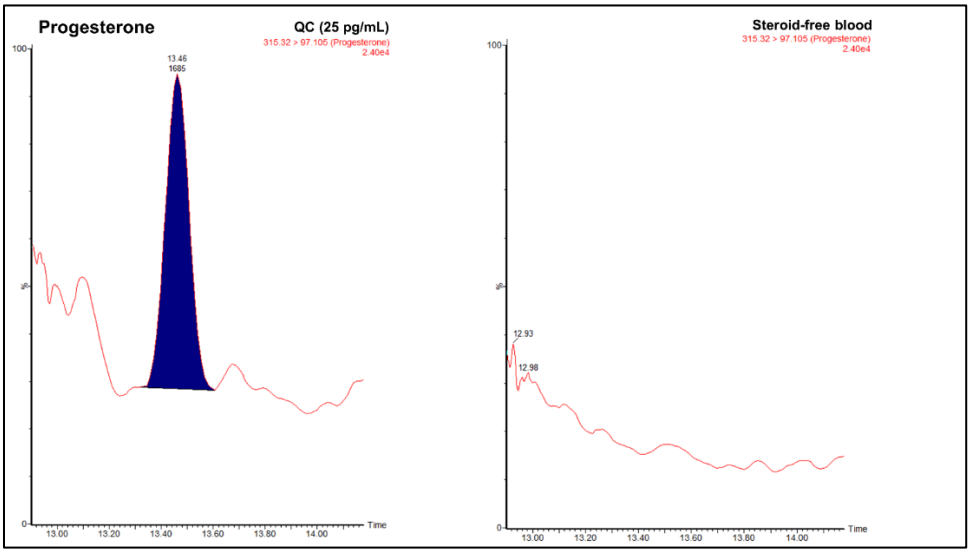

Testosterone
QC (20 pg/mL)
289.241 > 97.118 (Testosterone)
9.60e4
9.84
9339
Steroid-free blood
289.241 > 97.118 (Testosterone)
9.60e4
100
%
0
Time
9.60
9.80
10.00
10.20
10.40
10.60

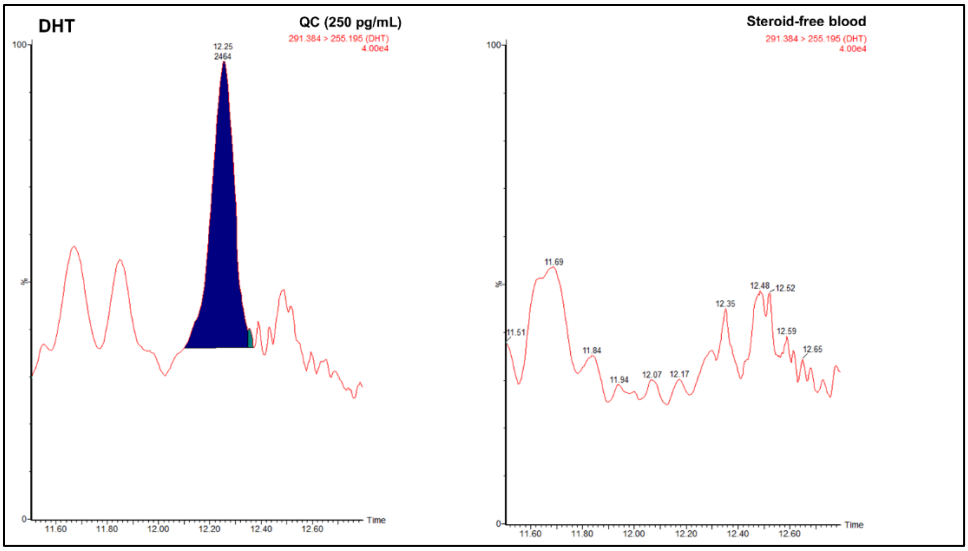

Epitestosterone
QC (50 pg/mL)
289.241 > 97.118 (Epitestosterone)
6.38e4
11.72
4746
Steroid-free blood
289.241 > 97.118 (Epitestosterone)
6.50e4
11.73
11.43
100
%
0
Time
11.20
11.40
11.60
11.80
12.00
12.20

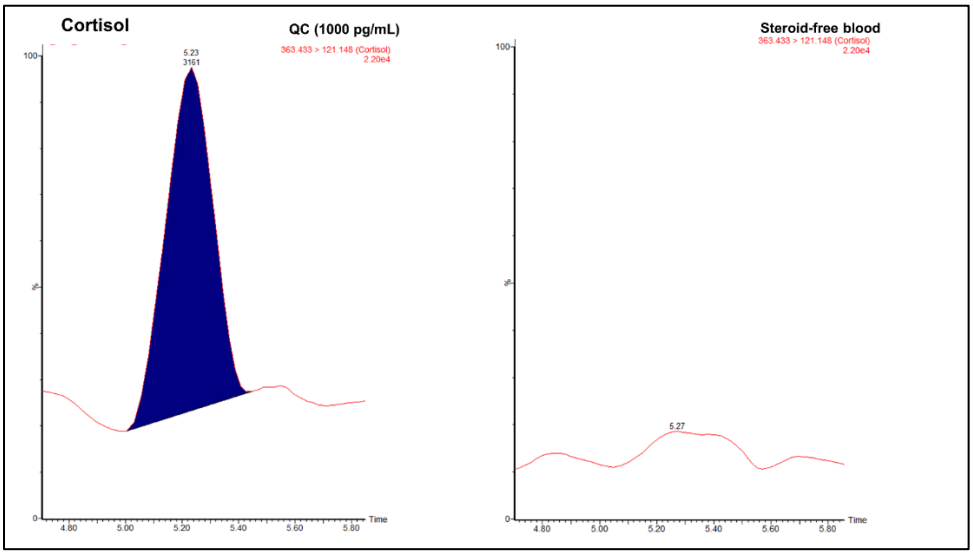

Androstenedione
QC (100 pg/mL)
287.353 > 97.116 (Androstenedione)
1.20e6
8.77
15278
Steroid-free blood
287.353 > 97.116 (Androstenedione)
1.20e5
100
%
0
Time
8.40
8.60
8.80
9.00
9.20
9.40

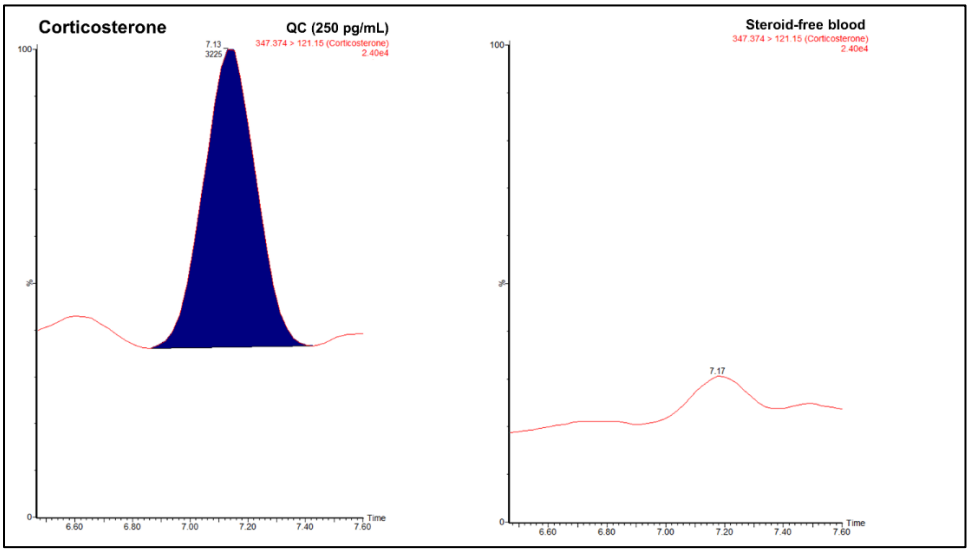

DHEA
QC (2500 pg/mL)
253.156 > 197.155 (DHEA)
1.72e5
10.80
10405
Steroid-free blood
253.156 > 197.155 (DHEA)
1.75e5
10.80
11.32
10.25
Time

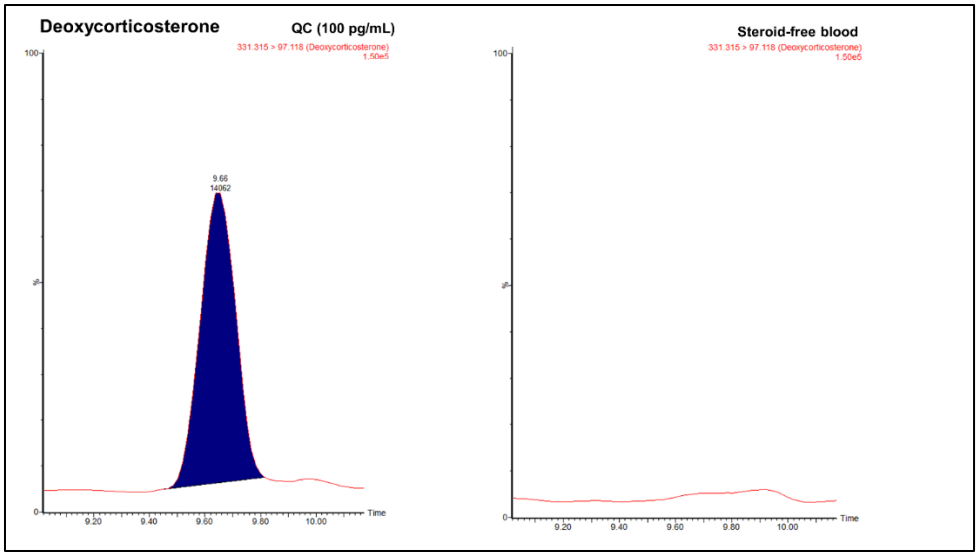

17α-OHProgesterone
QC (100 pg/mL)
331.188 > 97.109 (17OH-progesterone)
4.90e4
10.64
4225
Steroid-free blood
331.188 > 97.109 (17OH-progesterone)
4.90e4
10.73
10.99
Time

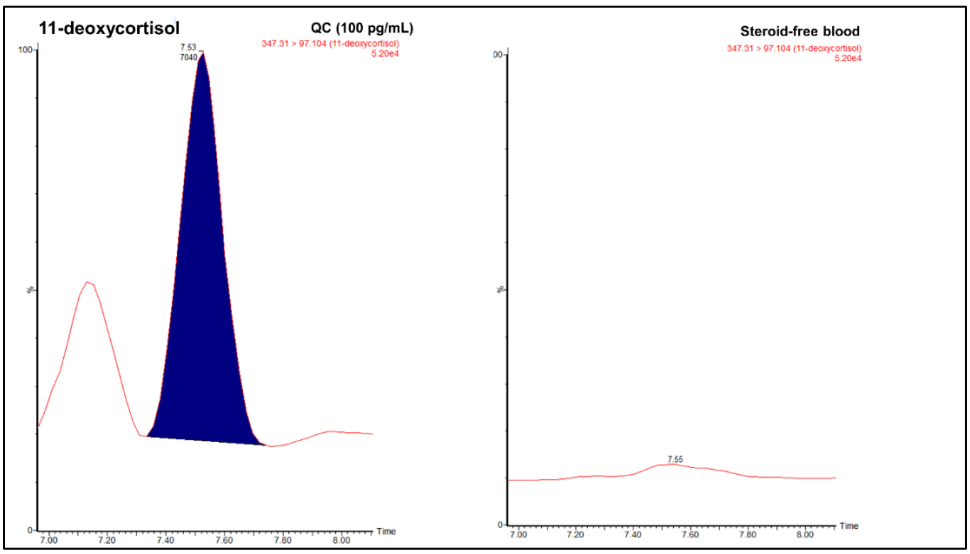

Progesterone
QC (25 pg/mL)
315.32 > 97.105 (Progesterone)
2.40e4
13.46
1686
Steroid-free blood
315.32 > 97.105 (Progesterone)
2.40e4
12.93
12.98
Time

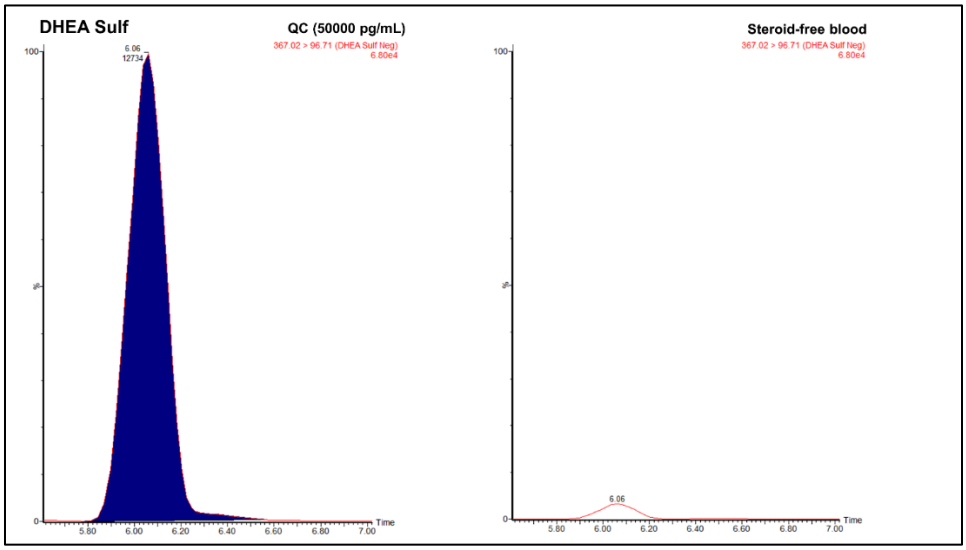

DHT
QC (250 pg/mL)
291.384 > 255.195 (DHT)
4.00e4
12.25
2464
Steroid-free blood
291.384 > 255.195 (DHT)
4.00e4
11.69
11.51
11.84
11.94
12.07
12.17
12.35
12.48
12.52
12.59
12.65
Time

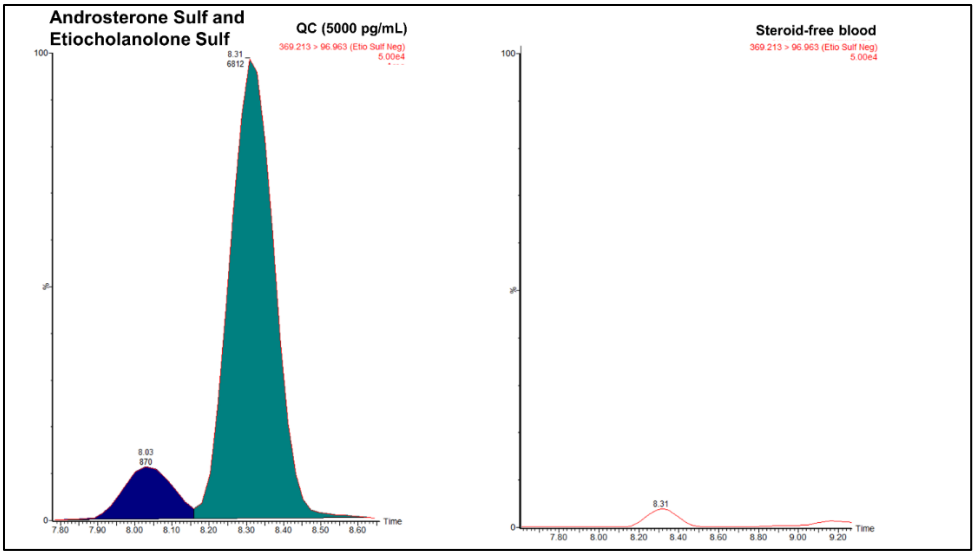

Cortisol
QC (1000 pg/mL)
363.433 > 121.148 (Cortisol)
2.20e4
5.23
3161
Steroid-free blood
363.433 > 121.148 (Cortisol)
2.20e4
5.27
Time

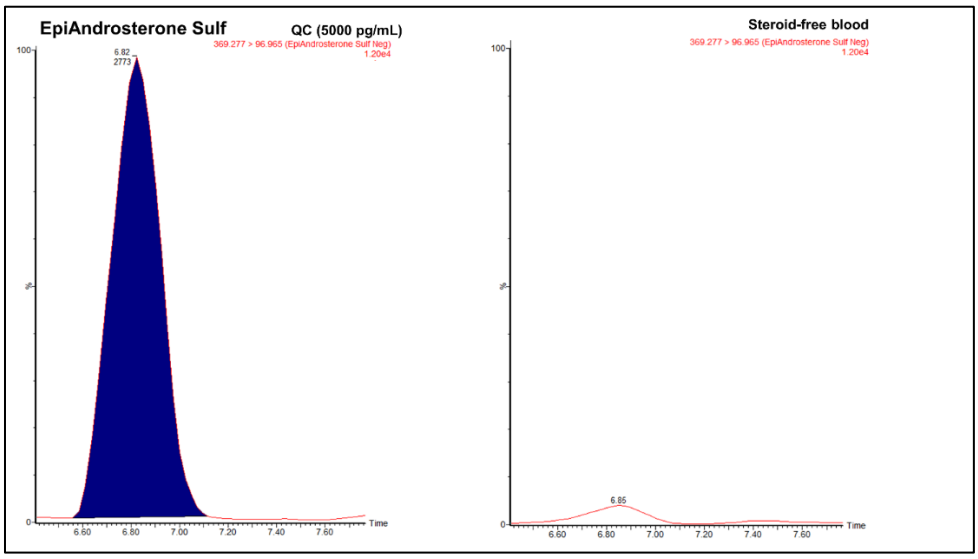

Corticosterone
QC (250 pg/mL)
347.374 > 121.15 (Corticosterone)
2.40e4
7.13
3225
Steroid-free blood
347.374 > 121.15 (Corticosterone)
2.40e4
7.17
Time

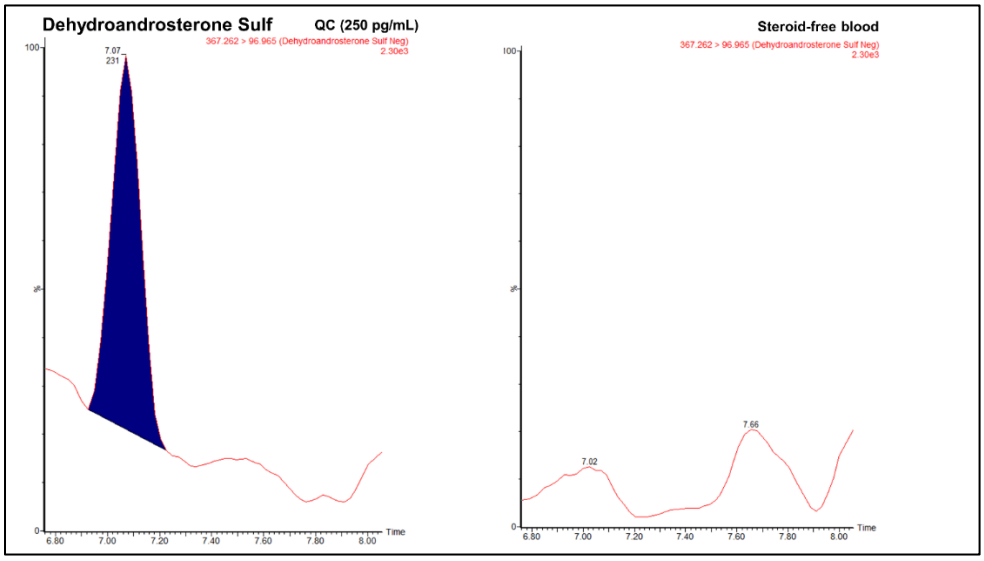

Deoxycorticosterone
QC (100 pg/mL)
331.315 > 97.118 (Deoxycorticosterone)
1.50e5
9.66
14062
Steroid-free blood
331.315 > 97.118 (Deoxycorticosterone)
1.50e5
Time


11-deoxycortisol
QC (100 pg/mL)
347.31 > 97.104 (11-deoxycortisol)
5.20e4
7.53
7040
Steroid-free blood
347.31 > 97.104 (11-deoxycortisol)
5.20e4
7.55
Time


DHEA Sulf
QC (50000 pg/mL)
367.02 > 96.71 (DHEA Sulf Neg)
6.80e4
6.06
12734
Steroid-free blood
367.02 > 96.71 (DHEA Sulf Neg)
6.80e4
6.06
Time


Androsterone Sulf and Etiocholanolone Sulf
QC (5000 pg/mL)
369.213 > 96.963 (Etio Sulf Neg)
5.00e4
8.31
6812
8.03
870
Steroid-free blood
369.213 > 96.963 (Etio Sulf Neg)
5.00e4
8.31
Time


EpiAndrosterone Sulf
QC (5000 pg/mL)
369.277 > 96.965 (EpiAndrosterone Sulf Neg)
1.20e4
6.82
2773
Steroid-free blood
369.277 > 96.965 (EpiAndrosterone Sulf Neg)
1.20e4
6.85
Time


Dehydroandrosterone Sulf
QC (250 pg/mL)
367.262 > 96.965 (Dehydroandrosterone Sulf Neg)
2.30e3
7.07
231
Steroid-free blood
367.262 > 96.965 (Dehydroandrosterone Sulf Neg)
2.30e3
7.66
7.02
Time

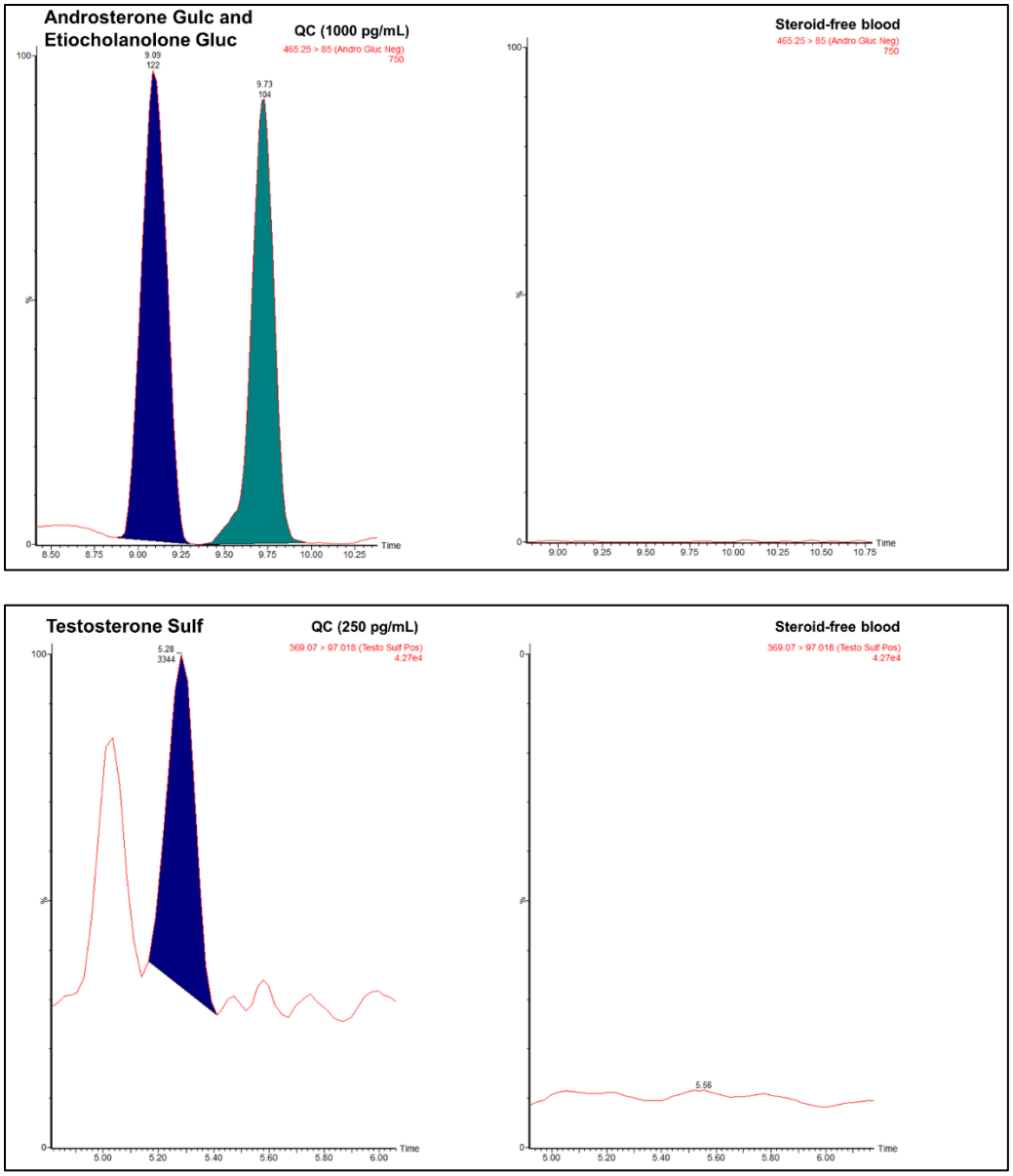



**Figure S3**. Chromatograms of the analytes with the quantifier in DBS samples obtained from QC at the LOQ and steroid-free blood.

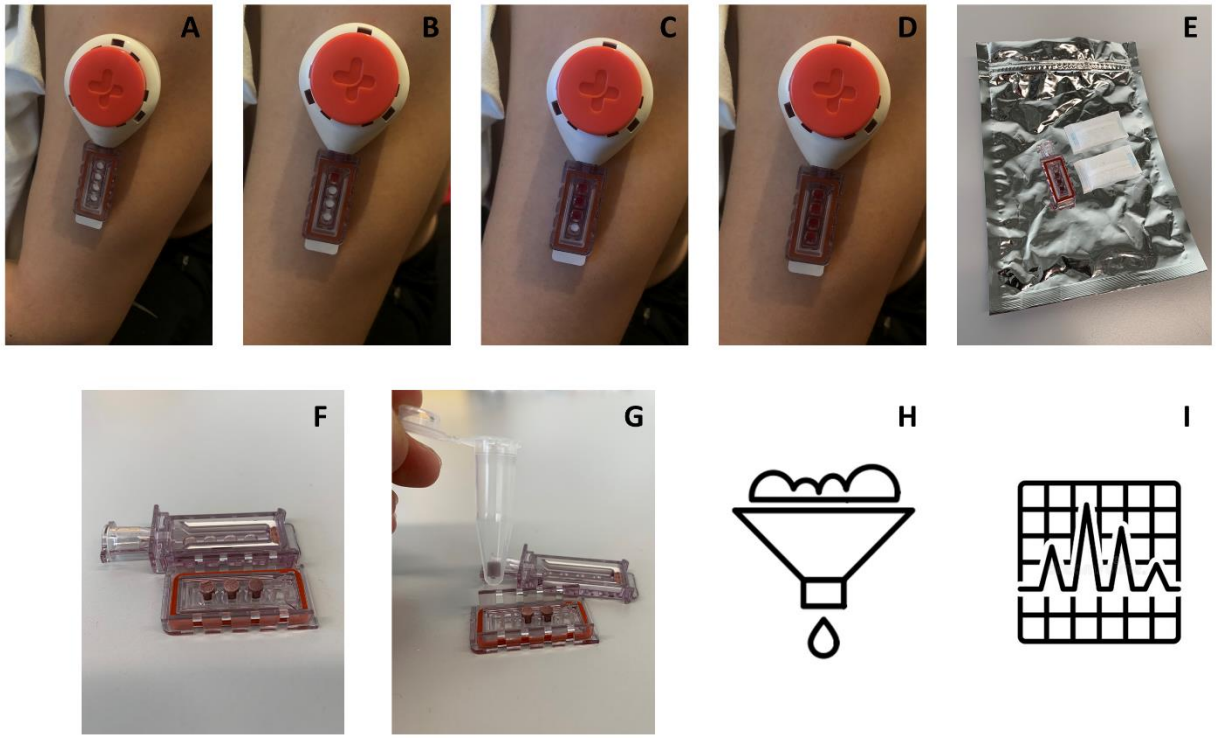



**Figure S4**. Collection procedure used for Tasso-M20 DBS collection until extraction (A-G)

**Table S1**. Calibration samples composition and concentration (final conc. in DBS)

| Analyte | Concentrations (pg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cal1 | Cal2 | Cal3 | Cal4 | Cal5 | Cal6 | Cal7 | Cal8 |
| **Testosterone** | 20 | 50 | 100 | 500 | 1000 | 5000 | 10000 | 25000 |
| **Epitestosterone** | | 50 | 100 | 500 | 1000 | 2500 | 5000 | 10000 |
| **Androstenedione** | | 100 | 500 | 1000 | 2500 | 5000 | 10000 | 25000 |
| **DHEA** | | 2500 | 5000 | 10000 | 25000 | 50000 | 100000 | 150000 |
| **DHT** | | 250 | 500 | 750 | 1000 | 2500 | 5000 | 10000 |
| **Progesterone** | | 25 | 50 | 100 | 500 | 2500 | 10000 | 25000 |
| **17α-hydroxyprogesterone** | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 | 25000 |
| **Cortisol** | 1000 | 2500 | 10000 | 25000 | 100000 | 200000 | 300000 | 400000 |
| **Corticosterone** | | 250 | 500 | 1000 | 5000 | 25000 | 50000 | 100000 |
| **Deoxycorticosterone** | | 100 | 250 | 500 | 1000 | 2500 | 5000 | 10000 |
| **11-Deoxycortisol** | | 100 | 250 | 500 | 1000 | 2500 | 5000 | |
| **Androsterone Glucuronide** | | | | 1000 | 5000 | 10000 | 50000 | 100000 |
| **Etiocholanolone Glucuronide** | | | | 1000 | 5000 | 10000 | 50000 | 100000 |
| **Testosterone Sulfate** | | | 250 | 500 | 1000 | 5000 | 10000 | 25000 |
| **Androsterone Sulfate** | | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |
| **Etiocholanolone Sulfate** | | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |
| **DHEA Sulfate** | 50000 | 100000 | 250000 | 500000 | 1000000 | 2500000 | 5000000 | 10000000 |
| **Dehydroandrosterone Sulfate** | | 250 | 500 | 1000 | 5000 | 10000 | 50000 | 100000 |
| **Epiandrosterone Sulfate** | | 5000 | 25000 | 50000 | 250000 | 500000 | 1000000 | 2500000 |

**Table S2**. Internal Standard Mix composition and concentration (final conc. in extraction solution)

| Compound | Concentration (pg/mL) |
|---|---|
| **Testosterone-d3** | 5 |
| **Epitestosterone-d3** | 12.5 |
| **DHT-d3** | 50 |
| **DHEA-d5** | 125 |
| **Corticosterone-d4** | 125 |
| **Androstenedione-d7** | 125 |
| **17α-hydroxyprogesterone-d8** | 250 |
| **11-deoxycortisol-d2** | 12.5 |
| **Deoxycorticosterone-d8** | 50 |
| **Progesterone-d9** | 12.5 |
| **Cortisol-d4** | 2500 |
| **Androsterone glucuronide-d4** | 500 |
| **Etiocholanolone glucuronide-d5** | 500 |
| **Androsterone sulfate-d4** | 6250 |
| **Etiocholanolone sulfate-d5** | 6250 |
| **DHEA sulfate-d6** | 37500 |
| **Testosterone sulfate-d3** | 50 |

**Table S3**. Mass spectrometric conditions for each analyte.

| Compound | Ionization mode | Precursor ion (m/z) | Product ion (m/z) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|---|
| Testosterone | Pos | 289.2 | **97.1;** 109.1 | 28 | 18;22 |
| Testosterone-d3 | Pos | 292.3 | 97.1 | 20 | 20 |
| Epitestosterone | Pos | 289.2 | **97.1**; 109.3 | 30; 34 | 18; 22 |
| Epitestosterone-d3 | Pos | 292.3 | 97.1 | 42 | 20 |
| Androstenedione | Pos | 287.4 | **97.1**; 109.1 | 44 | 26 |
| Androstenedione-d7 | Pos | 294.2 | 113.1 | 42 | 26 |
| DHEA | Pos | **253.2**; 271.3[a] | **197.2**; 253.2 | 56; 32 | 20; 12 |
| DHEA-d5 | Pos | 276.2 | 258.2 | 8 | 14 |
| DHT | Pos | 291.4 | 159.2; **255.2** | 22 | 26; 18 |
| DHT-d3 | Pos | 294.3 | 258.3 | 36 | 14 |
| Progesterone | Pos | 315.3 | **97.1**; 109.1 | 28 | 45 |
| Progesterone-d9 | Pos | 324.4 | 113.1 | 16 | 26 |
| 17α-hydroxyprogesterone | Pos | 331.2 | **97.1**; 109.1 | 22 | 22; 28 |
| 17α-hydroxyprogesterone-d8 | Pos | 339.2 | 100.1 | 26 | 24 |
| Cortisol | Pos | 363.4 | 91.1; **121.1** | 38 | 25; 55 |
| Cortisol-d4 | Pos | 367.0 | 121.0 | 38 | 26 |
| Corticosterone | Pos | 347.4 | 91.1; **121.1** | 40 | 44; 45 |
| Corticosterone-d4 | Pos | 351.3 | 121.0 | 28 | 28 |
| Deoxycorticosterone | Pos | 331.3 | **97.1**; 109.1 | 48 | 22; 24 |
| Deoxycorticosterone-d8 | Pos | 339.3 | 113.1 | 42 | 28 |
| 11-Deoxycortisol | Pos | 347.3 | **97.1**; 109.1 | 26 | 22; 24 |
| 11-deoxycortisol-d2 | Pos | 349.0 | 97.0 | 26 | 22 |
| Androsterone Glucuronide | Neg | 465.3 | 75.0; **85.0** | 74 | 34 |
| Androsterone Glucuronide-d4 | Neg | 469.2 | 84.8 | 74 | 34 |
| Etiocholanolone Glucuronide | Neg | 465.3 | 75.0; **85.0** | 74 | 34 |
| Etiocholanolone Glucuronide-d5 | Neg | 470.1 | 84.8 | 40 | 30 |

| | | | | | |
|---|---|---|---|---|---|
| Testosterone Sulfate | Pos | 369.1 | **97.0**; 109.1 | 46 | 24; 28 |
| Testosterone Sulfate-d3 | Pos | 372.2 | 97.1 | 48 | 20 |
| Androsterone Sulfate | Neg | 369.2 | **97.0** | 88 | 46 |
| Androsterone Sulfate-d4[b] | Neg | 373.1 | 98.0 | 88 | 34 |
| Etiocholanolone Sulfate | Neg | 369.2 | **97.0** | 88 | 46 |
| Etiocholanolone Sulfate-d5 | Neg | 374.1 | 98.0 | 88 | 34 |
| DHEA Sulfate | Neg | 367.0 | **96.7** | 66 | 60 |
| DHEA Sulfate-d6 | Neg | 373.0 | 98.0 | 66 | 50 |
| Dehydroandrosterone Sulfate | Neg | 367.3 | **97.0** | 78 | 40 |
| Epiandrosterone Sulfate | Neg | 369.3 | **97.0** | 90 | 46 |

Ion transitions used for quantification in bold

[a] Precursors with loss of (2) $H_2O$

[b] Used as IS for Epiandrosterone Sulf and Dehydroandrosterone Sulf

**Table S4**. Quantitative validation results for all analytes at each QC concentration level.

| Compound | Concentration (pg/mL) | Bias (%) | Intermediate Precision (%) | Repeatability (%) | Combined Uncertainty (%) |
|---|---|---|---|---|---|
| **Testosterone** | 20 | 3.2 | 4.0 | 2.7 | 5.5 |
| | 100 | 3.9 | 4.6 | 3.3 | 6.5 |
| | 5000 | 2.6 | 2.1 | 2.1 | 3.6 |
| | 25000 | 2.6 | 2.6 | 2.6 | 4.2 |
| **Epitestosterone** | 50 | 3.0 | 3.2 | 3.2 | 4.8 |
| | 100 | 3.3 | 3.2 | 3.2 | 5.1 |
| | 2500 | 3.8 | 2.6 | 1.5 | 5.2 |
| | 10000 | 2.4 | 2.9 | 2.4 | 4.1 |
| **Androstenedione** | 100 | 6.2 | 2.8 | 1.8 | 7.3 |
| | 500 | 2.5 | 2.0 | 1.7 | 3.5 |
| | 2500 | 2.8 | 3.6 | 2.3 | 4.8 |
| | 10000 | 4.3 | 1.6 | 1.3 | 4.8 |
| **DHEA** | 2500 | 2.1 | 2.7 | 2.7 | 3.7 |
| | 5000 | 2.4 | 3.2 | 2.3 | 4.4 |
| | 25000 | 3.6 | 3.9 | 2.9 | 5.7 |
| | 150000 | 3.6 | 2.6 | 2.6 | 4.7 |
| **DHT** | 250 | 1.9 | 2.5 | 2.5 | 3.5 |
| | 750 | 2.5 | 1.9 | 1.8 | 3.4 |
| | 2500 | 2.6 | 2.5 | 2.1 | 4.0 |
| | 10000 | 2.4 | 2.8 | 2.8 | 4.1 |
| **17a-OHProgesterone** | 100 | 2.2 | 2.2 | 1.7 | 3.4 |
| | 500 | 3.7 | 3.1 | 3.1 | 5.3 |
| | 2500 | 3.2 | 3.9 | 3.0 | 5.4 |
| | 25000 | 3.1 | 2.5 | 2.0 | 4.1 |
| **Progesterone** | 25 | 3.0 | 3.2 | 2.8 | 4.9 |
| | 100 | 2.1 | 2.1 | 2.1 | 3.1 |
| | 2500 | 4.7 | 2.8 | 2.8 | 6.0 |
| | 25000 | 2.1 | 2.9 | 2.3 | 4.0 |
| **Cortisol** | 1000 | 5.2 | 5.5 | 5.0 | 9.0 |
| | 2500 | 4.2 | 2.9 | 2.8 | 5.8 |
| | 100000 | 3.5 | 2.9 | 2.5 | 4.9 |
| | 300000 | 4.1 | 1.6 | 1.5 | 4.7 |
| **Corticosterone** | 250 | 4.6 | 4.9 | 4.9 | 7.5 |
| | 500 | 3.5 | 3.1 | 2.5 | 5.2 |
| | 5000 | 4.0 | 1.8 | 1.5 | 4.7 |
| | 50000 | 3.8 | 2.9 | 2.0 | 5.4 |
| **Deoxycorticosterone** | 100 | 3.3 | 4.1 | 1.9 | 5.7 |
| | 500 | 3.4 | 3.8 | 2.9 | 5.6 |
| | 2500 | 5.9 | 2.4 | 2.4 | 6.6 |
| | 10000 | 2.6 | 2.9 | 2.9 | 4.4 |

| | | | | | |
|---|---|---|---|---|---|
| **11-Deoxycortisol** | 100 | 3.6 | 4.5 | 3.0 | 6.1 |
| | 500 | 2.2 | 2.5 | 2.5 | 3.6 |
| | 2500 | 3.6 | 2.2 | 2.2 | 4.5 |
| | 5000 | 3.5 | 4.5 | 2.4 | 6.0 |
| **Androsterone Gluc** | 1000 | 8.6 | 11.8 | 7.2 | 16 |
| | 5000 | 7.0 | 4.9 | 4.5 | 9.2 |
| | 50000 | 5.4 | 5.6 | 4.4 | 8.7 |
| | 100000 | 3.7 | 4.8 | 3.6 | 6.5 |
| **Etiocholanolone Gluc** | 1000 | 9.6 | 14.1 | 13.3 | 20.4 |
| | 5000 | 4.3 | 4.7 | 4.6 | 7 |
| | 50000 | 4.2 | 4.9 | 4.9 | 7.2 |
| | 100000 | 3.4 | 4.8 | 4.8 | 6.7 |
| **Testosterone Sulf** | 250 | 3.4 | 3.9 | 2.6 | 5.8 |
| | 500 | 2.7 | 3.6 | 3.6 | 5.4 |
| | 5000 | 2.8 | 3.6 | 3.5 | 5.1 |
| | 25000 | 3.3 | 3.5 | 2.8 | 5 |
| **Androsterone Sulf** | 5000 | 3.0 | 3.0 | 3.0 | 4.6 |
| | 25000 | 4.3 | 3.1 | 2.1 | 5.6 |
| | 250000 | 5.6 | 3.4 | 3.0 | 6.9 |
| | 1000000 | 2.4 | 2.8 | 2.8 | 4.2 |
| **Etiocholanolone Sulf** | 5000 | 2.7 | 3.0 | 3.0 | 4.5 |
| | 25000 | 4.8 | 5.8 | 3.7 | 7.8 |
| | 250000 | 3.8 | 1.9 | 1.9 | 4.6 |
| | 1000000 | 3.3 | 3.8 | 2.2 | 5.4 |
| **Dehydroandrosterone Sulf** | 250 | 7.3 | 6.4 | 6.1 | 11 |
| | 500 | 2.4 | 3.0 | 3.0 | 4.4 |
| | 5000 | 3.0 | 3.3 | 3.3 | 4.7 |
| | 50000 | 2.6 | 3.3 | 2.5 | 4.6 |
| **Epiandrosterone Sulf** | 5000 | 9.4 | 8.2 | 8.2 | 13.9 |
| | 25000 | 5.4 | 5.9 | 5.9 | 8.3 |
| | 250000 | 5.5 | 5.5 | 5.5 | 8.3 |
| | 1000000 | 4.4 | 5.0 | 3.9 | 7.6 |
| **DHEA Sulf** | 50000 | 4.5 | 5.8 | 5.0 | 8.3 |
| | 100000 | 6.3 | 4.0 | 2.9 | 8.3 |
| | 500000 | 3.5 | 5.0 | 2.3 | 6.6 |
| | 5000000 | 2.7 | 2.9 | 2.9 | 4.4 |

# Chapter IV

Discussion and perspectives

# 4. Discussion and perspectives

The implementation of ABP was a milestone in the fight against doping as a new paradigm uncovering the use of prohibited substances or methods through individual and longitudinal profiling of indirect doping biomarkers on a solid scientific and legal basis. Although this tool demonstrated a significant deterrent effect and a better sensitivity for targeting specific analyses, athletes consequently adapted their doping scheme, using rather low doses of pseudo-endogenous substances such as T or rHuEPO and returning to archaic methods such as ABT, so as not to generate important fluctuations in the values of the ABP biomarkers remaining within natural variability. To reinforce the discriminative performance of the ABP and to provide further evidence of the "Use or Attempted Use of a Prohibited Substance or a Prohibited Method" under the 2.2 Article of the WADC, additional and complementary biomarkers are thus required. The strategy of the presented work took benefit from previous knowledge and, therefore, targeted approaches were employed from the different –omics layers instead of holistic untargeted approaches for the investigation of blood-based doping biomarkers.

### 4.1 Detection of blood doping using transcriptomic biomarkers

The first part of the thesis was focused on the evaluation of transcriptomic biomarkers of blood doping, namely ABT and rHuEPO injections. Using the Nanostring® nCounter technology and a subset of 45 pre-selected genes, three candidate genes (*ALAS2, CA1* and *SLC4A1*) could be highlighted as promising markers of altered erythropoiesis following an autologous blood transfusion. These genes were related to functional and structural components of RBCs. This approach benefited from the use of color-coded molecular barcodes that can hybridize directly to hundreds of target molecules (mRNA) with high sensitivity and high precision, and free from any amplification steps. Furthermore, the use of Tempus tubes have some advantages with the stabilization of the genomic material up to 5 days at RT and for years when kept frozen [214,215]. Unlike EDTA whole blood specimens, these tubes can therefore be transported and stored over 7 days at RT, reducing considerably the pre-analytical costs. Moreover, the gene expression analysis from Tempus tubes can be performed in batches and retrospective analyses can be carried out on the total extracted RNA. As demonstrated in Appendix VI, HGB could also be measured in Tempus tubes. To confirm the results of this profiling approach, an alternative quantitative method using RT-qPCR, considered as gold standard in diagnostic testing, was performed on the same extracts and on additional samples and volunteers for a longitudinal evaluation of the expression of the three candidate genes after an ABT. This study confirmed and consolidated that ABT triggers a down-regulation of genes encoding proteins

implicated in functional and structural processes of immature RBCs with a magnitude of changes more significant than that of IRF or RET%. In this study, the reinfused volume of RBCs concentrate corresponded to approximately 280 mL and can therefore be considered as a moderate-volume transfusion [216]. When the hematological data of the 15 subjects were entered into the ADAMS training environment, only one flag for a high HGB and OFF-Score was generated for one subject (unpublished data). Although RET% had a tendency to decrease in the subjects between 6 and 9 days following the reinfusion while staying within range, the paired HGB concentration did not increased significantly and remained within natural variability. On the contrary, abnormal values in the relative expression for at least one of the three candidate genes were reported for 10 subjects (unpublished data) when the data were longitudinally evaluated using the values from the control phase (saline injection) for individual limits calculation (mean ± 3SD). Therefore, the altered transcriptomic information originating from the immature RBCs would be a valuable additional evidence to establish a doping scenario with blood transfusion. An example of an individual hematological profile integrating the follow-up of the expression of the three candidate genes is depicted in Fig. 11.

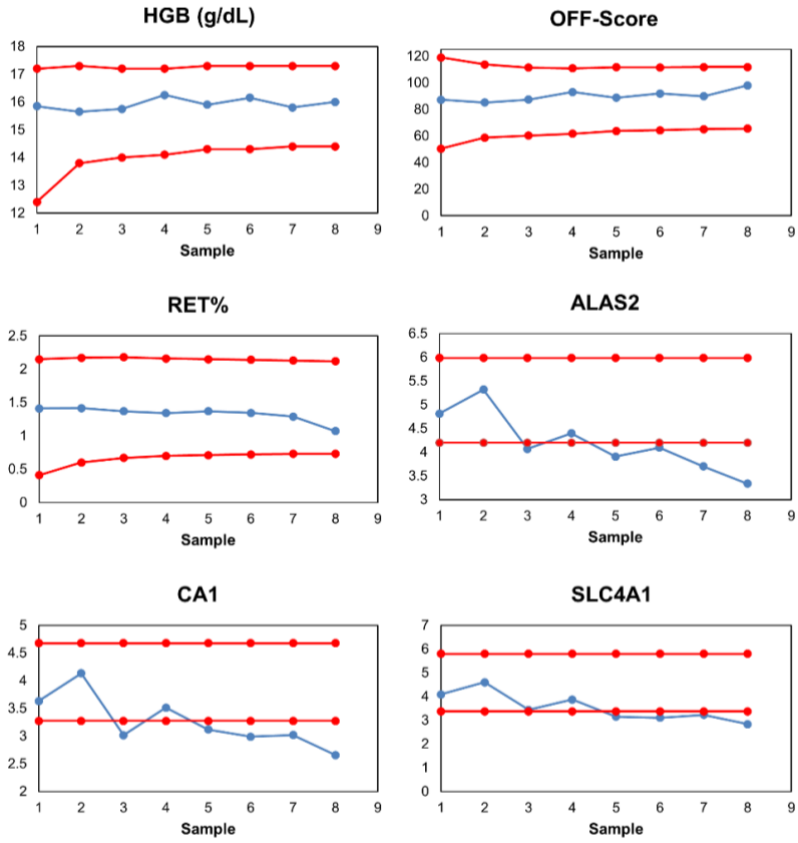



**Figure 11.** Example of an individual hematological profile, including the three transcriptomic biomarkers for ABT detection (between sample 2 and 3). Y-axis of the genes represents the relative expression, normalized to the corresponding levels of housekeeping genes.

These results support the use of transcriptomic biomarkers as surrogates indicative of blood manipulation as previously reported for rHuEPO administration [196]. To further evaluate this approach, a method for monitoring the erythropoiesis using the RNA-based *ALAS2* biomarker in DBS was developed. This study demonstrated that the number of transcripts was stable in DBS independent from the storage conditions and that the integration of the circular RNA form into the analysis improved the consistency of *ALAS2* biomarker in DBS stored under non-refrigerated conditions. When the method was applied on samples collected after stimulated erythropoiesis (endogenously by blood withdrawal or exogenously by rHuEPO injections), the response observed in the number of *ALAS2* transcripts was more marked than for conventional hematological parameters such as RET% or IRF confirming results from ABT study. The results between venous (Tempus tubes) and capillary blood (DBS samples) demonstrated high correlation suggesting that DBS is a suitable matrix for monitoring gene expression such as *ALAS2*. In addition, the normalization of *ALAS2* to endogenous housekeeping genes allowed for correction to changes in plasma volume that can influence concentration-based markers such as HGB.

The collection of DBS benefits from minimal invasiveness, simplicity of sample collection and facilitated transport and storage conditions which could allow for more frequent testing and therefore better targeting of the tested athletes and of specific analyses to perform [217]. To increase the specificity and sensitivity of the approach, additional genes also affected by altered erythropoiesis such as *CA1* or *SLC4A1* could be added to the method. Indeed, Loria *et al.* demonstrated that both additional genes demonstrated a similar response than *ALAS2* to rHuEPO injections in DBS [218]. Moreover, in an integrative "-omics" approach, proteins coded by those genes and others specific to immature RBCs could also be integrated in a longitudinal monitoring. For example, Band 3 protein coded by *SLC4A1* gene has been demonstrated with CD71 proteins as potential biomarkers of autologous blood transfusion measured in DBS [219]. Recently, mitochondrial membrane proteins (FECH and CPOX) involved in heme synthesis similar to ALAS2 were proposed as additional markers of blood doping [220]. The indirect approach could also be coupled to the direct detection of a prohibited substance in DBS such as ESAs [221].

Ultimately, transcriptomic biomarkers of blood doping may be combined with other emerging biomarkers of altered erythropoiesis originating from -omics approaches to identify molecular signature of blood doping [222,223]. The combination of biomarkers with short- and long-term response and from different matrices would improve the general detection window of blood doping and the specificity-sensitivity relationship. Some emerging biomarkers of ABT and

rHuEPO administration have been reviewed in the articles presented in Appendices I and II. Figure 12 represents the range of tests possible for the screening of blood doping using various matrices and potential biomarkers.

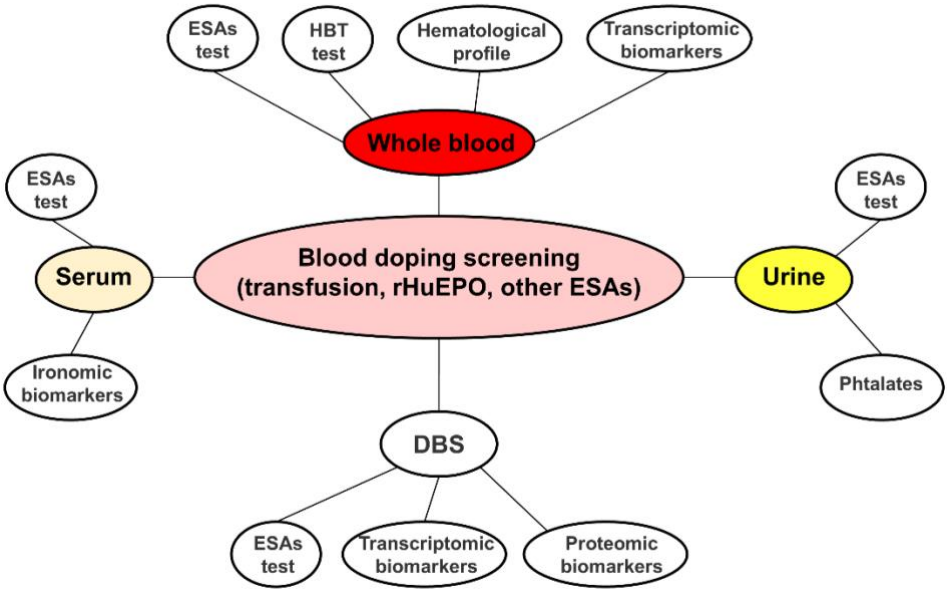



**Figure 12**. Ranges of matrices and tests or biomarkers that could be used in combination to underline blood doping.

Despite the considerable advantages presented here, the application of transcriptomic biomarkers to anti-doping displays some limitations. First, quantitative RNA methods are not part of accredited methods in anti-doping laboratories. Therefore, the implementation of these methods would require more thorough validation experiments including linearity, response to increasing HCT [217], choice of SYBR Green or TaqMan methods [224], primer sequences, or selection of housekeeping genes. Similar to hematological parameters, as the transcriptomic biomarkers would be longitudinally evaluated, the major obstacle is related to the harmonization between the different anti-doping laboratories to avoid any potential bias in the values reported for longitudinal monitoring. Automation of the RNA extraction process would therefore improve the accuracy of the analyses by avoiding human error and sample contamination as reported by Loria *et al*. [218]. An additional confirmation procedure would be required to guarantee the results reported. The use of external and internal QC controls in every assay would also be necessary to avoid any bias of the analysis, to exclude false negative results due to interference of inhibitors, and to ensure performance of the nucleic acid extraction procedure [225]. Finally, as described in the management of the profiles, the subjective

evaluation of individual profile by external experts is required to provide assistance in planning appropriate target testing schemes or to possibly open a passport case [141]. These experts should be qualified to evaluate the data and interpret them for the influence of doping substance, analytical issues and/or the influence of exercise or pathological conditions. Therefore, the data on transcriptomic biomarkers of blood doping are nowadays too scarce to allow external review by experts even though their fluctuations are believed to be related to those of RET% and IRF. Consequently, studies on large athlete populations should be carried out to establish reference ranges for transcriptomic biomarkers and to estimate between- and within-subjects variability. The confounding factors known to influence RET% and IRF should also be investigated for their effect on transcriptomic biomarkers. In a first approach, Wang *et al.* investigated the effect of strenuous exercise and moderate altitude exposure when validating the transcriptomic signature of rHuEPO [226]. Our group also evaluated the effect of hypoxic exposure on the expression of the three candidate genes (*ALAS2, SLC4A1* and *CA1*) when measured in DBS and reported moderate influence (article in revision).

### 4.2 Detection of testosterone doping using steroidomic biomarkers

The transcriptomic approach using circulating miRNAs for the detection of T administration displayed mitigate results due to specificity issues related to the expression pattern of miR-122 indicative of liver function. Furthermore, those results could not be replicated in another study implicating administration of a single 100 mg dose of testosterone gel [145].

Besides, our group developed a promising blood steroid profiling strategy to further improve the steroidal module (Federico Ponzetto thesis). Using targeted and untargeted steroidomics (metabolomics targeted to the steroids compartment), a subset of potential serum biomarkers could be highlighted for the detection of T administration in male subjects [210,212].

Based on these findings, a UHPLC-MS/MS method for the serum profiling of a large panel of steroids, including potential T biomarkers, was developed and validated, and a clinical trial involving healthy female subjects and T gel administration was carried out to obtain important complementary information on the blood steroid profiling in the prospect of a future implementation. Indeed, the current urinary ABP biomarkers have been shown to be influenced by the hormonal fluctuations of the menstrual cycle which may disrupt their sensitivity, and limited GC-MS(/MS) sensitivity can be encountered with urine samples of female athletes due to lower urinary T and other EAAS concentrations. Therefore, a study investigating the sensitivity of the current urinary ABP biomarkers as well as serum steroid biomarkers for the

monitoring of T gel administration in combination with the impact of menstrual cycle in female subjects represented a fundamental step forward for the detection of T.

A placebo-controlled study has recently demonstrated that a moderate increase of T concentration consecutive to 10 weeks of treatment with 10 mg of testosterone cream improved significantly physical performance of young, physically active women [106]. Using the same dosage although for a shorter period, the design of our clinical trial was adapted for the discovery and evaluation of present and novel biomarkers of doping practises at the individual, not population level [187]. Robust baseline sampling and measurement of within-subject changes in the different biomarkers evaluated over time using longitudinal approach could be obtained with the control phase allowing to establishing basal levels of the various biomarkers with 15 sample collections over a 4-week period. The similar sample collection design allowed discriminating between natural within-subject variability and treatment-induced variability of the evaluated biomarkers. In this manner, each subject was individually evaluated and the changes in biomarker pattern were assessed at the individual level.

The results emanating from this clinical study confirmed that urinary epitestosterone concentration is deeply impacted by menstrual cycle fluctuations as reported in previous studies [168,169]. The cyclic fluctuations were repeatable over the three consecutive menstrual cycles and demonstrated strong correlation with the female sex hormones suggesting that urinary E concentration is not influenced by exogenous T administration due to the absence of negative feedback in females. Combined with the previous studies evaluating the effects of contraceptives, pregnancy or PCOS, it suggests a possible control of E production or excretion by the HPG axis. These fluctuations had a major impact on the E-dependent ratios, T/E and 5αAdiol/E, which reduced their detection capacity notably with a high intra-individual variability. Consequently, as both ratios were the ones to be significantly affected by T gel administration, only half of the urine samples collected during the treatment phase generated abnormal values in the urinary ABP biomarkers.

In serum, T and DHT were highlighted as the most promising biomarkers confirming previous studies with male subjects [145,210]. While T demonstrated a slight increase in the ovulatory phase due to effect of the LH surge, the increase remained within natural variability (~16%) and its association with androstenedione, which reacts similarly, into a ratio dissipated this effect and demonstrated a high stability during a regular menstrual cycle. This ratio has also already been used in clinical endocrinology to characterize the excess of androgens [227]. The individual longitudinal monitoring of the serum biomarkers, namely T, DHT and T/A4, demonstrated high sensitivity with more than 90% of the serum samples displaying abnormal

values during T gel administration. Similar results between urine and blood steroid profile were also reported after 10 weeks of T cream administration in women [228]. Using the developed and validated method for extended serum steroid profiling including phase II metabolites, the number of potential biomarkers could be expanded with androsterone and epiandrosterone sulfate increasing after 28 days of T gel administration. Similar to sulfo-conjugated metabolites of prohibited substances, these biomarkers are supposed to increase the detection window and to reflect the long-term use of a prohibited substance. This hypothesis was further confirmed with transgender patients (female-to-male) treated with exogenous T over 12 months. Contrary to the steroidomics study conducted by Ponzetto *et al.* [212], the glucuro-conjugated steroids were not significantly influenced by the administration of transdermal T and seem to rather react to oral route of administration due to the first-pass effect. Notably, the quantification of T glucuronide for longitudinal monitoring purposes seems limited for female subjects due to the low basal concentration, which was mostly below LLOQ in our study. Based on the presented findings of this work and on the previous knowledge acquired with male subjects, a novel version of ABP steroidal module including a list of biomarkers that could be integrated into the blood steroid profile as a complement module to the current urinary steroid profile is proposed in Fig. 13.

To take advantage of the assets of DBS in a manner analogous to the transcriptomic biomarker approach, a UHPLC-MS/MS method was developed for the quantification of a panel of steroids in DBS, including the proposed T biomarkers. DBS collected during the control phase demonstrated good agreement with serum concentrations for most compounds. Excessive T concentrations were measured in capillary DBS (from finger prick) in the samples collected during and after T phase, while concentration values reported in venous DBS (DBS generated with EDTA whole blood) were similar to those of serum matrix. This finding suggests a possible local recovery of exogenous T on the finger(s) used for gel application generating high T values in capillary blood. A previous study supports this hypothesis and showed that considerable amounts of testosterone remained on the intact skin for several hours after T gel application and evaporation of the alcohol vehicle while the risk of interpersonal contamination was very low [229]. To overcome this potential pitfall, the Tasso-M20 push button device was also evaluated as alternative DBS collection method. The device allows collecting capillary blood on the upper arm using volumetric absorptive microsampling. Steroid concentrations were comparable between finger-prick DBS and Tasso DBS suggesting that this alternative device might be a relevant option.

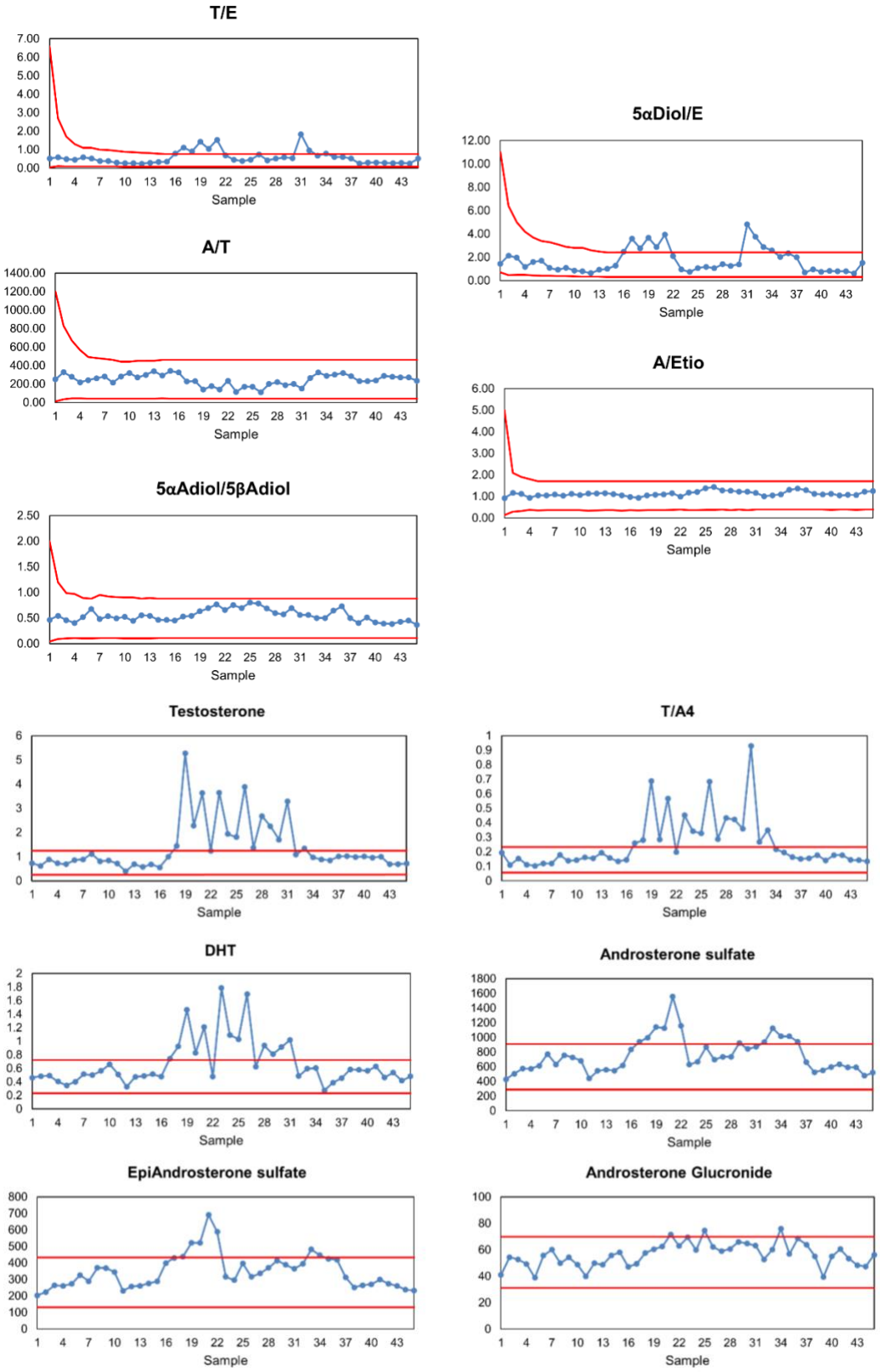



**Figure 13**. Example of a novel version of the steroidal module, including the serum markers (bottom of the figure) capable of increasing the sensitivity for transdermal testosterone administration (intake between sample 16 and 31).

The other limitation of the steroid profiling in DBS was related to the quantification of DHT which could not reach the sensitivity required (LLOQ at 250 pg/mL) for measuring DHT in samples of female subjects. Ultimately, DBS may be used as screening method allowing for more frequent sampling and for targeting blood (serum) and urine sample collection that would be used for a full steroid profile and for GC/C/IRMS analysis. The increased sampling would provide a better estimation of natural baseline variability of a given athlete and would provide a better resolution of a doping picture [217].

Currently, the actions taken in relation to a suspicion for T doping mainly relies on the outcomes of the confirmatory GC/C/IRMS analysis and not on the sole basis of the ABP urinary steroid profile contrary to the hematological module. Furthermore, the presence of high ethyl glucuronide concentration reflective of alcohol consumption generating abnormal urinary markers vaues very often lead to GC/C/IRMS analyes that are in many cases reported as negative. In our study, GC/C/IRMS demonstrated a limited sensivitiy for the detection of T gel in female subjetcs on a defined subset of urine samples (one urine/week during treatment phase for 8 subjects). Less than one third of the tested samples fullfilled the criteria for positivity confirming previous results reporting low GC/C/IRMS sensitivity for the detection of transdermal T in male subjects [145]. Among the samples that were considered as negative (not fullfilling any of the WADA criteria, 48%), several would still be reported as inconclusive (ATF; atypical finding) because the $\Delta\delta^{13}C$ values reported were not consistent with the endogenous origin of the TC(s). The blood steroid profile demonstrated better performance for targeting positive GC/C/IRMS analysis with a flag in all corresponding serum samples, while the urinary steroid profile was flagged for half of GC/C/IRMS positive urine samples. Regarding the samples evaluated negative by GC/C/IRMS, 42% produced abnormal values in the urinary profile while 80% of the serum samples exceeded individual thresholds. These findings highlight the benefit of combining the urinary and serum steroid profile data to increase the discriminative performance of ABP and to provide further evidence of the use of exogenous T (art. 2.2 of the WADC), independent from GC/C/IRMS and similar to a forensic approach. In the event of an inconclusive GC/C/IRMS result, the information origintating from the blood steroid profile could also provide additional piece of evidence for the T administration. Furthermore, the blood profile seemed less affected by alcohol consumption than the urinary ABP biomarkers, which would assist for a more efficient targeting of confirmatory GC/C/IRMS analysis. The ranges of tools emerging from the present work for the screening of T doping are presented in Fig. 14.

The utility of the blood steroid analysis has already been recognized following the suspension of two female athletes by the court of arbitration for sport (CAS) confirming that "*the analysis of blood samples taken from both athletes established that such samples collected shortly before the Rio 20156 Olympic Games were found to contain an excessive concentration of testosterone"*. Interestingly, the urinary steroid profile of one athlete did not generate abnormal values that would have triggered a confirmatory GC/C/IRMS, while for the other athlete, low urinary T concentrations made GC/C/IRMS analysis impossible [230]. These cases combined with the additional results of our study reinforce the concept of a blood steroid profile that would be used alongside the current urinary steroid profile and in the same way as the hematological module that can be used to sanction athletes on the sole basis of variations in its biomarkers. It also underlines the need for a shift of paradigm of the current situation, mainly dependent on the outcomes of the GC/C/IRMS analysis.

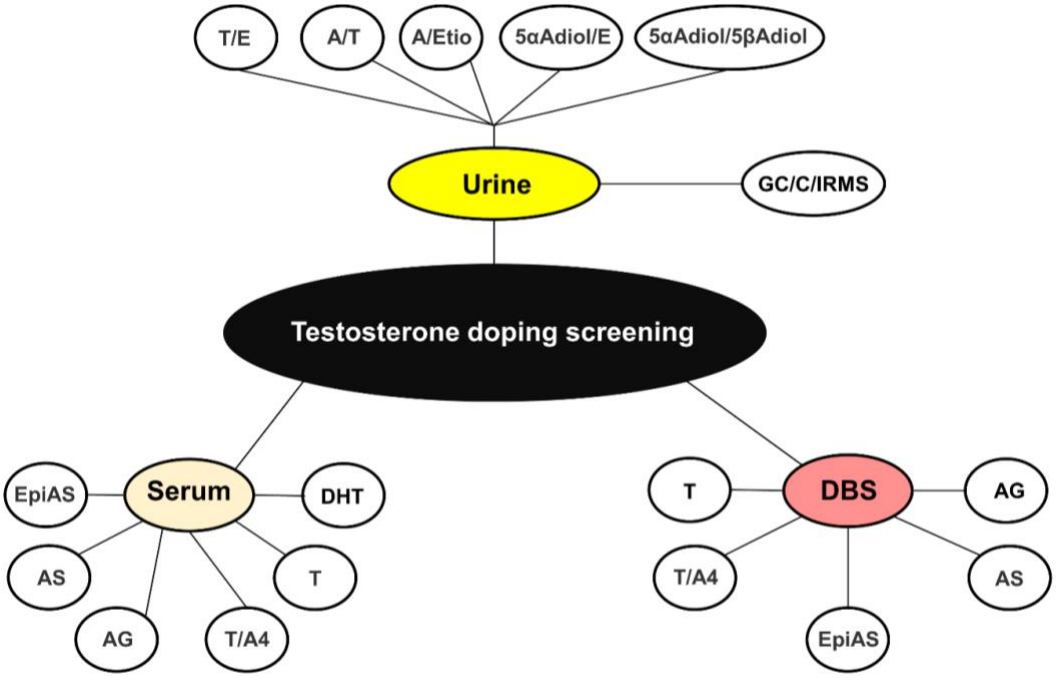



**Figure 14**. Ranges of current and emerging tools to detect testosterone doping. AS: androsterone sulfate; AG: androsterone glucuronide; EpiAS: epiandrosterone sulfate.

Contrary to the transcriptomic approach, the longitudinal monitoring of blood steroid biomarkers benefit from the large knowledge of endocrinology experts regarding the steroid concentration values that could be reported, notably for T. Furthermore, the biological interpretation of the steroid concentrations in blood is more robust and more informative of the physiological responses than in urine, which rather provides excretion information. The expert

evaluation of a passport, by weighing the likelihood that the passport is the results of the use of a prohibited substance against the likelihood that the passport is the result of a normal physiological or pathological condition, will be facilitated using blood information and will assist to establish a doping scenario.

Compared to RNA quantitative methods, the quantification of serum or DBS steroid concentrations is an easier method to implement in anti-doping laboratories, which benefit from prior expertise in LC-MS/MS methods already entrenched in the field activity. Similar to quantitative methods already implemented, identification criteria will need to be established and quality controls will need to be used within each batch of analysis. External quality controls for T, cortisol or progesterone are already available on the market for clinical laboratories and could easily be used by anti-doping laboratories to assess their analytical performance.

Finally, upstream of an implementation of the blood steroid profile, the stability of the proposed panel of markers will need to be assessed by studying the effect of different confounding factors on their concentration values. While extensive information originating from the clinical field is already available for serum T, the influence of potential confounding factors such as exercise, circadian rhythm, alcohol consumption or oral contraception among others on the concentration values of the other proposed steroid biomarkers, notably phase II metabolites, is not well documented and should hence be thoroughly evaluated and clearly understood. To limit the effect of some confounding factors on the serum concentration such as plasma volume variation, different ratios and combination scores will be investigated. In the present study, a first ratio combining T and A4 was proposed to limit the effect of the menstrual cycle. Moreover, the quantification of serum androgen concentrations in a large population of high-level athletes could also allow for establishing normative values as well as estimating the prevalence of T doping in the specific population as has already been performed to estimate the prevalence of hyperandrogenism in elite female athletes [231]. Such a study would also highlight putative differences in steroid metabolism between males and females. The use of an extended steroid profile would also help to characterize different populations with excess of androgens such as PCOS or congenital adrenal hyperplasia in a clinical context.

### 4.3 Conclusions and future perspectives

The interpretation of an individual biological passport is a difficult task. The addition of evidence with supplementary biomarkers would help for the evaluation and would provide further proofs of non-physiological variation. In the present work, novel biomarkers have been proposed for the improvement of the two modules of the ABP, namely transcriptomic biomarkers for blood doping and serum/blood steroid biomarkers for testosterone misuse.

Nevertheless, for a future implementation, these biomarkers need to be fully characterized. Namely, the effects of known and potential confounding factors will need to be evaluated on the longitudinal follow-up of these markers. In order to integrate them into the Bayesian adaptive model, the intra- and inter- individual variability as well as the variability of the measurement (analytical variability) will need to be assessed for the calculation of individual limits. For that, large-scale testing on athlete populations and inter-laboratories studies are required. Using artificial intelligence and machine-learning, the combination of multiple parameters involving current and emerging markers into scores will also be considered to increase the sensitivity of detection and limit the effect of confounding factors. For example, in Article 5, we showed that using binary logistic regression, the combination of urinary and serum steroid biomarkers improved the general predictive accuracy for the detection of T administration.

Regarding T detection, alternative studies will also be conducted. The GC/C/IRMS in serum is definitely something that will be investigated in few years to support the blood steroid profile. The major limitation of this approach will be the choice of target compounds which are sufficiently concentrated using a limited volume of serum. Similar to blood doping, the transcriptomic signature of testosterone gel administration in women will be investigated in a project funded by the Partnership for Clean Competition. Using a novel bulk RNA barcoding and sequencing (BRB-seq) approach, differentially expressed genes in whole blood will be identified and the most promising transcriptomic biomarkers will be validated using RT-qPCR. To benefit from their advantages, DBS will also be assessed for the monitoring of the differentially expressed genes.

In conclusion, the blood matrix is a massive reservoir for biomarker discovery, which can be fully exploited using the emergence of sensitive –omics technologies. In addition, the rise of DBS as potential matrix for anti-doping purposes offers a valuable alternative for longitudinal blood collection and measurement of doping biomarkers abundantly present in the circulation using sensitive methods. Similar to a forensic approach, the combination of information originating from multiple biomarkers (emerging and current) could ultimately be used as a

bundle of evidence to increase the likelihood ratio that the observed fluctuations result from the use of a prohibited substance or method rather than from a normal physiological or pathological condition. Finally, the characterized and longitudinally monitored doping biomarkers could also be applied in some clinical contexts for the diagnosis, monitoring, or prognosis of diseases in a personalized medicine approach.

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# APPENDICES

# Appendix I

Autologous Blood Transfusion in Sports: Emerging Biomarkers.

*Salamin O, De Angelis S, Tissot JD, Saugy M, Leuenberger N. Transfu Med Rev. 2016 Jul; 30(3):109-15*

Transfusion Medicine Reviews 30 (2016) 109–115

Contents lists available at ScienceDirect

Transfusion Medicine Reviews

journal homepage: www.tmreviews.com

Original Articles

# Autologous Blood Transfusion in Sports: Emerging Biomarkers

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ARTICLE INFO

Available online 21 May 2016

*Keywords:*
Athlete biological passport
Blood transfusions
Blood doping
— omics methods
Biomarkers

ABSTRACT

Despite being prohibited by the World Anti-Doping Agency, blood doping through erythropoietin injection or blood transfusion is frequently used by athletes to increase oxygen delivery to muscles and enhance performance. In contrast with allogeneic blood transfusion and erythropoietic stimulants, there is presently no direct method of detection for autologous blood transfusion (ABT) doping. Blood reinfusion is currently monitored with individual follow-up of hematological variables via the athlete biological passport, which requires further improvement. Microdosage is undetectable, and suspicious profiles in athletes are often attributed to exposure to altitude, heat stress, or illness. Additional indirect biomarkers may increase the sensitivity and specificity of the longitudinal approach. The emergence of "-omics" strategies provides new opportunities to discover biomarkers for the indirect detection of ABT. With the development of direct quantitative methods, transcriptomics based on microRNA or messenger RNA expression is a promising approach. Because blood donation and blood reinfusion alter iron metabolism, quantification of proteins involved in metal metabolism, such as hepcidin, may be applied in an "ironomics" strategy to improve the detection of ABT. As red blood cell (RBC) storage triggers changes in membrane proteins, proteomic methods have the potential to identify the presence of stored RBCs in blood. Alternatively, urine matrix can be used for the quantification of the plasticizer di(2-ethyhexyl)phthalate and its metabolites that originate from blood storage bags, suggesting recent blood transfusion, and have an important degree of sensitivity and specificity. This review proposes that various indirect biomarkers should be applied in combination with mathematical approaches for longitudinal monitoring aimed at improving ABT detection.



**Contents**



Increasing oxygen delivery capacity to exercising muscles to enhance aerobic performance is a well-established concept. Blood manipulation may be used by athletes pursuing a rapid increase in red blood cells (RBCs) and hemoglobin. According to the World Anti-Doping Agency, this approach comprises the reintroduction of any quantity of blood, RBC products, artificial enhancement of oxygen delivery, and intravascular manipulation of blood [1]. These methods are covered by blood transfusion and the administration of recombinant human erythropoietin (rHuEPO) and are the most common means used by athletes attempting to manipulate their blood. After its global commercialization

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http://dx.doi.org/10.1016/j.tmrv.2016.05.007
0887-7963/

between 1987 and 1989, widespread abuse of rHuEPO in athletes during the 1990s/2000s was observed and attributed to its easy access and its tremendous impact on performance. Although the drug was banned by sporting authorities, there was no detection method available at that time. A detection method based on isoelectric focusing was developed in 2000 [2,3]. Since then, there has been an increase in the use of blood transfusions in athletes. In 2006, "Operacion Puerto" revealed the presence of refrigerated blood bags from professional athletes associated with sophisticated calendars of blood reinfusion, suggesting that blood transfusion is still abused by athletes in an attempt to boost performance [4].

Flow cytometry can be used to detect allogeneic transfusion by detecting variations in blood group antigens [5,6]. Nevertheless, a group reported that the number of occurrences of two individuals sharing an identical phenotype in the same sport was 5 times higher than the theoretical probability [7]. Autologous blood transfusion (ABT), reinfusion of own blood or red cell concentrates, is, however, undetectable using this approach. The absence of direct detection of ABT is an important challenge facing antidoping laboratories in the fight against doping.

Autologous blood transfusion is currently monitored using indirect markers in a longitudinal profile via the hematological module of the athlete biological passport (ABP) [8]. Although the introduction of the ABP was a breakthrough toward the detection of blood transfusion and blood doping, the transition to microdose regimens of rHuEPO and blood transfusion has raised questions concerning the efficiency of the hematological module [9,10]. The addition of new indirect biomarkers in the ABP would improve the detection of blood transfusion and blood manipulation. As blood doping alters erythropoiesis, it may cause variations at the transcriptomic level, which may be more sensitive than classical hematological parameters. Iron metabolism is involved in the production of RBC and hemoglobin, and quantification of proteins involved in iron regulation may offer a valid alternative for the detection of ABT. Red blood cell storage results in changes in the membrane or shape of RBCs, which may indicate blood transfusion. Finally, urinalysis may indicate the presence of plasticizers leaked from blood storage bags, suggesting recent transfusion.

## The ABP and Hematological Markers

Implementation of the hematological module of the ABP by the International Cycling Union in 2008 was a small revolution in the antidoping world. Rather than the direct detection of the prohibited substance, this new paradigm aimed to investigate the effects of doping methods on metabolism [11]. The ABP relies on an individual and longitudinal monitoring of specific biomarkers of doping. This new approach offers the great advantage of being independent of the marketing of new pharmaceutical doping drugs [12]. Moreover, the longitudinal follow-up of athletes can be used to suspend those from competition due to doping and can be a powerful tool to establish targeted testing of suspicious profiles [13].

Blood withdrawal and reinfusion cause characteristic alterations in several markers of erythropoiesis, leaving a characteristic fingerprint on the biology of the athlete [14] (Table 1). This concept is the underlying basis of the ABP in detecting blood doping. Furthermore, instead of setting population-based cutoffs such as 50% hematocrit, individual references are defined by an adaptive model [15]. Biomarkers of erythropoiesis (hemoglobin concentration [Hb] and reticulocytes percentage [Ret%]) are monitored over time and analyzed using a mathematical model based on Bayesian inference that considers previous values and identifies patterns of blood manipulation [16]. A suspicious case can be reported if a particular value lies outside the defined range.

Although the ABP met a certain success, blood doping remains omnipresent among cheating athletes. This analytical tool must thus be continuously refined and correlated with the introduction of new markers of altered erythropoiesis. In addition to standard blood parameters (Hb and Ret%), the total mass of hemoglobin ($Hb_{mass}$) appeared as a sensitive indicator of blood transfusion and was evaluated as a marker of the adaptive model in a longitudinal blinded study [17]. A new score ($Hb_{mr}$) that included $Hb_{mass}$ and Ret% was introduced and demonstrated superior sensitivity in detecting the highest dosage of blood transfused, but 0% when 1 U was reinfused [18]. Moreover, $Hb_{mass}$ measurement can detect ABT performed with frozen RBCs [19]. The potential of $Hb_{mass}$ for the detection of rHuEPO misuse was also assessed in another study [20]. The main advantage of this variable over other parameters is its independence of plasma fluctuations and lower variability [17,21]. However, the primary drawback of this promising parameter is that the measurement of $Hb_{mass}$ is based on the carbon monoxide (CO) rebreathing method [22]. CO is toxic and may reduce exercise capacity. Furthermore, the CO rebreathing method requires athletes to fully cooperate, which is unlikely in cheating athletes [23]. Therefore, research is focused on the indirect modeling of $Hb_{mass}$ from indirect markers.

## Transcriptomics

The genome represents the genetic material of an organism and is organized in genes. Each gene codes for a protein that is first transcribed into RNA. The transcriptome is the set of all RNA transcripts and also includes noncoding RNA. In contrast to the genome, which is invariable, the transcriptome is subject to environmental variations. Doping substances or methods have been recognized to influence messenger RNA expression. The apparition of high-throughput techniques such as microarray or polymerase chain reaction has allowed an easier application of transcriptomics and offered a promising alternative in the research of biomarker for the detection of blood transfusion.

Through these tools, and based on the hypothesis that exposure of cell detritus originating from stored blood induces a cellular and molecular immune response, a pilot study demonstrated that blood reinfusion altered the expression profile of T lymphocytes [24]. At 72 and 96 hours posttransfusion, the expression of more than 700 genes was altered, particularly in genes coding for proteins regulating surface receptor endocytosis, the Toll-like receptor pathway and the adaptive immune response. The aforementioned study had several limitations including a limited number of subjects. Furthermore, the approach used is susceptible to false-positive results caused by infection or hemolysis [25].

Reticulocytes still retain quantities of functional residual nucleic acid material, even after expelling their nucleus [26,27]. Blood doping influences the production of immature RBC and may alter their gene expression. Consistent with this idea, Varlet-Marie et al [28] identified and confirmed 95 genes that were differentially expressed after administration of high and microdoses of rHuEPO using serial analysis of gene expression and quantitative real-time polymerase chain reaction. Recently, Durussel et al [29] reported the whole-blood transcriptional signature of rHuEPO in 2 distinct and independent groups composed of endurance-trained Caucasian males at sea level and Kenyan endurance runners at moderate altitude who received rHuEPO injections for 4 weeks. On the basis of this study, our laboratory demonstrated that ABT altered the expression of genes whose functions are related to RBC metabolism using digital multiplexed gene expression. Interestingly, the variations in the number of transcripts were more significant than those of the percentage of reticulocytes (unpublished results). This promising approach has the potential to be a powerful complement and appears more sensitive to small variations than classic hematological biomarkers. Athletes often combine ABT with rHuEPO injections to avoid fluctuations and are thereby undetectable with the ABP.

As previously mentioned, the transcriptome also includes noncoding RNAs. MicroRNAs (miRNAs) play a crucial role in gene expression regulation. Cell-free miRNAs are detectable in blood plasma or serum and can be used as specific and sensitive markers of various pathophysiological processes. The ability of circulating miRNAs to serve as biomarkers of ABT was investigated by Leuenberger et al [30]. Blood reinfusion triggered a distinct change in the pattern of circulating miRNAs whose

**Table 1**
Variations of hematological and iron-related parameters after various methods of blood manipulations

| | Hb | Ret% | HCT | s-EPO | Free iron | Ferritin | sTfR | Hepcidin | ERFE |
|---|---|---|---|---|---|---|---|---|---|
| rHuEPO | ↗ [25,35,69] | ↗ [35] | ↗ [35] | ↗ [35,36] | ↘ [36,70] | ↘ [36,70] | ↗ [35,36] | ↘ [36,70][a] | ↗ [46] |
| Iron | ↔/↗ [71–77] | ↗ [78] | ↔ [73,75,79] | ↔ [b] | ↔/↗ [71,75] | ↗ [41,71–76,80,81] | ↘ [79] | ↗ [41,71] | ↘ [c] |
| Iron + rHuEPO | ↗ [82,83] | ↗ [82,84] | ↗ [82,84] | ↗ [82,84] | ↔ [d] | ↘/↗ [82-84][e] | ↗ [82,84] | ↔ [b] | ↗/↔ [c] |
| ABT (reinfusion) | ↗ [19,25,37,85,86] | ↘ [37,85,86] | ↗ [85] | ↔/↘ [32,37,85] | ↗ [32,44,85] | ↗ [32,85] | ↘ [37,85] | ↗ [32] | ↘ [87] |
| ABT (withdrawal) | ↘ [14,23,85,86,88,89] | ↗ [14,85,86] | ↘ [37] | ↗ [85] | ↘ [85,90] | ↘ [85,88,91] | ↗ [85] | ↘ [88,89,91] | ↗ [46] |
| ABT (withdrawal) + iron | ↘/↗ [37,76,92] | ↗ [37] | ↘ [37] | ↗ [37] | ↔ [b] | ↔/↗ [41,76,92] | ↔ [b] | ↔/↗ [41,76,92] | ↔ [c] |

Abbreviations: HCT, hematocrit; s-EPO, serum erythropoietin.
[a] Hepcidin levels significantly increased 4 hours after rHuEPO injection and significantly decreased after 12 and 24 hours.
[b] Unknown.
[c] The variations in ERFE are based on the preliminary results from our laboratory.
[d] Personal communication.
[e] Plasma ferritin levels significantly increased in the intravenous iron + EPO group and significantly decreased in the EPO + oral iron group.

origin was related to pulmonary and liver tissues. The observed changes were detectable up to 3 days posttransfusion. Interestingly, these miRNAs could be combined with erythropoietin (EPO) concentration in a mathematical model to enhance the efficiency of ABT detection. These results provide the basis for using a combination of transcriptomic and hematological biomarkers in longitudinal measurements to detect ABT.

## Ironomics

Iron plays a key role in hemoglobin synthesis and in erythrocyte production. Iron metabolism is tightly regulated, and iron-related variables are therefore likely to be influenced by blood manipulation (Table 1). Transferrin is the main protein involved in the transport of iron in the circulation, and serum transferrin is a common biomarker of iron-related disorders [31]. When blood is transfused, the concentration of iron tends to increase and is associated with a rapid increase of transferrin saturation [32,33]. Iron required for the synthesis of hemoglobin is taken up by erythroblasts through the transferring receptor (TfR). Measurement of soluble TfR (sTfR) is proportional to the total mass of TfR cellular expression. This parameter is an interesting candidate for blood doping because it reflects the erythropoietic activity of the body [34]. Increased erythropoiesis caused by rHuEPO injections or blood withdrawal results in an increase in sTfR [35,36], and a decrease in erythropoietic activity, which is characteristic in blood transfusion, implicates a decrease in sTfR [37] (Table 1). On the other side, ferritin is an iron storage protein and is inversely correlated with transferrin. Small quantities of ferritin are also present in human serum and can serve as a potential biomarker of ABT and, in particular, blood withdrawal [32,38].

Hepcidin, a hepatic peptide that regulates the availability of iron to erythropoiesis by adaptation of iron absorption and recirculation [39], has been investigated as a new potential marker of blood transfusion [32]. Blood transfusion significantly increased hepcidin concentrations at 12 hours and 1 day postreinfusion by 7- and 4-fold, respectively, with no increase in inflammatory markers (C-reactive protein and white blood cells). This 25–amino acid peptide hormone is easily quantified in human plasma or serum using liquid chromatography combined with high-resolution mass spectrometry (LC-MS/MS), a technique implemented in most accredited laboratories [40]. Furthermore, hepcidin is correlated with ferritin and may serve as a marker of the iron repletion required for erythropoiesis [41].

Storage of RBC promotes the accumulation of so-called storage lesions that reduce their survival posttransfusion [42]. After transfusion, these impaired RBC are digested, and their iron content is released in blood, exhausting the iron binding capacity of plasma transferring receptor and inducing a transient increase of free iron [39,43]. Measurement of iron in serum or EDTA-plasma has thus been proposed as a rapid screening method for the detection of blood transfusion [32,44,45]. Recently, the newly discovered protein erythroferrone (ERFE), which is involved in iron metabolism and erythropoiesis, has emerged as an interesting candidate biomarker of blood manipulation [46]. Erythroferrone is produced by erythroblasts upon hypoxia or EPO stimulation and suppresses hepcidin expression; however, ERFE functions were exclusively investigated in a mouse model, and comparative studies in humans are currently underway in our laboratory.

Because all of these players in iron metabolism demonstrate high interindividual variation, individual follow-up of these variables in an "ironomics-based" approach appears to be an appropriate strategy in detecting blood manipulation. Nevertheless, before implementation of such a follow-up, the effects of confounding factors such as high-altitude training, physical exercise, or iron injections on the measurements of iron parameters should be explored. Differences in iron metabolism between men and women should also be well defined before any potential implementation of an ironomic passport.

## Plasticizers in Urine Matrix

In 2010, quantification of the plasticizer molecule di(2-ethyhexyl)phthalate (DEHP) and its metabolites in urine was proposed as an indirect method for the detection of ABT [47]. Blood bags used in the storage of RBCs are composed of polyvinyl chloride, to which softening substances such as DEHP are added. Long-term storage of blood in these bags leads to a diffusion of the phthalates into stored blood, which are subsequently transfused with blood. In the human body, DEHP is hydrolyzed to mono(2-ethylhexyl)phthalate (MEHP) and is subsequently oxidized and glucuronidated to mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP), mono-(2-ethyl-5-oxohexyl)phthalate (5oxo-MEHP), mono-(2-ethyl-5-carboxypentyl)phthalate (5-cx-MEPP), and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP) (Fig 1A), which are all markers of DEHP exposure [48]. Various studies have demonstrated the detection of these substances in urine for a short period of time after transfusion using LC-MS/MS [47,49,50]. The metabolites 5cx-MEPP and 2cx-MMHP are of particular interest due to their long half-life [48]. Although most metabolites are detectable several hours after transfusion (12 hours), these two molecules extend the detection window for up to 1 to 2 days [47,50].

Measurement of DEHP metabolites as a screening method for blood transfusion is the first approach to use urine matrix, which can be easily collected at antidoping controls. In contrast to flow cytometry, the LC-MS/MS–based method is cost-efficient and less time-consuming [23]. Furthermore, because plasticizers are exogenous compounds, the

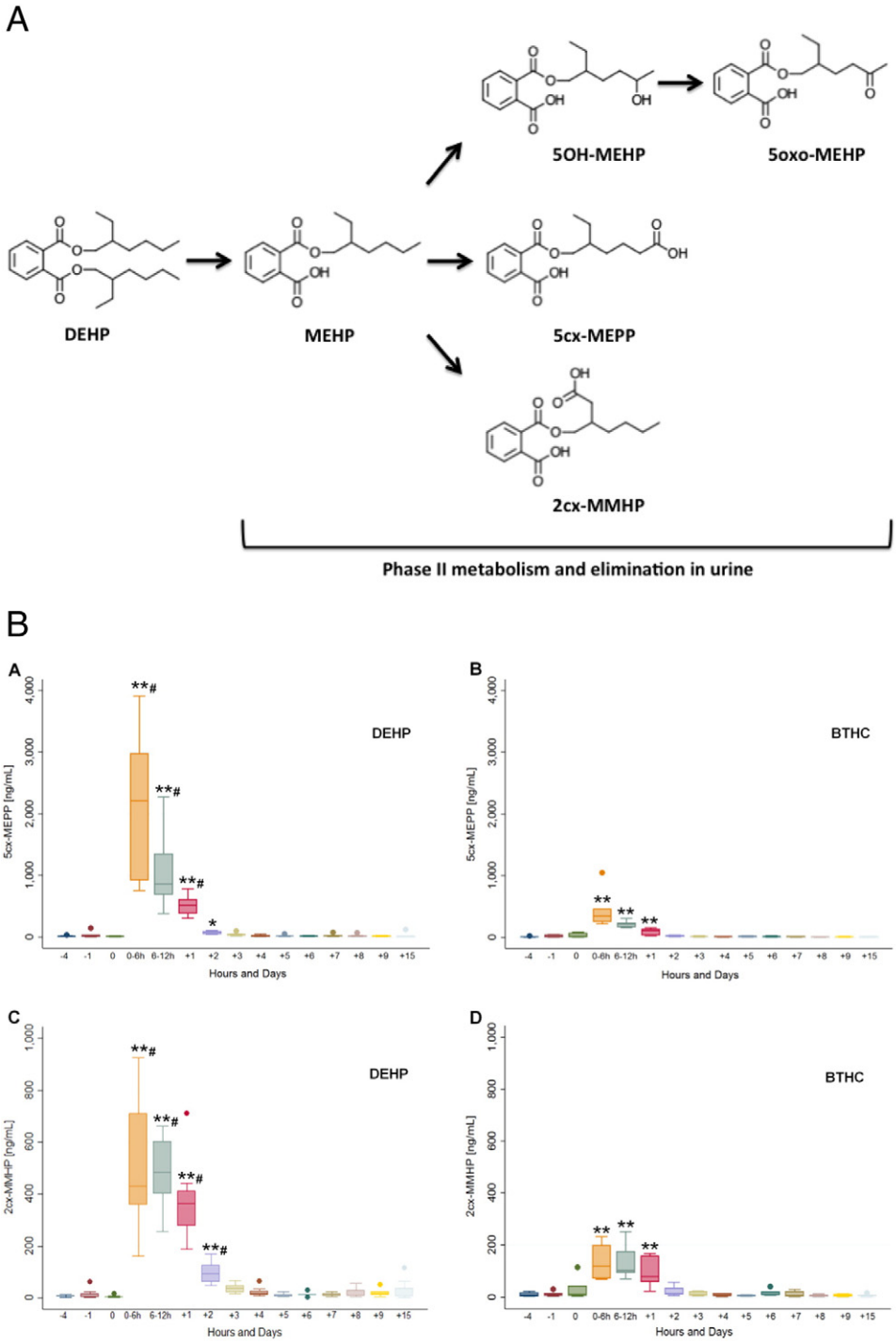


**Fig 1.** A, Di(2-ethyhexyl)phthalate metabolism. B, Di(2-ethyhexyl)phthalate metabolite concentrations before and after transfusion: 5cx-MEPP and 2cx-MMHP. Data were log transformed, and all concentrations were adjusted for specific gravity. 5cx-MEPP and 2cx-MMHP concentrations after transfusion with autologous blood stored in blood bags containing DEHP (A,C) or BTHC (B,D) are depicted in the box plots. The collection time point is indicated on the x-axis. $^{*}P < .05$; $^{**}P < .01$ compared to baseline (mean of the −4-, −1-, and, 0-hour data). $^{\#}P < .05$ compared with BTHC results at the same time point [50].

qualitative "dilute-and-shoot" screening approach can be applied and easily implemented in accredited laboratories [51–53].

Nevertheless, the main drawback of this detection method is that phthalates are ubiquitous and can be found in urine after occupational or dietary exposures [54]. Consequently, different studies have investigated normal daily exposure to plasticizers to define a threshold for each metabolite in athletes [50,51,55,56]. Using a control phase with saline infusion, Leuenberger et al [50] investigated the specificity of urinary DEHP quantification for the detection of blood transfusion. With the exception of 1 volunteer, metabolite concentrations were low, and the mean and median values of the 5 metabolites were similar to concentrations reported in a Spanish cohort [49], suggesting that contamination of DEHP from environmental exposure is uncommon.

With the recent commercialization of polyvinyl chloride blood bags containing *n*-butyryl-tri-(*n*-hexyl)-citrate (BTHC) instead of DEHP, athletes may prefer to use these plasticizer-free blood bags to avoid detection of DEHP metabolites after recent blood transfusion [57]. Leuenberger et al [50] measured considerable levels of urinary DEHP

metabolites up to 1 day after blood transfusion with BTHC blood bags (Fig 1B). The two long-term metabolites 5cx-MEPP and 2cx-MMHP were particularly sensitive for the detection of ABT with BTHC bags. Levels of DEHP were high in BTHC bags (6.6%), tubing of the transfusion kit (25.2%), and the white blood cell filter (22.3%). This suggests that urinary DEHP quantification is still effective even in cases where an athlete may shift to using labeled DEHP-free blood bags.

Longitudinal studies of plasticizers similar to those of the ABP appear to be an appropriate approach instead of setting absolute thresholds. This follow-up would indicate the usual degree of exposure of each athlete and provide additional evidence of suspicion when a sudden elevated value is found. Because blood transfusions are likely to occur shortly before important cycling events such as the Olympics or World Championships, a strategy implicating urine collection before and after the race with the quantification of plasticizers appears to be of practical relevance. Finally, athletes often justify an abnormal profile with a specific condition, such as fluid loss caused by gastroenteritis [10]. The addition of data from urinary phthalates to the adaptive model would, therefore, provide additional evidence of blood transfusion and decrease the probability that the observed variations are caused by natural processes or specific conditions. It has been proposed that urinary DEHP metabolites should be measured for each urinary antidoping analyses and that confirmatory analyses should be performed for each suspicious sample [50].

## Study of Storage Lesions Using Proteomics

The storage of RBCs triggers a series of biochemical and biomechanical changes in the cell, reducing subsequent in vivo survival and function [58]. These alterations include 2,3-diphosphoglycerate and adenosine triphosphate depletion, morphologic changes (crenation and spicule formation), accumulation of extracellular potassium, loss of membrane phospholipid, protein oxidation, and lipid peroxidation [58–60]. These changes may cause negative effects on transfusion, and their identification is imperative. Although hemolysis is a simple parameter to monitor, it does not reflect the profound molecular changes that affect RBC during storage [60]. Concerning the notion of "omics"-based methods [61], proteomics has been proposed as a potential tool to assess and identify RBC changes at the protein level during storage [62]. Alterations of RBC due to storage based on proteomics have been suggested as an indication of the use of blood transfusion for doping control purposes [63].

Using 2-dimensional electrophoresis gel and isobaric tags for relative and absolute quantification, Nikolovski et al [63] observed changes in 2 main protein groups composed of proteins decreasing and increasing during 42 days of storage. These 2 complexes included proteins that were primarily located in the cytoskeleton such as spectrin $\beta$, band 4.2, anykrin-1, tropomodulin-1, $\beta$ adducing, band 4.9 (dematin), tropomyosin, and transmembrane proteins (glycophorin C, aquaporin-1, and band 3). Oxidative degradation has been observed to be prevalent in band 4.2, to a minor extent in bands 4.1 and 3, and in spectrin after 7 days of storage [59]. Extending RBC storage to 14 days resulted in the identification of new fragments from $\beta$-actin, glyceraldehydes-3-phosphate dehydrogenase, band 4.9, and ankyrin. Peroxiredoxin 2 was also identified as a candidate biomarker of oxidative stress of stored RBC [64,65]. This antioxidant protein is normally located in the cytosol of RBC of healthy volunteers. During storage, Prdx2 migrates to the RBC membrane and may be used as a biomarker of blood transfusion if the blood bags have been stored for a sufficient period of time. The protein content of RBC-derived microvesicles may also reveal blood that has been stored for a certain period. A recent study highlighted differences between calcium-stimulated and storage-induced RBC-derived microvesicles and identified few differences in terms of lipid raft proteins, notably stomatin and flotillin-2 [66]. However, the detection window of these potential biomarkers has not been investigated yet.

Nevertheless, such phenomena are unlikely to occur when blood is stored under anaerobic conditions such as helium [59,67]. Furthermore, these hypotheses should be verified with individuals subjected to blood transfusion to determine the potential of proteomic-based methods to detect blood transfusion in athletes. Given that the reinfused blood constitutes only 4% (when 1 U is reinfused) of the circulating pool of RBCs and that approximately 20% of transfused RBCs are removed within the first 24 hours, changes based on proteomic methods will be difficult to detect [21].

## Combination of Biomarkers

The hematological module of the ABP relies on a combination of specific hematological parameters including [Hb], Ret%, OFF-score ($[Hb] - 60 \times \sqrt{Ret\%}$), and the abnormal blood profile score. On the basis of this model, future research using mathematical methods and algorithms should be conducted on possible combinations of the emerging markers mentioned in this review, which often demonstrate good sensitivity and poor specificity. Different biological sources such as urine, plasma, or whole blood will provide complementary data and increase the specificity and the discriminative performance of the ABP. The combination of longitudinal biomarkers improves diagnostic accuracy compared with use of a single marker and provides a powerful tool in personalized medicine [68].

The various emerging biomarkers of blood transfusion react differently and have a diverse range of detection windows (Fig 2). Markers that are measured several hours posttransfusion generally vary with important amplitude, whereas parameters that react days after blood reinfusion have weaker variations. Combining short-term and long-term biomarkers would thus improve the general detection window and the specificity-sensitivity relationship. Therefore, the combination of omics-based technologies with classic hematological variables will allow the identification of the biological fingerprint of blood transfusion (allogenic/autologous) and blood doping in general. Finally, this strategy will provide a powerful tool for targeting and intelligent testing strategies [13,61].

## Conclusion

Since the apparition of a direct detection method for rHuEPO, there is increasing evidence that athletes have returned to blood transfusions to artificially increase oxygen delivery to exercising muscle. Although homologous blood transfusion can be effectively detected by flow cytometry, there is no validated methodology currently available for the detection of ABT. The introduction of the hematological module of the ABP in 2008 based on longitudinal monitoring of blood-based parameters has provided an instrument for the detection of ABT and blood doping in general. This approach allows for the detection of both autologous and homologous blood transfusion and represents an advantage of being independent from the doping technique or the marketing of new pharmaceutical drugs.

This promising tool reaches its limits in situations where the athlete justifies abnormal blood values with specific conditions such as a recent stay at high altitude. Additional data would therefore be required to reveal whether an athlete has undertaken blood transfusion. Omics technologies provide a multitude of promising markers that may be used in an individual subject-based model and have the potential to significantly improve the ABP approach. Furthermore, urine DEHP metabolites offer a powerful complement to blood-based biomarkers in case of doubtful results. This strategy would be easily implemented in doping control laboratories. Furthermore, plasticizers may be integrated into the ABP.

## Conflict of interest

The authors have disclosed no conflict of interest.

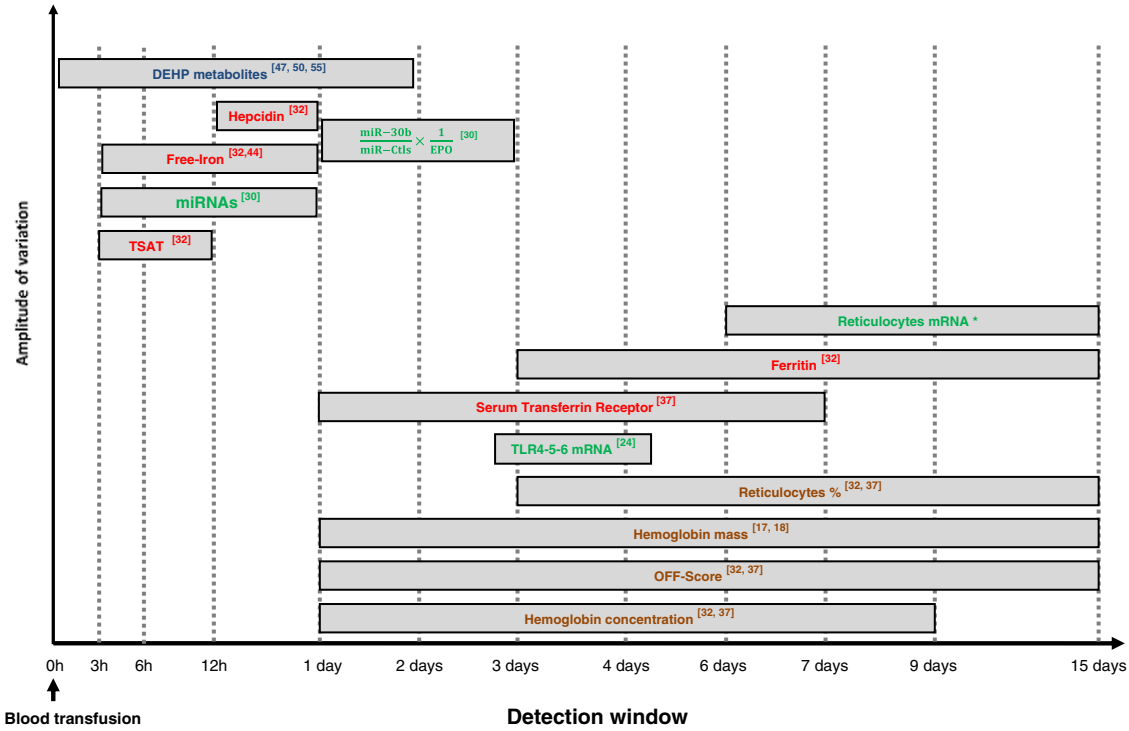


**Fig 2.** Detection window of emerging and implemented biomarkers of autologous blood transfusion. Biomarkers are classified according to their amplitude of variations and their detection window after blood reinfusion. Plasticizers are indicated in blue; iron metabolism, hematological, and transcriptomic variables are indicated in red, brown, and green, respectively. *Unpublished data. Abbreviations: mRNA, messenger RNA; TLR, Toll-like receptor; TSAT, transferrin saturation.

## Acknowledgments

Our researches on blood transfusion biomarkers were supported by Partnership for Clean Competition, World Anti-Doping Agency, and Département Universitaire de Médecine et Santé Communautaire.

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# Appendix II

Erythropoietin as a performance- enhancing drug: Its mechanistic basis, detection, and potential adverse effects

*Salamin O, Kuuranne T, Saugy M, Leuenberger N. Mol Cell Endocrinol. 2018 Mar 15; 464:75-87*

Molecular and Cellular Endocrinology 464 (2018) 75–87

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

# Erythropoietin as a performance-enhancing drug: Its mechanistic basis, detection, and potential adverse effects

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ARTICLE INFO

*Article history:*
Received 10 October 2016
Received in revised form
20 January 2017
Accepted 20 January 2017
Available online 22 January 2017

*Keywords:*
Erythropoietin
Doping detection
Athlete biological passport
Biomarkers
Omics technologies

ABSTRACT

Erythropoietin (EPO) is the main hormone regulating red blood cell (RBC) production. The large-scale production of a recombinant human erythropoietin (rHuEPO) by biotechnological methods has made possible its widespread therapeutic use as well as its misuse in sports. Since the marketing of the first epoetin in 1989, the development has progressed to the third-generation analogs. However, the production of rHuEPO is costly, and the frequent administration of an injectable formula is not optimal for compliance of therapeutic patients. Hence, pharmaceutical industries are currently developing alternative approaches to stimulate erythropoiesis, which might offer new candidates for doping purposes. The hypoxia inducible factors (HIF) pathway is of particular interest. The introduction of new erythropoiesis-stimulating agents (ESAs) for clinical use requires subsequent development of anti-doping methods for detecting the abuse of these substances. The detection of ESAs is based on two different approaches, namely, the direct detection of exogenous substances and the indirect detection, for which the effects of the substances on specific biomarkers are monitored. Omics technologies, such as ironomics or transcriptomics, are useful for the development of new promising biomarkers for the detection of ESAs. Finally, the illicit use of ESAs associates with multiple health risks that can be irreversible, and an essential facet of anti-doping work is to educate athletes of these risks. The aim of this review is to provide an overview of the evolution of ESAs, the research and implementation of the available detection methods, and the side effects associated with the misuse of ESAs.



## 1. Introduction

During the Mexico City 1968 Olympics, it became evident that the oxygen transport capacity of exercising muscles was the limiting factor for aerobic performances (Jokl et al., 1969). The performances were significantly affected by altitude-impaired oxygen delivery. Consistent with this phenomenon, Ekblom et al. reported that an increase in the hemoglobin (Hb) concentration can ameliorate the maximal oxygen uptake ($VO_{2max}$) and consequently athletic performance (Ekblom et al., 1972). Shortly thereafter, athletes started to experiment with blood transfusions to increase Hb for improved performance. However, this practice requires a sophisticated methodology and carries the risk of adverse effects. Following its global commercialization between 1987 and 1989, recombinant human erythropoietin (rHuEPO) use surged in the 1990s/2000s due to its easy access and its significant impact on performance. The drug was readily placed on the International Olympic Committee (IOC) list of banned substances in 1990, although no validated detection method was available. A decade later, a direct test to effectively differentiate between endogenous and recombinant EPO based on isoelectric focusing (IEF) was developed by Lasne and de Ceaurriz (Lasne and de Ceaurriz, 2000). Concurrently, the indirect detection of rHuEPO via blood markers of erythropoiesis was also established (Parisotto et al., 2000). In accordance with the indirect detection of blood doping, the Athlete Biological Passport (ABP) was developed and implemented in 2008 (Sottas et al., 2011). The hematological module of the ABP aims to assess the effects of blood manipulators, such as erythropoiesis-stimulating agents (ESAs), on selected biological parameters. The implementation of this tool provoked a trending change of cheating athletes that now use microdoses of rHuEPO to avoid large

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http://dx.doi.org/10.1016/j.mce.2017.01.033
0303-7207/

fluctuations in the levels of blood markers in the ABP and to reduce the detection window for classic direct detection (Ashenden et al., 2011). These microdoses are hardly detectable with classical hematological parameters, and new markers need to be developed to increase the sensitivity of the diagnostic tool. Recently, the pharmaceutical industry has developed new ESAs, which provide good candidates for doping. Alarmingly, some athletes have tested positive for some of these agents, although many are still in clinical trials. Consequently, anti-doping laboratories have to prepare for this situation and to develop new methods for the detection of ESAs. Omics technologies, such as transcriptomics and ironomics, offer promising candidates for the refinement of the hematological module. Although the microdosing trend has decreased the occurrence of death related to ESAs, their long-term misuse associates with significant adverse effects that can be irreversible. Therefore, anti-doping authorities play a crucial role in educating athletes about these risks.

## 2. Erythropoietin (EPO) and ESAs

### 2.1. Erythropoietin (EPO)

Human erythropoietin (HuEPO) is a glycoprotein hormone that is mainly produced by peritubular fibroblasts in the kidney. It stimulates the survival, proliferation, and differentiation of erythroblasts (Jelkmann, 2011). The molecule exerts its effects by binding to its receptor (EPOR), which triggers its activation, followed by the activation of different kinases and intracellular signaling pathways such as the Janus kinase (JAK)-2 and signal transducer and activator of transcription (STAT)-5 pathways (Debeljak and Sytkowski, 2012). Besides its hematopoietic effects, EPO-EPOR signaling functions in several tissues and systems, including the central nervous system, heart, kidney, gastrointestinal system, reproductive tract, and endothelium (Ogunshola and Bogdanova, 2013; Arcasoy, 2008). Moreover, the pleiotropic effects of erythropoietin (EPO) are not only mediated by an endocrine, but also by autocrine and paracrine mechanisms (Sytkowski, 2007).

Endogenous HuEPO production is mainly regulated by hypoxia and this regulation occurs at the transcriptional level (Jelkmann, 2011). When the oxygen supply of the human body decreases, transcriptional factors defined as hypoxia-inducible factors (HIFs) are activated, inducing the expression of several genes that include the *Epo* gene. Under normoxic conditions, GATA-2 and NF-κB suppress the *Epo* promoter, and HIF prolyl hydroxylases (HIF-PH) degrade the HIFα subunit (La Ferla et al., 2002). Under hypoxic conditions, on the other hand, the oxygen-dependent activity of HIF-PH shuts down, which stabilizes HIFα and triggers its dimerization with HIFβ and *Epo* transcription.

The resulting mature EPO protein consists of 165 or 166 amino acids and contains three N-linked and one O-linked oligosaccharide side chains. The molecular weight of the protein is approximately 34 kDa, with the glycans representing 35–40% of the molecular mass of the glycoprotein. N-glycosylation is particularly important for the biological activity of EPO, because it regulates its affinity for the receptor, clearance, and serum half-life (Franz, 2009).

### 2.2. First-generation recombinant EPO

The human *Epo* gene was first isolated and cloned in 1985, which enabled the transfection of mammalian cell lines, including Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, for the production of recombinant human EPO (Lin et al., 1985; Jacobs et al., 1985). Because post-translational modifications depend on the enzymatic make-up of the cells performing the synthesis, the use of various cell lines results in differences in glycosylation and sulfation of the protein (Skibeli et al., 2001; Kawasaki et al., 2001). As a result, the biochemical and biological properties can differ between epoetins produced in different cells. Depending on the composition and nature of the glycosylation pattern, commercially produced epoetins are followed by a Greek letter (α, β, δ, ω, ζ).

In 1989, epoetin alfa was the first recombinant EPO to become commercially available. CHO cell-produced epoetin alfa was subsequently approved by the United States Food and Drug Administration (FDA) for the treatment of anemic patients with chronic kidney disease (CKD). It was then approved for the treatment of anemia associated with chemotherapy in cancer patients or with antiviral treatment in HIV patients and for the preparation of autologous blood donation prior to surgery. The treatment decreases the risks of recurrent blood transfusions and iron overload, and improves the quality of life (Bonomini et al., 2016). The serum half-life of epoetin alfa is approximately 8 and 24 h when the product is administered intravenously or subcutaneously, respectively (Franz, 2009). The bioavailability of epoetin alfa is low, and up to three injections per week are needed to attain therapeutic effects (Macdougall et al., 1991). For doping uses, the short elimination time is advantageous, because the detection window is narrowed, whereas the effect is sustained (Fig. 1).

In 1990, epoetin beta became commercially available. Although epoetin beta was also expressed in CHO cells, its glycosylation and biological activity were somewhat different compared to those of epoetin alfa. These properties did not significantly improve the clinical characteristics of the product. In the 1990s, and particularly in 2004 when the patents for epoetin alpha and beta expired, several companies started to produce copies of rHuEPO with different post-translational modifications. For example, epoetin omega was produced in BHK cells, whereas epoetin delta was expressed in the HT-1080 human cell line (Sikole et al., 2002; Smith et al., 2007). These differences in the manufacturing processes created differences in glycosylation and yielded several EPO isoforms. However, none of these products succeeded in improving the biological activity or the half-life of rHuEPO.

### 2.3. Second-generation recombinant EPO

To overcome these pharmacological limitations and reduce the frequency of troublesome administrations, the pharmaceutical industry urged researchers to develop a modified recombinant EPO with a longer half-life. This goal was reached when the novel erythropoiesis-stimulating protein (NESP or darbepoetin alfa) became available. Five amino acids in the protein backbone of EPO were mutated, enabling the addition of two more N-linked carbohydrate chains with terminal sialic acid residues. These changes increased its molecular mass (while lowering its receptor affinity), circulating half-life by 3-fold, and *in vivo* potency (Egrie and Browne, 2001; Egrie et al., 2003). Due to the longer half-life, the dosing intervals were decreased to once weekly or monthly. However, this was not desirable for illicit doping use, because it increased detection window and the risk of being tested positive (Fig. 1). The launching of darbepoetin alfa resulted in the first collaboration between a pharmaceutical industry (Amgen) and anti-doping laboratories.

### 2.4. Third-generation recombinant EPO

The third-generation rHuEPO continuous erythropoietin receptor activator (CERA) was launched on the European market in 2007 (Jelkmann, 2012). The drug consists of CHO cell-produced epoetin beta linked to methoxy polyethylene glycol (PEG). Pegylation results in a molecular size of approximately 60 kDa, leading

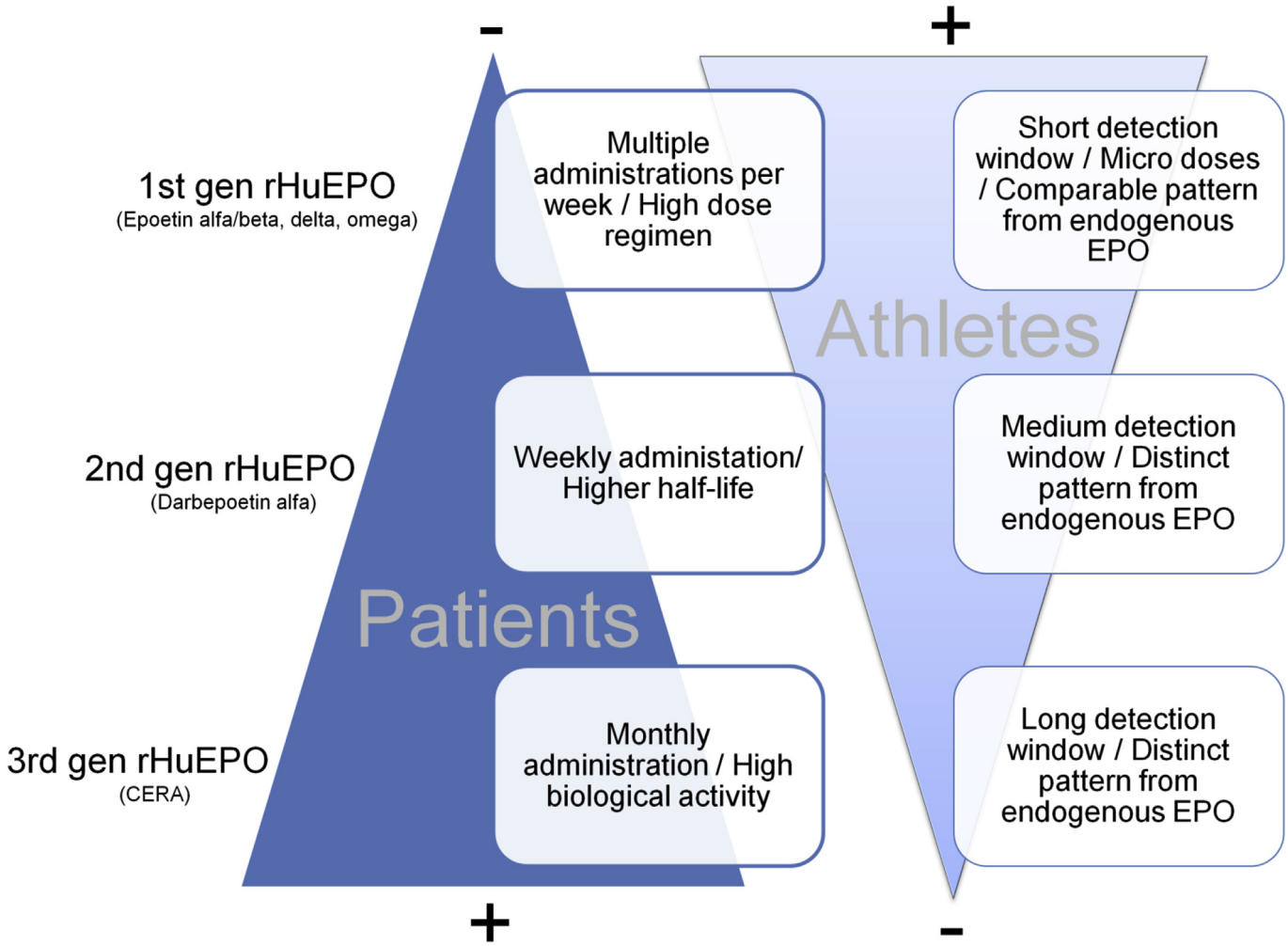


**Fig. 1. Advantages and disadvantages of the three different generations of recombinant human EPO for athletes and patients**. The benefits are inversely correlated between patients suffering from chronic anemia and cheating athletes for the different generations of rHuEPOs. The first generation is advantageous for athletes, because of its short detection window and its similarity to endogenous EPO. However, high doses are needed to treat anemia, and it requires multiple uncomfortable injections per week. The benefits are reversed with longer half-life rHuEPOs.

to a lower affinity for the EPO receptor, hindering its glomerular filtration by the kidneys, and increasing its serum half-life (Macdougall, 2005). The exceptional stability of CERA in the blood results in a mean serum half-life of 70–122 h, which decreases its administration to once monthly or less frequently (Macdougall et al., 2006). Because of its wider detection window, CERA is less likely to be used for doping purposes (Fig. 1). The manufacturer (Roche) developed a CERA-specific ELISA for pharmacokinetic studies and provided it to the World Anti-Doping Agency (WADA) before the marketing of the drug.

### 2.5. Erythropoietin-mimetic peptides (EMPs)

Another strategy to stimulate erythropoiesis is the use of EPO biomimetic peptides (Wrighton et al., 1996). Peginesatide (trade name: Hematide, Affymax/Takeda) is a synthetic pegylated dimeric peptide-based ESA that was approved by the FDA in 2012 for the treatment of anemia caused by CKD (Fan et al., 2006; Macdougall et al., 2013). The molecule has no sequence homology with EPO, and is therefore unlikely to induce a cross-reactive immune response against endogenous or recombinant EPO (Macdougall et al., 2009). Although the structure of peginesatide differs from that of EPO, it can stimulate EPOR dimerization and activate similar intracellular signaling pathways to induce erythropoiesis (Fig. 2) (Green et al., 2012).

A study on the safety and pharmacodynamics of peginesatide reported a prolonged half-life and delayed clearance (Stead et al., 2006). The same study demonstrated that the injection of peginesatide was associated with an increase in Hb in healthy patients, whose level was sustained for longer than a month. The monthly administration of the drug was able to maintain the Hb level in anemic patients with CKD undergoing hemodialysis and those not receiving dialysis (Macdougall et al., 2013; Fishbane et al., 2013). However, Affymax and Takeda voluntarily recalled peginesatide from the market 1 year after its commercialization due to serious, life-threatening reactions (Takeda, 2013).

Before the drug was licensed, peginesatide was included on the Prohibited List of the WADA in 2009 (WADA, 2009). There were indeed rumors that cheating athletes were using unlicensed substances, including peginesatide (Benkimoun, 2009). Nevertheless, despite its withdrawal from the market, there are still suspicions about the compound being used for doping purposes (Leuenberger et al., 2012).

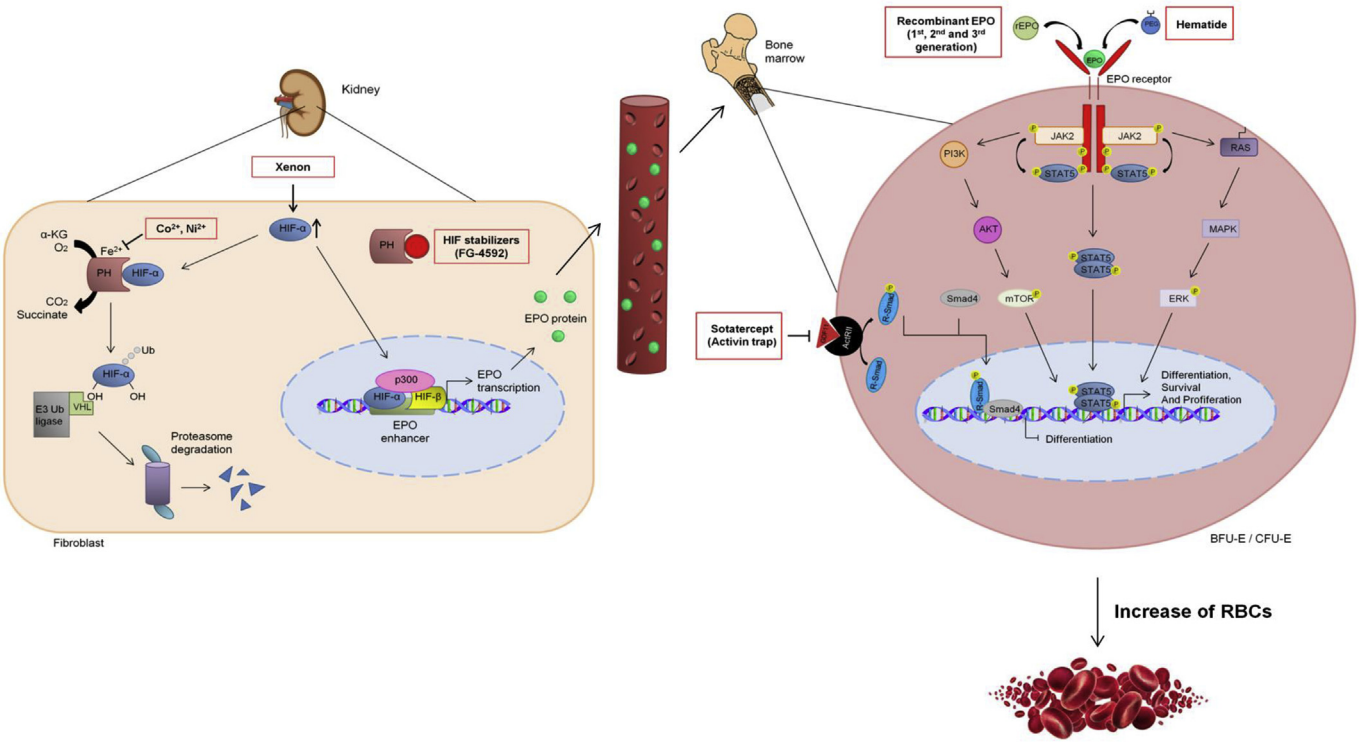


**Fig. 2. Schematic model of the control of red blood cell production and the sites of action of erythropoiesis-stimulating agents**. EPO is produced in the kidney under the control of HIFs which can be modulated by different substances. As a circulating endogenous hormone, EPO enters the bone marrow and activates the EPO receptor in BFU-E and CFU-E cells. Signal transduction through the JAK2/STAT5 pathway induces the differentiation, survival, and proliferation of red blood cell precursors. Recombinant human EPO and EPO biomimetic peptides also activate EPOR. Sotatercept prevents the binding of GDF-11 to its receptor and blocks the inhibition of differentiation of erythroblasts (Shenoy et al., 2014).

### 2.6. HIF stabilizers

HIFs are transcription factors that coordinate the physiological response to hypoxia, resulting in increased EPO production in the liver and kidney, enhanced iron uptake and utilization, and changes in the bone marrow (BM) environment that facilitates erythroid progenitor maturation and proliferation (Haase, 2013). As discussed in section 2.1, HIF levels are regulated by the hydroxylation state of the HIFα subunit under the control of prolyl hydroxylases (HIF-PH). The catalytic activity of HIF-PH depends on the presence of 2-oxoglutarate and oxygen among others (Fe(II)) (Bruegge et al., 2007).

To block HIF-PH activity and stabilize the HIFα subunit, chemical competitors of 2-oxoglutarate have been developed (Mole et al., 2003). These compounds are defined as HIF stabilizers, and they mimic the hypoxia-driven expression of endogenous EPO in the kidney (Fig. 2) (Bonomini et al., 2016; Rabinowitz, 2013). The unique feature of HIF stabilizers is that they are administered orally to stimulate erythropoiesis with EPO at physiological concentrations compared to the high concentrations observed with standard ESA therapy (Bonomini et al., 2016; Unger et al., 2010). Therefore, they are interesting compounds for pharmaceutical companies but also for doping purposes. Although HIF stabilizers are in the clinical trial phase of development, they are available on the black market. For these reasons, HIF stabilizers were classified as prohibited substances by the WADA in 2011 (WADA, 2016a, b).

Several HIF stabilizers are currently being developed by different pharmaceutical companies (FG-4592 and FG-2216 by FibroGen, AKB-6548 by Akebia Therapeutics, GSK 1278863 by GlaxoSmithKline, BAY 85-3934 by Bayer Pharmaceuticals). FibroGen has made the most progress in this area (Buisson et al., 2016). Both oral drugs are intended to treat anemia in patients with CKD. FG-2216 was the first promising molecule in this category, but it was withdrawn after a case of fatal hepatitis (Bonomini et al., 2016). Currently, roxadustat (FG-4592) is in a phase 3 clinical trial for the treatment of anemia in patients with CKD (FibroGen, 2016). In a phase 2 randomized placebo-controlled study, roxadustat at doses of 0.7–2.0 mg/kg administered orally two or more times weekly increased the Hb level in non-dialysis-dependent subjects without intravenous iron supplementation (Besarab et al., 2015). The drug was well tolerated without adverse effects normally associated with EPO analogs. Moreover, oral administration of the compound reduced the serum hepcidin level, increasing the availability of iron for erythropoiesis.

Products labeled “FG-4592” are already available on the black market and represent a threat to be used for doping purposes. This was recently substantiated by the first reported case of doping with FG-4592 (Buisson et al., 2016).

In addition to oxygen and 2-oxoglutarate, Fe (II) is an essential co-factor for HIF-PH activity. Fe (II) is also reversibly linked to the active site of the metalloenzyme. Metal ions, such as $Co^{2+}$ or $Ni^{2+}$, can reduce iron availability by competitive substitution, stabilize HIF transcription factors, and increase EPO gene expression (Fig. 2) (Beuck et al., 2012). Another mechanism of action involves the direct binding of cobalt to HIFα. Nevertheless, chronic cobalt exposure can lead to severe toxic effects, and its use as a hypoxia-mimetic agent is thus limited to experimental applications.

Recently, xenon gas was identified as an activator of the HIF pathway and therefore a stimulator of red blood cell (RBC) production. It came to the public's attention during the past Olympics (2014), following claims that some athletes inhaled this gas for performance-enhancing purposes. Xenon exerts its effects by

stimulating HIF-1α translation, rather than preventing its degradation (Fig. 2) (Jelkmann, 2014). Although there are no human data on the effects of xenon on the serum level of EPO, xenon was classified as a HIF activator on the Prohibited List by the WADA in 2014.

### 2.7. Activin traps

The current ESAs are not effective for all anemic patients and have limitations, including undesirable and adverse effects. There is thus the need for agents that treat anemia for which conventional ESAs are not effective such as thalassemia. Members of the transforming growth factor beta (TGF-β) family, such as activins, growth differentiation factors (GDFs), or bone morphogenic proteins (BMPs), are potential modulators of adult erythropoiesis via the intracellular SMAD signaling pathway (Fig. 2) (Maguer-Satta et al., 2003).

Sotatercept (ACE-011, Acceleron and Celgene Corp) is a dimeric fusion protein consisting of the extracellular domain of the human activin receptor IIA (ActRIIA) linked to the Fc portion of the human immunoglobulin G1 (IgG1) antibody. It interferes with downstream signaling cascades, in particular the SMAD pathway, by sequestering activin (Fig. 2) (Raje and Vallet, 2010). Sotatercept was mainly developed to increase bone mineral density. In a phase 1 clinical trial involving healthy postmenopausal women, a single dose of ACE-011 resulted in a dose-dependent increase in the biochemical markers of bone formation and a decrease in the biochemical markers of bone resorption. Surprisingly, the treatment provoked an increase in Hb and hematocrit (Hct) levels and RBC number (Ruckle et al., 2009). These increases persisted for approximately 3 months. Using a murine ortholog of ACE-011 (RAP-011), preclinical studies explored the potential cellular and biochemical mechanisms by which sotatercept regulates erythropoiesis (Carrancio et al., 2014). Mice treated with RAP-011 exhibited a rapid increase (within 24 h) in Hct and Hb levels, and RBC number, accompanied by a rapid stimulation of late-stage erythroid precursors in the BM. RAP-011 at a dose of 30 mg/kg induced a significant increase in erythroid burst-forming units and EPO.

In a phase 2 trial involving healthy postmenopausal women, sotatercept rapidly increased erythropoiesis dose-dependently, which persisted for up to 4 months, suggesting that ActRIIA ligands are important negative regulators of erythrocyte levels in healthy individuals (Sherman et al., 2013). This compound has a long half-life, which contributes to the sustained erythropoietic response and allows a less frequent dosing schedule compared to classic ESAs. These results indicate that the mechanism underlying the erythropoietic effects of sotatercept, which is an alternative for the treatment of anemia associated with various pathologies, is different from that of conventional ESAs (Fields et al., 2013). Recent phase 2 clinical trial demonstrated its benefits in chemotherapy-induced anemia (Raftopoulos et al., 2016). This approach also provides a new product for athletes to artificially increase their Hb content.

### 2.8. EPO gene doping

Advances in DNA manipulation have extended the benefits of gene therapy to various common diseases such as anemia. The treatment of anemia with recombinant HuEPO is relatively expensive, involving continuous monitoring and repeated administration. In addition, the doses often overly raise or lower the serum EPO (s-EPO) level. Nevertheless, EPO gene therapy constitutes an attractive approach for continuous secretion where a single administration of the EPO gene would ensure long-term delivery and a steady-state EPO level.

Vectors (Repoxygen) in which murine *Epo* expression was under the control of the Oxford BioMedical hypoxia response element (OBHRE) were developed for the treatment of anemia in mice (Binley et al., 2002). Mindful of the potential of this approach to increase the endogenous EPO levels and RBC production, gene therapy was placed on the Prohibited List by the WADA in 2006.

An autologous *ex vivo* strategy was used in the first *Epo* gene therapy trial on patients with CKD (Jelkmann and Lundby, 2011). Dermal cores (Biopump) were transfected with an adenovector designed to express EPO under the control of the cytomegalovirus (CMV) promoter (Lippin et al., 2005). The dermal cores were harvested and then implanted into CKD patients, resulting in a significant increase in the s-EPO level for up to 14 days.

In conclusion, it was demonstrated that *Epo* gene therapy is effective for the treatment of anemia in CKD patients, suggesting that gene doping is possible. However, the efficacy, safety, and immunogenicity of *Epo* gene transfer have not yet been explored in detail. It seems therefore unlikely that this practice is currently being used by doping athletes.

## 3. Testing procedures

### 3.1. Direct detection methods

#### 3.1.1. IEF and sodium dodecyl sulfate (SDS)/sarcosyl (SAR)-polyacrylamide gel electrophoresis (PAGE)

The first method to detect rHuEPO in specimens from doping athletes was developed in 2000 by Lasne et al., more than 10 years after the beginning of rHuEPO abuse in sports (Lasne and de Ceaurriz, 2000; Lasne et al., 2002). IEF, a separation technique, exploits the differences in carbohydrate composition between rHuEPO and endogenous HuEPO. These modifications result in a change of the final net charge of the molecule at a certain pH that can be observed by IEF, in which proteins are separated according to their isoelectric point (pI) (Pascual et al., 2004). Indeed, there are fewer acidic isoforms of rHuEPO than urinary HuEPO (Reichel, 2011). Consequently, the overall charge of rHuEPO is less negative than that of HuEPO, and the isoforms of rHuEPO migrate differently from those of HuEPO in the electric field (Pottgiesser and Schumacher, 2013).

Due to the large volume of urine and the low EPO concentration in this specimen, the urine is first concentrated by ultracentrifugation and the resulting retentate is then separated on an IEF gel with a pH gradient ranging from 2 to 6 (Franz, 2009). This separation produces a distribution of various isoforms, which generate the profile of a particular EPO analog. After double-blotting using the monoclonal AE7A5 antibody, the isoforms are visualized by chemiluminescence. The result of this analysis is an image of the IEF pattern of the EPO molecules present in the urine (Lasne and de Ceaurriz, 2000). Due to its greater overall negative charge, HuEPO migrates in a more acidic region of the gel, whereas the main rHuEPO (α, β, ω) isoforms migrate in a more basic area (Fig. 3). With its additional N-linked carbohydrate chains, darbepoetin alfa has a greater negative charge than HuEPO and migrates in a more acidic region of the IEF gel (Catlin et al., 2002) (Fig. 3). However, the method is not useful for the detection of epoetin delta, which is produced in human cell line, because its IEF pattern partially overlaps with that of endogenous HuEPO (Franz, 2009). Proteinuria, either disease-related or exercise-induced, can also affect the IEF distribution leading to an atypical IEF profile (Lamon et al., 2009; Beullens et al., 2006).

Another approach based on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the mobilities of target analytes was developed to complement the IEF assay (Kohler et al., 2008). SDS-PAGE was developed in the 1970s for the

separation of proteins according to their molecular weights. Using mass spectrometry, it was observed that the molecular weights of rHuEPOs were higher than those of HuEPO due to differences in the glycosylation pattern, and these differences could be detected in the urine and/or blood using SDS-PAGE (Leuenberger et al., 2012; Reichel, 2011; Kohler et al., 2008). Two internal standards were used, enabling the calculation of the relative mobility values and the discrimination of the analytes in the specimens (Fig. 4) (Reichel, 2011; Tsitsimpikou et al., 2011). Negative feedback of rHuEPO-administration could be detected as the absence of HuEPO-bands on the gel, and consequently during washout period, an intense rHuEPO band evolved in a glycoform smear above the HuEPO band (Fig. 4) (Leuenberger et al., 2012).

Both methods generally follow the same workflow (concentration by ultracentrifugation, separation, single/double blotting, chemiluminescent detection), except for the different principles of separation (charge versus molecular mass). Unlike in IEF-PAGE, urine and blood samples analyzed by SDS-PAGE must be first immunoaffinity purified (an ELISA plate or anti-EPO monolithic disk) due to the high protein content (Lonnberg et al., 2010). Vogel et al. proposed an alternative purification method using EPO receptor-coated magnetic beads (Vogel et al., 2014).

The two complementary methods can easily detect first- and second-generation recombinant EPO. They can even detect gene therapy-derived EPO isoforms that are different from the natural hormone (Lasne et al., 2004). CERA is also different from the endogenous molecule with respect to its IEF pattern and relative mobility due to its pegylated structure. However, the main disadvantage for detecting CERA is that it is too large (~60 kDa), not filtered by the kidneys, and not excreted in the urine, except in cases of exercise-associated proteinuria (Lamon et al., 2009; Bellinghieri et al., 2008). Therefore, the detection method required amendment regarding the matrix of choice; by adding a step of immunopurification, Lasne et al. were able to study the isoelectric profiles of EPO and CERA in human serum samples (Lasne et al., 2007). To separate proteins according to their molecular weight, SDS was replaced with sarcosyl (SAR) because it does not bind to the PEG moiety of CERA. Contrary to SDS, SAR only binds to the amino acid chain of the PEGylated protein, thus leading to enhanced antibody binding and a sharper electrophoretic band (Reichel, 2012; Reichel et al., 2009).

### 3.1.2. *Membrane-assisted isoform immunoassay (EPO WGA MAIIA)*

Researchers in Sweden have developed a new test called the membrane-assisted isoform immunoassay (EPO WGA MAIIA) (Franco Fraguas et al., 2008; Lonnberg et al., 2012). The test combines a chromatographic separation of the glycosylated isoforms of EPO with wheat germ agglutinin (WGA) and a sensitive lateral flow immunoassay using anti-EPO carbon black nanostrings. This assay is executed on a dipstick (Lonnberg et al., 2012). Lectins are proteins possessing at least one domain with carbohydrate binding properties. They can interact reversibly and specifically with carbohydrate groups in different glycocompounds (Franco Fraguas et al., 2008). Thus, this assay enables researchers to differentiate endogenous EPO from recombinant EPO based on the affinity of different isoforms with lectin, which is due to the differences in terminal sialic acid residues and poly-N-acetyl lactosamine (LacNAc) residues (Reichel, 2011; Dehnes et al., 2013). rHuEPO isoforms interact more strongly with WGA than HuEPO. Darbepoetin alfa has the strongest affinities for WGA, while the affinity of CERA for WGA is lower than that of HuEPO (Franco Fraguas et al., 2008; Dehnes et al., 2013).

Lectin-bound EPO isoforms are eluted with sugar N-acetyl glucosamine (GlcNAc) at two different concentrations (low/high) for each sample. A low concentration of GlcNAc buffer will elute only EPO isoforms with a low affinity for WGA, whereas a high concentration will elute all EPO isoforms The eluted EPO isoforms react with an immobilized anti-EPO antibody in the capture zone, and the EPO isoforms are detected with another anti-EPO labeled antibody with carbon black nanostrings (Dehnes et al., 2013). The scanner image is used for quantification, and the intensity of the signal is proportional to the concentration of bound EPO.

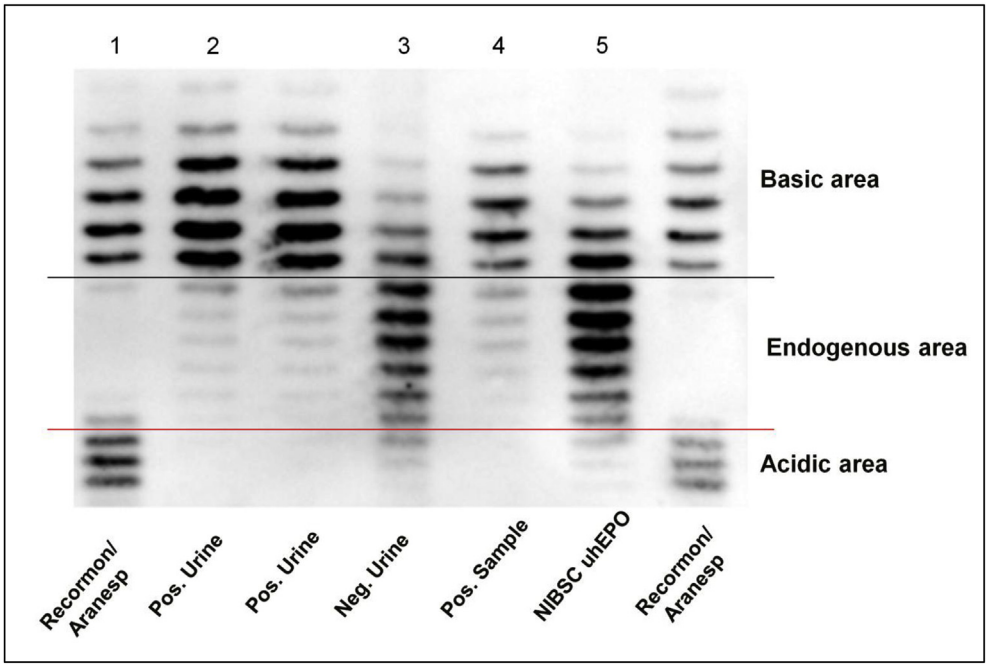


**Fig. 3. Example of isoelectric focusing analysis of a sample positive for rhEPO.** Lane 1: Reference marker corresponding to a mix of epoetin beta (Recormon) and darbepoetin alfa (Aranesp). Lane 2: human urine spiked with epoetin beta. Lane 3: endogenous urinary EPO. Lane 4: sample positive for epoetin alfa/beta. Lane 5: urinary EPO standard (National Institute for Biological Standard and Control; NIBSC).

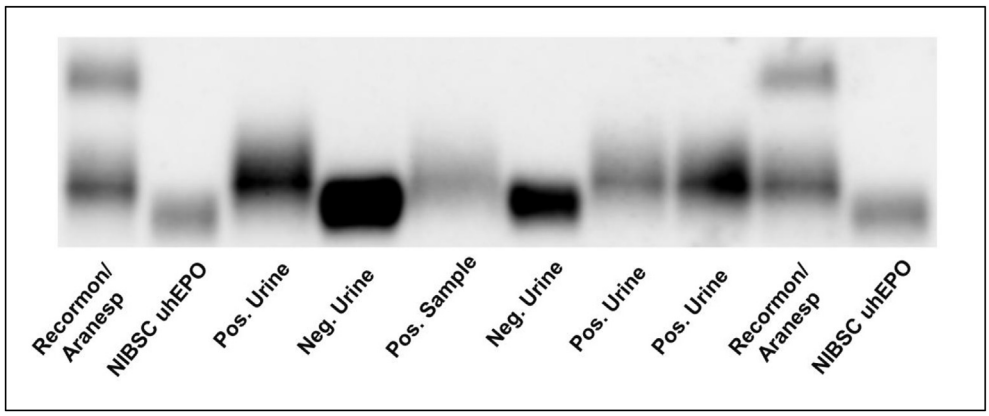


**Fig. 4. Example of SDS-PAGE analysis of a sample positive for epoetin alpha/beta**. A mix of epoetin beta (Recormon) and darbepoetin alfa (Aranesp) is used as reference standard. Urinary EPO standard (NIBSC) and endogenous EPO (negative urine) are used as negative control. The positive sample is characterized by mixed bands creating a glycoform smear above the endogenous band. Positive urine corresponds to urine spiked with epoetin beta.

Urine and serum/plasma specimens are compatible with the EPO WGA MAIIA assay but require immunoaffinity purification using anti-EPO monolith columns. Compared to the current WADA-accredited IEF and SDS/SAR-PAGE methods, the MAIIA assay can quickly screen a batch of 15 immunopurified samples (controls included) within 1 h (Dehnes et al., 2013). Therefore, the EPO WGA MAIIA has the sensitivity and potential required for a high-throughput screening test to complement IEF and SDS/SAR-PAGE. In their study evaluating the MAIIA dipstick test for the detection of microdose quantities of rHuEPO in plasma, Ashenden et al. proposed a longitudinal evaluation of the percentage migration isoforms (PMI) score and to interpret the results against the subject's historical values rather than a population-based threshold similar to that of the ABP for an optimal sensitivity (Ashenden et al., 2012). Compared to SDS-PAGE, the scores from the MAIIA test are more difficult to interpret than the bands from the electrophoresis gel, and they are more difficult to defend in court. Although the method needs further improvement, the dipstick test seems to be an interesting complement to the existing antidoping tests.

#### 3.1.3. *Liquid chromatography-tandem mass spectrometry (LC-MS/MS)*

As previously discussed, the pharmaceutical industry is focusing on the development of small molecules that directly and indirectly stimulate RBC production, such as HIF stabilizers and erythropoietin-mimetic peptides (EMPs), in addition to their efforts in recombinant EPO production. These peptides are readily filtered by the kidney and are present in the urine, and they can be detected with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although most of these new molecules are still under investigation, they are available on the black market. For these reasons, the use of these agents by athletes was prohibited by the WADA, and accredited anti-doping laboratories have taken preventive measures and developed methods for doping control (Beuck et al., 2011; Moller et al., 2011).

Based on the collaboration between the pharmaceutical industry and anti-doping laboratories, Hematide/peginesatide detection assay was developed and validated using ELISA and immunopurification followed by SDS-PAGE for the confirmation of results (Leuenberger et al., 2011a; b). Together with this approach, a LC-MS/MS method incorporating proteolytic digestion was introduced for the detection of Hematide in plasma and then further developed for the detection of the compound in dried blood spots (DBSs) (Moller et al., 2011; Moller et al., 2012). After being prohibited for 5 years, the first case of an athlete testing positive for the HIF stabilizer FG-4592 was reported (Buisson et al., 2016). LC-MS/MS was used to detect and confirm the presence of FG-4592 in the urine, while there were no profound alterations in the hematological parameters of the ABP. This case highlights the sensitivity and efficiency of the direct LC-MS/MS measurement of an exogenous substance, but also the limitations of the ABP. Regarding the activin traps and the potential misuse of sotatercept as a new doping agent, a research project was recently funded by the WADA to develop initial testing and confirmatory procedures using LC-MS/MS (Reichel, 2016). Although the direct identification of exogenous agents by LC-MS/MS was shown to be very efficient, the main disadvantage is that the method requires reference material, which is often available only from the pharmaceutical companies developing these products. The analytical task of the rHuEPO, especially for the first-generation compounds, is in the specificity of LC-MS/MS, as the differences with HuEPO are restricted to only a few sugar moieties. However, natural or artificial (darbepoetin alfa) changes in the amino acid sequence can be distinguished by mass spectrometry (Guan et al., 2009). This was successfully used in the detection of rHuEPO in horse plasma and urine specimens (Yu et al., 2010; Bailly-Chouriberry et al., 2012). Indeed, the method exploits the species-specific differences between human and horse EPO for detecting the presence of rHuEPO, NESP, and CERA in equine plasma where the specific task cannot be compared to the context of human doping control (Guan et al., 2010).

### 3.2. *Indirect detection methods*

#### 3.2.1. *Athlete biological passport*

During the development of the IEF direct detection method for recombinant EPO, Parisotto et al. indirectly detected the drug using different blood markers of altered erythropoiesis (Parisotto et al., 2000; Parisotto et al., 2001). By combining several indirect hematological variables, two models (ON/OFF-models) were created to detect rHuEPO during the administration phase and the wash-out period. The ON-model took into account reticulocyte hematocrit (RetHct), s-EPO, serum soluble transferring receptor (sTFR), and Hct values as well as the macrocyte percentage (%Macro), while the OFF-model took into account RetHct, s-EPO, and Hct levels. With the availability of direct detection methods and the potential availability of indirect detection methods, the doping habits of endurance athletes shifted to low doses of rHuEPO (i.e.,"microdosing") and forced researchers to propose new models.

Consequently, Gore et al. introduced second-generation blood tests, in which the OFF-hr score was analyzed (Gore et al., 2003).

This score derived from an algorithm ($Hb - 60\sqrt{(Ret\%)}$) is useful, because it can detect rHuEPO many weeks after the injections have stopped. Thereafter, researchers investigated the possibility of comparing an athlete's hematologic values against his/her own historical baseline, rather than a population-derived threshold to enhance the ability to detect rHuEPO (Sharpe et al., 2006; Robinson et al., 2002). This third-generation test aimed to distinguish the effects of rHuEPO abuse from the natural biological fluctuations of Hb and OFF-hr. In 2006, Sottas et al. updated the ABP concept by introducing wider set of data from a single blood profile of an athlete in a multiparametric score called the abnormal blood profile score (ABPS) (Sottas et al., 2006). The ABPS could incorporate 3 to 12 different blood markers influenced either by rHuEPO administration or blood transfusion.

In 2008, the Union Cycling International (UCI) was the first sports organization to implement the hematological module of the ABP to detect blood doping, followed 1 year later by the WADA. The hematological module involves the longitudinal monitoring of eight markers of erythropoiesis including Hct, Hb, RBC count, Ret%, Ret count (Ret#), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), from which OFF-hr and ABPS scores can be calculated (Saugy et al., 2014). The profile of each athlete is longitudinally evaluated using an adaptive model based on Bayesian inference techniques that considers previous values and identifies the pattern of blood manipulation (Saugy et al., 2014). A case is deemed suspicious if a particular value lies outside of the defined range, requiring review by a panel of experts, which may then lead to an anti-doping rule violation (WADA, 2016a, b). Finally, a longitudinal follow-up of the athlete's blood markers should demonstrate that the athlete is in a healthy physiological condition that is unaltered by performance-enhancing drug (Sottas et al., 2011).

The hematological module enables more sensitive and extended detection of EPO doping than non-targeted direct methods that often suffer from a short detection window (Fig. 5) (Reichel, 2011). Indeed, the ABP benefits from the fact that the effects of a drug on the metabolism last longer than its presence in the urine or blood. Furthermore, this new paradigm bears the advantage of already being sensitive to the marketing of any future compound that could potentially increase performance. Finally, the longitudinal monitoring of hematological parameters, Hb, Ret%, and the OFF-score is much more sensitive than the no-start rule such as 50% Hct introduced by the UCI in the mid-1990s.

Although the hematological module of the ABP is valuable, it is often criticized, requiring constant revision to accommodate the introduction of new markers of altered erythropoiesis. Ashenden et al. evaluated the sensitivity of the hematological module to flag microdoses of rHuEPO administered to athletes. They concluded that it was still possible for athletes to use rHuEPO and bypass abnormal changes in the blood variables currently monitored by the ABP (Ashenden et al., 2011). These limits were confirmed in the athlete that used HIF stabilizer FG-4592, whose hematological parameters demonstrated no profound alterations (Buisson et al., 2016). Moreover, it was recommended that seasonal changes in the hematological parameters due to training and competition should be taken into account (Banfi, 2011; Sanchis-Gomar et al., 2011). In addition to Hb and Ret%, a new marker of altered erythropoiesis caused by rHuEPO or blood transfusion, the total mass of hemoglobin ($Hb_{mass}$) was proposed especially for its independence of plasma fluctuations. Nevertheless, the variability observed over time when assessing $Hb_{mass}$ was not justified in an anti-doping setting (Lundby and Robach, 2010), and the measurement of the variable based on carbon monoxide (CO) rebreathing method was too complicated to be implemented.

### 3.2.2. Ironomics

The production of RBC is tightly linked to the metabolism of iron, an element essential for Hb synthesis and erythroid proliferation. Furthermore, endogenous EPO regulates RBC production by inducing the proliferation and differentiation of erythroid progenitor cells and controlling cellular iron metabolism (Weiss et al., 1997). Hence, the exogenous administration of rHuEPO or ESAs greatly affects the physiology of iron metabolism and iron-related variables.

Transferrin (Tf) and ferritin are the main cargo proteins of blood iron in the organism. Serum Tf and ferritin are common biomarkers of iron-related disorders (Szoke and Panteghini, 2012). The two variables are inversely correlated; transferrin is the transport protein of iron in the circulation, while ferritin is the iron storage protein. Their production is regulated by the iron response element/iron response protein (IRE/IRP) regulatory system. Erythroblasts take up iron required for Hb synthesis via the transferrin receptor (TfR). The soluble form of TfR (sTfR) reflects the total cellular expression of TfR and represents an interesting candidate for blood doping, because it reflects the erythropoietic activity of the organism (Salamin et al., 2016a, b, c). Thus, the administration of ESAs results in an increase of sTfR through the activation of IRP-1 (Parisotto et al., 2000; Weiss et al., 1997; Dehnes and Hemmersbach, 2011). On the other hand, serum ferritin decreases after the administration of ESAs due to the mobilization of iron reserves for the production of new RBCs. It is another potential biomarker of altered erythropoiesis (Fig. 5). The re-infusion of an athlete's own blood also affects erythropoiesis and iron-related variables such as sTfR and serum ferritin. The monitoring of these two parameters could therefore assist in targeting this doping method for which no direct detection method is available (Salamin et al., 2016a, b, c).

Hepcidin, a peptidic hormone secreted by the liver, regulates the availability of iron for erythropoiesis by altering iron absorption and recirculation (Waldvogel-Abramowski et al., 2014). The peptide binds to the iron exporter ferroportin, and induces its internalization and degradation, thus blocking iron efflux (Robach et al., 2013). Erythroid cell proliferation dramatically alters iron metabolism to satisfy the high demand for iron during the synthesis of Hb, and thus influences the secretion of hepcidin by the liver. Indeed, rHuEPO decreases hepcidin expression 24 h after subcutaneous or intravenous injections (Robach et al., 2013; Laine et al., 2012). Other ESAs, such as HIF stabilizers or sotatercept, also decrease hepcidin expression (Buisson et al., 2016; Jelkmann, 2015) Therefore, hepcidin may be a new potential marker for the detection of ESAs (Fig. 5). The size of the peptide hormone (25 amino acids) allows for its quantitation in human plasma or serum using LC-MS/MS, a technique that is available in all accredited anti-doping laboratories. Furthermore, the hepcidin level strongly correlates with the ferritin level, and may serve as a marker of iron repletion that is required for erythropoiesis (Salamin et al., 2016a, b, c).

For some time, the factor responsible for the inhibition of hepcidin by erythropoietic signals in humans remained unknown. A previous study identified erythroferrone (ERFE) as the erythroid regulator of iron metabolism (Kautz et al., 2014). This protein, which is produced by erythroblasts upon hypoxia or EPO stimulation, suppresses hepcidin expression. ERFE may be a key regulatory protein in the release of stored iron release and appears to be an interesting candidate biomarker of blood manipulation. A recent study demonstrated an association between ERFE and biomarkers of erythropoiesis and iron metabolism after ESA treatment in patients on hemodialysis (Fig. 5) (Honda et al., 2016). The ERFE levels significantly increased from day 3 of treatment with darbepoetin alfa and CERA. Detailed comparative studies in healthy humans are currently ongoing.

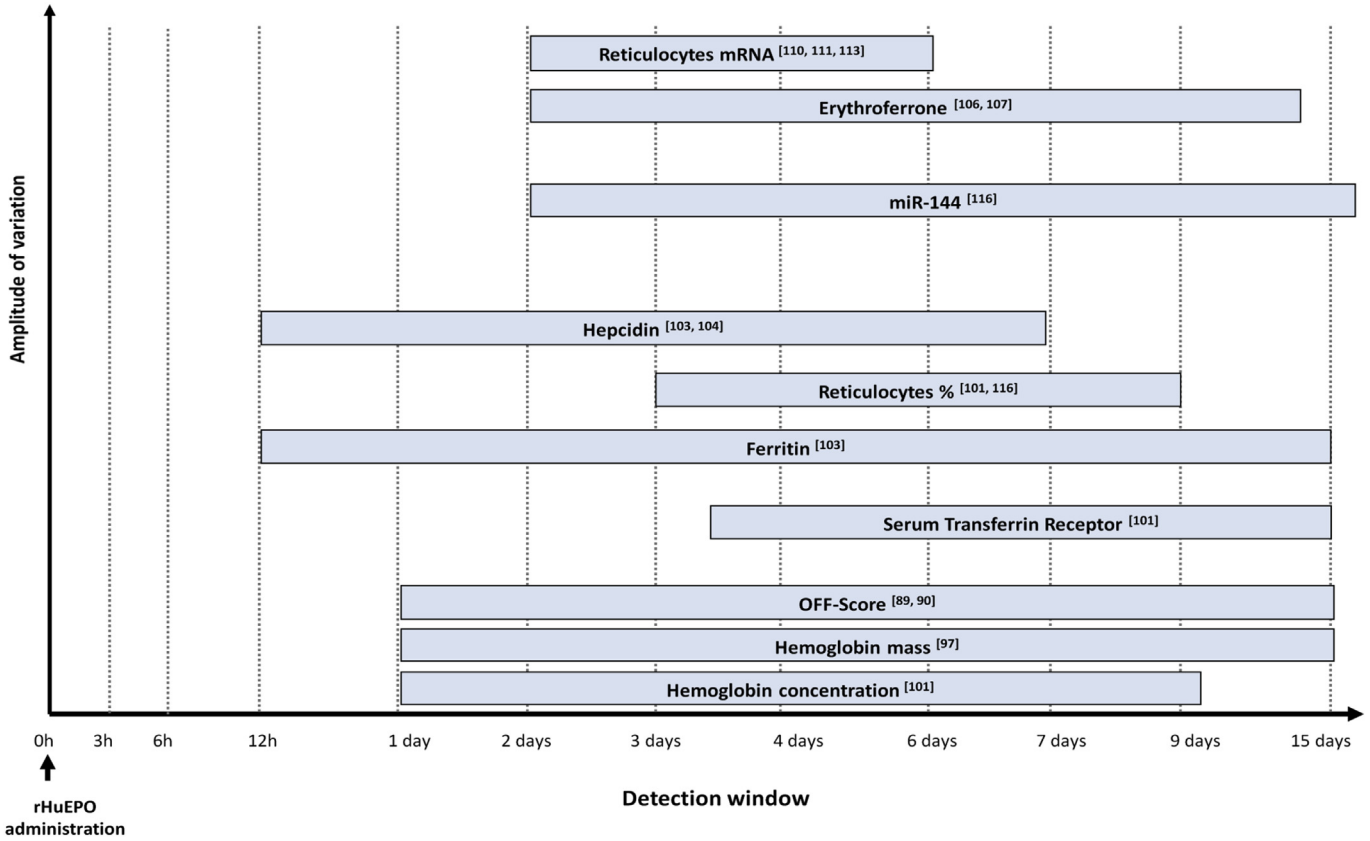


**Fig. 5. Detection window of validated and emerging biomarkers of rHuEPO abuse**. The different biomarkers are classified according to their fold-change and the duration of their detection window after rHuEPO administration.

Similar to the changes in hematological parameters after altered erythropoiesis, iron-related variables demonstrate high inter-individual variability, suggesting that individual longitudinal monitoring of these parameters by an "ironomics-based" approach may supplement the hematological module of the ABP for the detection of blood manipulation with ESAs. However, the effects of heterogenous factors, such as exercise, altitude-training, iron injections, or gender on these variables should be studied before any implementation of such a follow-up.

### 3.2.3. *Transcriptomics (messenger RNA (mRNA), microRNA (miRNA))*

Anti-doping authorities are continuously forced to revise the existing assays and develop new tests to reach the athletes that successfully bypass anti-doping detection methods. The genome is the genetic material of the organism, and includes genes and non-coding DNA. Genes code for proteins and are first transcribed into RNA. The transcriptome represents the set of all RNA transcripts, including non-coding RNA, and the quantification of the total RNA complement in a cell, tissue, or organism is defined as transcriptomics. Although the genome is unchanging, the transcriptome is constantly in a state of flux and is highly reflective of the cell milieu and metabolic activity (Rupert, 2009). Therefore, considering that doping agents affect messenger RNA expression, the transcriptional profiling of erythroid target genes may be a promising tool for the discovery of new biomarkers for the detection of blood doping (Fig. 5).

ESAs stimulate the production of RBCs and immature RBCs known as reticulocytes. After these immature cells expel their nucleus, they still contain functional residual nucleic acid material that can be analyzed (Lee et al., 2014). Varlet-Marie et al. investigated changes in the expression of reticulocyte genes after rHuEPO administration (Varlet-Marie et al., 2004). Using a serial analysis of gene expression (SAGE) library of human reticulocytes, three erythroid gene markers (hemoglobin subunit beta (*HBB*), ferritin light chain (*FTL*) and ornithine decarboxylase antizyme (*OAZ*)) were selected and their levels were quantified by real time-polymerase chain reaction (RT-PCR) in whole blood during and 3 weeks after drug administration. The selected genes showed a characteristic profile in subjects using rHuEPO compared to the placebo group. The *OAZ* mRNA expression profile was strongly indicative of rHuEPO treatment. Thereafter, 95 genes differentially expressed after the administration of high and microdoses of rHuEPO were identified using three SAGE libraries and confirmed by quantitative RT-PCR (Varlet-Marie et al., 2009). The microarray-based significance analysis identified 33 markers genes for the detection of rHuEPO among which five remained differentially expressed during the microdose regimen. A similar approach was applied for the long-term detection of rHuEPO abuse in horseracing (Bailly-Chouriberry et al., 2010).

Recently, Durussel et al. identified, replicated, and validated the whole blood transcriptional signature of rHuEPO in two distinct populations comprising Caucasian endurance-trained males at sea level and Kenyan endurance runners at moderate altitude, all of whom received rHuEPO injections for 4 weeks (Durussel et al., 2016). Transcriptional profiling showed that hundreds of transcripts were altered by rHuEPO in both cohorts. The expression pattern was characterized by a rebound effect, with a profound upregulation during rHuEPO injections and a subsequent down-regulation for up to 4 weeks after administration. A transcriptomic-

based approach was also applied to autologous blood transfusion (ABT) with some success. Salamin et al. measured the expression of a subset of genes involved in RBC metabolism using digital multiplex mRNA counting technology after ABT and demonstrated that changes in transcript number were more significant than those in hematological parameters (Salamin et al., 2016a, b, c). Another pilot study demonstrated that blood reinfusion altered the expression profile of T lymphocytes. This was based on the hypothesis that exposure of cell detritus derived from stored blood can induce a cellular and molecular immune response (Pottgiesser et al., 2009).

Non-coding RNAs can also serve as potential biomarkers of doping methods. MicroRNAs (miRNAs) are small non-protein coding RNAs that function in the post-transcriptional modulation of gene expression. These molecules can be detected in the blood plasma or serum, and they have emerged as sensitive markers of various pathophysiological conditions. The usefulness of the circulating miRNAs as markers of altered erythropoiesis was first investigated by Leuenberger et al. (Leuenberger et al., 2011a, b). Using miRNA microarrays and quantitative RT-PCR, a significant change was observed in the plasma levels of several miRNAs in subjects receiving CERA injections. In particular, a specific miRNA, miR-144, exhibited a significant increase that lasted 27 days after CERA injection (Fig. 5). Different studies demonstrated that circulating miRNAs could also be used as biomarkers of ABT (Leuenberger et al., 2013), testosterone administration (Salamin et al., 2016a, b, c), and growth hormone treatment (Kelly et al., 2014).

A longitudinal monitoring of these transcriptomic biomarkers can complement the hematological parameters of the ABP, although further studies are needed to address the effects of possible confounding factors. The combination of transcriptomic, iron-related, and hematological biomarkers could reflect the general effects of ESAs on athletes and provide a longer detection window than direct detection methods (Fig. 5).

## 4. Adverse effects of ESAs

The public often forgets that ESAs are first intended to ameliorate the quality of life in patients suffering from a particular syndrome. Molecules designed to increase erythropoiesis are mainly used to treat anemia in patients with CKD or undergoing chemotherapy. Under normoxic physiological conditions, Hb levels are endogenously regulated by the blood oxygen level via the HIF system. ESA injections shock the system, and increase the erythrocyte number and the transport capacity of oxygen, which increases blood viscosity and the probability of thromboembolic events. Although professional athletes gravitate toward microdosing regimens that require medical supervision, amateur athletes may use high doses of these compounds and often according to anecdotal information. Besides increasing blood viscosity, long-term use of ESAs can result in various side effects such as red cell aplasia and heart failure (Tsitsimpikou et al., 2011; Locatelli and Del Vecchio, 2003). In individuals with an iron deficiency, epoetin can elevate thrombocyte counts and increase the risk of cardiovascular problems, including cardiac arrest, seizures, arrhythmia, hypertension, congestive heart failure, vascular thrombosis, myocardial infarction, and edema (Franz, 2009; Tsitsimpikou et al., 2011; Streja et al., 2008). Moreover, EPO is also involved in angiogenesis (Hardee et al., 2007), and EPO withdrawal may lead to neocytolysis (Trial et al., 2001). Furthermore, the combination microdoses of rHuEPO with other substances, such as testosterone or transfused blood, can have harmful consequences for the individual. The administration of ESAs to subjects with naturally high endogenous levels of EPO can also cause serious adverse effects. Finally, endogenous EPO is depleted upon the administration of rHuEPO through a negative feedback mechanism and this inhibition can be irreversible after long-term treatment (Tsitsimpikou et al., 2011).

Another major concern is the availability of non-approved compounds on the black market for which clinical studies are not completed. The use of these drugs in healthy individuals may show unexpected side effects (Franz, 2009). For example, HIF stabilizers may possess tumorigenic potential, because HIFs activate hundreds of genes coding proteins involved in carcinogenesis and neovascularization. The administration of sotatercept may also induce adverse effects in healthy individuals, because the mechanism of action for erythropoiesis is still not completely understood.

Last but not least, the source of the drugs used by athletes may raise some concerns. Indeed, epoetins produced from non-controlled sources lack sufficient quality control and may contain impurities such as bacterial endotoxins (Tsitsimpikou et al., 2011). In some compounds, aggregated proteins were detected, which may trigger the onset of anti-EPO antibody-induced pure red cell aplasia (PRCA) (Jelkmann, 2015). Moreover, subcutaneous injections are not recommended for several biosimilar epoetins and the lack of information on these compounds may lead to serious health risks.

Human nature is marked by the will to win, often without regard for the risks involved and their consequences on health. It is fitting to assume that athletes can become addicted to performance-enhancing drugs (Gil et al., 2016). From a psychological point of view, an athlete performing well in a doped-state will be tempted to repeat the doping in future competitions. It is therefore important to educate athletes on the risks of performance-enhancing drugs.

## 5. Conclusions and perspectives

Increasing the Hb level is one of the most effective ways of enhancing performance in endurance sports. rHuEPOs can markedly enhance oxygen availability to muscles. However, they constitute a major challenge for anti-doping authorities. The availability of a direct test to detect rHuEPO has revolutionized the fight against this dangerous substance and provoked a change in the doping habits of cheating athletes. Currently, athletes gravitate toward microdosing to decrease the detection window and to avoid large fluctuations in their hematological parameters. The development of novel ESAs has provided new ways of boosting the Hb level, which has been met with the development of new direct detection methods, such as LC-MS/MS in addition to immunological analyses. Several ESAs that are still in clinical trials have been detected in athletes indicating that collaboration between pharmaceutical companies and anti-doping laboratories is key. The companies should inform anti-doping laboratories about potential performance-enhancing products in clinical trials and provide access to the molecules prior to marketing, which may support new doping research.

The introduction of the hematological module of the ABP has offered a new approach for the indirect detection of blood doping and ESAs. The individual longitudinal monitoring of the different parameters ensures that athletes compete in a non-doped state. The proper functioning of the ABP requires close cooperation between the Athlete Passport Management Unit (APMU), which associates with a WADA-accredited laboratory or a national anti-doping organization (NADO), and anti-doping organizations (ADOs), international sports federations (IFs), and the WADA. The APMU reviews the passports in the adaptive model and advises the ADO on intelligent testing strategies (Saugy et al., 2014). The main asset of this tool is that it is independent from new doping drugs on the market. This promising tool is pushed to the limits of sensitivity in the detection of rHuEPO use in microdoses. Additional data

would therefore supplement the ABP and offer new evidence of ESA use by athlete. Several markers originating from studies using omics technologies such as ironomics or transcriptomics could be introduced in an individual subject-based model to complement the ABP for the detection of ESA misuse.

## Conflict of interest

The authors disclose no conflict of interest.

## Funding

Our researches transcriptomics and ironomics were supported by World Anti-Doping Agency (12C14NL) and Département Universitaire de Médecine et Santé Communautaire (06/2015).

## Acknowledgements

The authors are grateful to Professor Marc Froissart for the critical reading of the manuscript.

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## Appendix III

Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competition

*Salamin O, Garcia A, González-Ruiz V, Rossi F, Bigard X, Déglon J, Daali Y, Faiss R, Saugy M, Rudaz S. Drug Test Anal. 2020 Nov;12(11-12):1649-1657*

Received: 3 July 2020 | Revised: 21 August 2020 | Accepted: 21 August 2020

DOI: 10.1002/dta.2923

SPECIAL ISSUE - RESEARCH ARTICLE

WILEY

# Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competitions

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**Funding information**
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## Abstract

Tramadol is a synthetic opioid drug used in the treatment of chronic and acute pain. An abnormal prevalence of its misuse in elite sport to overcome pain resulting from prolonged physical effort was recently reported. However, besides its antinociceptive effects, tramadol consumption is associated with negative effects such as numbness, confusion, and reduced alertness. This fact prompted the Union Cycliste Internationale to ban the use of tramadol in cycling competitions. Herein, we present the development of a dried blood spot (DBS) sample collection and preparation method followed by a liquid-chromatography mass spectrometry (LC-MS) analysis to rapidly determine the presence of tramadol and its two main metabolites in blood samples. The detection window of each analyte was evaluated and the analysis of performance on various MS platforms (HRMS and MS/MS) was assessed. Tramadol and its two main metabolites were detected up to 12 h after the intake of a single dose of 50 mg of tramadol in positive controls. In professional cycling competitions, 711 DBS samples collected from 361 different riders were analysed using the developed methodology, but all returned negative results (absence of parent and both metabolite compounds). In the context of professional cycling, we illustrate a valid method bringing together the easiness of collection and minimal sample preparation required by DBS, yet affording the performance standards of MS determination. The proposed method to detect tramadol and its metabolites was successfully implemented in cycling races with a probable strong deterrent effect.

**KEYWORDS**

cycling, DBS, metabolites, tramadol, UHPLC-MS/MS

## 1 | INTRODUCTION

Tramadol is a synthetic, centrally acting opioid used to treat moderate to moderately severe pain. This analgesic drug has a dual analgesic action via two complementary mechanisms: the binding to the μ-opioid receptor through its active metabolite exerts the opioidergic action, whereas inhibition of serotonin and norepinephrine reuptake via tramadol itself contributes to the euphoric effect.[1] Tramadol is actually a prodrug submitted to *O*- and *N*-desmethylation by the isoforms 2B6, 2D6 and 3A4 of cytochrome (CYP) P450. CYP2D6

  

catalyses the *O*-desmethylation reaction, which yields the active metabolite (M1—*O*-desmethyl tramadol). M1 has a higher affinity for the μ-receptor than the parent drug and is thus the principal contributor to the opioid effect of the drug. CYP2B6 and CYP3A4 are responsible for *N*-desmethylation of tramadol to the inactive *N*-desmethyl tramadol (M2). The large phenotypic variations of CYP2D6 enzyme among the population affect the pharmacokinetics of tramadol and its metabolites resulting in analgesic variability.[2]

Pain-alleviating effect of tramadol supplemented with a well-being feeling and euphoria could be particularly appealing in the context of sporting performance.[3] Indeed, it is extensively recognised that tramadol is widely used in sports to treat minor injuries or to reduce muscle and joint pain during and after exercise.[4] Although the analgesic effect might be favourable to improve resistance to exercise, use of tramadol might also alter attention and generate dizziness and drowsiness, affecting the safety of the athletes during the competition. The chronic use of tramadol for the management of exercise-induced pain also increases the risk of developing physical dependency and addiction.[5]

There is besides a large body of evidence underlining the misuse and abuse of this medication in cycling with testimonies from professional cyclists and cycling team doctors.[6] Although a direct causality is hard to prove, an altered alertness or dizziness may definitely increase the risk of crashes in cycling with a major concern for the safety of a peloton. The latter may also explain the concomitant use of caffeine and pseudoephedrine in addition to tramadol by cyclists to minimise side effects of the opioid and with a possible synergetic effect on nociception.[7,8] The Monitoring Program of the World Anti-Doping Agency (WADA) indicated a prevalence of use of tramadol in-competition of 4.3% in cycling accounting for 56% of all tramadol findings in urine (>50 ng/ml) for Olympic sports.[4] There is however only scarce and unequivocal evidence on a putative ergogenic effect of tramadol to enact a global interdiction by WADA.[3,9] Nevertheless, taken together, the available data supported the introduction by the Union Cycliste Internationale (UCI) of a medical rule banning tramadol in competition in force as of 1 March 2019 to preserve the safety of the cyclists in competitions with the set-up of its detection in dried blood spots (DBS).[10,11] The penalties in case of positive control for tramadol use are diversified and are applied according to a possible recurrence. A first offence by a rider is penalised with a disqualification from the event in addition to a fine of CHF5,000 if the rider is a member of a UCI-registered team (otherwise CHF1,000). A second offence is sanctioned by a disqualification from the event and a 5-month suspension. A further offence will result in a 9-month suspension. Finally, if two riders belonging to the same UCI-registered team are tested positive for tramadol within a 12-month period, the team is fined CHF10,000, and in case of further offence within the same period of 12 months, the team is suspended for a period of 1 to 12 months depending on the decision of the UCI Disciplinary Commission.

Results obtained from urine samples are somewhat biased by drug excretion rates, thus making it difficult to infer at what time the medication was administered and when it was exerting its effect. On the contrary, the detection of a given drug in blood is comparatively more informative of the pharmacokinetics status and would therefore be the matrix of choice to prove the usage during a sport competition. To overcome challenges related to the collection of whole blood/plasma samples in sports competitions, DBS after a single finger prick offer a convenient alternative in terms of sample collection, shipment and chemical stability at room temperature. In addition, compound recovery from DBS requires minimal sample preparation, and the resulting extracts can be directly analysed using liquid chromatography (LC) hyphenated to mass spectrometry (MS).[12]

The present article describes the development and application of a method for the determination of tramadol and its two major metabolites in human blood samples using DBS. The workflow for DBS analysis was developed with LC-high resolution MS (HRMS) and then transferred to LC-MS/MS for a sensitive detection of tramadol and its metabolites in DBS. While QqQ- or Qtrap-based instrumentation is widely represented and remains the gold standard for drug quantification in analytical laboratories, the use of HRMS detection allows the development of workflows that deliver informative untargeted data and that can be used for deciphering wide and finer biological answers in the case of doping.[13] Performance of either platform can be then assessed when working with the one or the other. Finally, we aim to present the results for all DBS samples collected in professional cyclists in competitions during the 2019 competitive season and analysed with the developed method between March and November 2019.

## 2 | MATERIAL AND METHODS

### 2.1 | Reagents

Water ($H_2O$), methanol (MeOH) and acetonitrile (MeCN) were obtained from Fisher Scientific (Loughborough, UK). Ammonium hydroxide, tramadol, *O*-desmethyl tramadol, *N*-desmethyl tramadol and *O*-desmethylvenlafaxine were purchased from Sigma-Aldrich (Wesel, Germany). Venlafaxine was obtained from Lipomed (Arlesheim, Switzerland). Internal standard (IS), tramadol-$^{13}$C-$d_3$ was obtained from Cerilliant (Round Rock, TX, USA). Fresh drug-free blood with EDTA as anticoagulant was supplied by the University of Lausanne (Lausanne, Switzerland).

A stock solution at 1 mg/ml of each compound was prepared in MeOH; intermediate solutions at appropriate concentrations were prepared through consecutive dilution of the stock solutions in MeOH. Working solutions containing mixes of standard compounds were also prepared in MeOH. Stock solutions, intermediate and working solutions were stored at −20°C.

Formic acid was from Biosolve (Dieuze, France). Ammonium formate buffer 2.5 mM was prepared with an adequate volume of ammonium hydroxide 28% and the pH adjusted to 9.0 with formic acid.

Spiked blood required for the calibration and QCs was prepared using fresh EDTA whole blood and then spotted onto filter card. DBS

calibration standards and QCs were prepared using separate working solutions. Peak areas of each analyte were corrected using IS.

## 2.2 | DBS sample pretreatment

Microsampling HemaXis DB10 kits were purchased from DBS System SA (Gland, Switzerland). These wallet-format kits incorporate a microfluidic chip and a standard Whatman® protein saver card 903™ (MA, USA), thus revealing very well adapted for on-field sampling and storage. This device, which is CE mark, allows to collect four whole blood spots with a standardised volume of 10 μl.

A 10-μl volume of real or spiked whole blood was spotted onto the filter card using either a micropipette or the HemaXis DB10 device. The DBS samples were let dry for at least 1 h at room temperature and then stored at 4°C in sealable plastic bags.

A paper disc comprising the entire DBS was punched with a 8-mm diameter hole puncher around the blood spot. The spot was folded using tweezers and inserted into a 300-μl vial with insert. For the extraction, 100 μl of MeOH containing internal standard at 2 ng/ml was added. The spot was incubated during 15 min, while vortexing for 5 s every 5 min. Following incubation, 50 μl of the supernatant was collected in a new vial and mixed with 50 μl of $H_2O$. The vial was then sealed and positioned in the LC rack.

## 2.3 | UPLC-HRMS conditions

DBS analysis was performed using a UPLC-HRMS set-up consisting of an H-Class UPLC system from Waters (Milford, MA, USA) and a Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation was performed using an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm, 130 Å, also from Waters) kept at 45°C. Mobile phase A was an aqueous solution containing 2.5-mM ammonium formate (pH 9), and mobile phase B was MeCN. Flow rate was set at 400 μl/min, and the gradient increased linearly from 10% to 80% B over 2.75 min. These conditions were maintained for 1 min before re-equilibration for 2 min at initial conditions.

Analytes were detected using positive electrospray ionisation (ESI) in full scan (FS) acquisition mode. A probe temperature of 150°C was used on the heated ESI source (HESI II). Sheath gas pressure, auxiliary gas pressure and weep gas flow were set to 5, 3 and 0 arbitrary units, respectively. The ion spray voltage was set to 3 kV, the capillary temperature was set to 250°C and the S-Lens RF level was 50%. Full-scan mass spectra (m/z 200–350) were acquired using a mass resolution of 70,000 (full width at half maximum) at m/z 200, with a maximum injection time of 100 ms and the automatic gain control (AGC target) set to $10^6$ ions. Chromatograms were extracted using a tolerance of ±2.5 ppm.

Parallel reaction monitoring tests (PRM) were performed with a HR MS/MS method to provide insights of the peaks corresponding to tramadol, *O*-desmethyl tramadol and *N*-desmethyl tramadol on two blood samples spiked with 100 or 10 ng/ml. The ionisation settings were kept identical as for the full-scan method. Injections of the samples with normalised collision energies ranging from 10 to 60 eV were performed for both concentrations. The resolution used for PRM was 35,000 FWHM, isolation window was set at 0.4 m/z, the AGC target was set to $5 \times 10^4$ ions and the injection time was limited at 50 ms.

## 2.4 | Haematocrit influence

Five whole blood samples drawn into 3.5-ml EDTA-coated tubes with haematocrit values between 36.4% and 46.1% were spiked with a mix solution at 100 ng/ml containing tramadol and its *O*-and *N*-desmethyl metabolites. Haematocrit in EDTA whole blood samples was previously measured using an automated Sysmex XN-1000 analyser. A 10-μl volume of each spiked sample was spotted onto the filter card using a micropipette and allowed to dry for a minimum of 1 h at room temperature. The spiked DBS were extracted in duplicate, and the extracts were randomly injected into the LC-HRMS system.

## 2.5 | HemaXis DB10 repeatability

Fresh EDTA whole blood was spiked with a solution containing tramadol and its two metabolites at 100 ng/ml. Spiked blood was then transferred from a micropipette to the filter card using the HemaXis DB10 device according to the manufacturer's instructions. In total, four HemaXis DB10 devices corresponding to 16 spots were used to assess the repeatability of the whole procedure. In order to get closer to the field procedure and assess the stability of tramadol and its metabolites in DBS, the prepared samples were then transported unrefrigerated to the laboratory before analysis.

## 2.6 | Pharmacokinetics study

To test the sensitivity of the developed method and to estimate a detection window, a proof-of-concept pharmacokinetics study was internally performed with one healthy volunteer that received an oral dose of Tramal® (50 mg tramadol chlorhydrate, Grünenthal Pharma AG, Glarüs, Switzerland). The volunteer was a 26-years-old Caucasian male of 74 kg with unknown genotype for the CYP450 isoforms, which metabolise tramadol; 10 μl of capillary whole blood were collected 0, 1, 2, 3, 4, 6, 8, 10, 12, 24 and 30 h after intake from a finger prick using HemaXis DB10 devices.

## 2.7 | Methodology transfer to UPLC-triple quadrupole MS/MS and evaluation of the method

Separation was performed on a 1200 series LC system from Agilent (Waldbronn, Germany) connected onto a QTrap 6500 from AB Sciex

(Darmstadt, Germany). The conditions of chromatographic separation were identical to the ones described above.

MS/MS analysis was operated in the positive ionisation mode. Nitrogen was used as the curtain and nebulizer gas and the turbo spray conditions were as follows: curtain gas: 40 psi; collision gas: 8 psi; ion spray voltage: 4500 kV; temperature: 450°C; ion source gas 1: 60 psi; ion source gas 2: 60 psi.

Standard solutions of each analyte were directly infused in positive mode at 100 ng/ml in reconstitution solvent to optimise MS/MS transitions, collision energy and declustering potential. Compound optimisation was performed using Analyst software (version 1.5.2; AB Sciex, Toronto, Canada). MRM transitions (Table S1) were included into the UPLC-MS/MS method, and standard solutions were injected to determine their retention time.

*Matrix effects* were assessed by comparing a standard solution to an extracted blank DBS sample that was then spiked with the same standard solution. Investigated concentrations for tramadol, *O*-desmethyl tramadol and *N*-desmethyl tramadol were 5, 50, 100 and 500 ng/ml and were produced in duplicates.

*Recovery* of the procedure was assessed by comparing postextraction spike blood samples containing tramadol and its metabolites to blood samples spiked prior to the deposit onto the DBS card and extracted following the described procedure. The investigated concentrations were 5, 50, 100 and 500 ng/ml and were produced in duplicates.

*Carry over* was tested by analysing a DBS sample spiked with 500 ng/ml of tramadol and its metabolites followed by a blank sample containing only MeOH:$H_2O$. The signal of tramadol and its metabolites were investigated in the blank sample.

*Cross contamination effect* was studied by proceeding to a DBS sample preparation while deliberating punching the spot throughout a blood spot spiked at 500 ng/ml with tramadol and its metabolites in order to allow physical contact between the hole puncher and the surface of the dried blood. The tool was reused without any cleaning step to punch a blank spot and possible contamination traces determined by LC-MS analysis.

### 2.8 | Implementation of DBS collection in professional cycling competitions

In parallel to the validation of the analytical method described above, a first step was to select a simple-to-use DBS collection device conforming with high health and safety standards (i.e., HemaXis DB10, DBS System SA, Gland, Switzerland). In addition to its capacity to collect four whole blood spots with a standardised volume of 10 µl, the kit has the advantage of being conditioned close to the UCI headquarters for a facilitated training of the blood collection officers to its utilisation. The collection devices were then packaged in single collection kits (tamper-evident sealed cardboard boxes) containing gloves, an isopropylic disinfection cloth, a contact-activated lancet (BD Microtainer, BD Europe, Eysins, Switzerland), a compression pad and self-adhesive plaster, labels with unique numbering and a tamper-evident plastic pouch (SteriBag Premium, Bürkle, Bad Bellingen Germany). The list of riders selected for tramadol DBS samples collection was determined by the UCI medical director. At a minimum, athletes notified for antidoping tests were also tested for tramadol, but riders could also be selected depending on the events of the race. The riders identified for tramadol control were registered on a separate list from that of doping controls. Concerning the high stability of DBS, samples were sent unrefrigerated by regular mail or courier services to the laboratory for the latter detection of tramadol and its metabolites.

## 3 | RESULTS AND DISCUSSION

### 3.1 | UHPLC-HRMS method evaluation

To assess method selectivity, venlafaxine and its *O*-desmethylated metabolite, *O*-desmethylvenlafaxine, were included as potential interfering compounds. Venlafaxine chemically differs from tramadol only by a single methylene and is also metabolised by CYP450 to *O*-desmethylvenlafaxine, which presents the same m/z ratio as tramadol and can produce similar fragments. Indeed, false tramadol positive results have been observed in case of patients being treated with venlafaxine.[14] Thus, the chromatographic separation of this potentially interfering analytes corresponds to a particular suitable indicator of method selectivity.

A previous UHPLC method was adapted[15] by optimising the gradient profile and length. An isocratic elution step with 80% ACN was added to allow the elution of the highly apolar interfering compounds originating from blood at the end of the run. Ammonium formate concentration in mobile phase A was decreased from 10 to 2.5 mM to reduce peak tailing. This allowed a good separation of all analytes while keeping a short analysis time (6 min per injection) (Figure S1). To avoid peak splitting, various injection solvents were evaluated, and MeOH:$H_2O$ 50:50 was found to be the most appropriate one. The injection volume was initially fixed at 5 µl to make room for a larger injection volume if needed to adapt the sensitivity upon method transfer to other laboratories.

For quantitative analysis, focus was put on tramadol and its two main metabolites *O*-desmethyl tramadol and *N*-desmethyl tramadol as qualitative indicators of a drug intake. The response function was considered as linear for each analyte (over the concentration range from 5 to 500 ng/ml). This range corresponds to the expected blood concentrations after administration of therapeutic doses of tramadol.[16] Calibration curves for each analyte (ranging from 5 to 500 ng/ml) were built using a weighting factor of 1/*x*. Using calibration curves in spiked blood, it was possible to assess the limit of detection (LOD), defined as the concentration leading to a signal-to-noise (S/N) ratio equal to 3. The lowest concentration (5 ng/ml) yielded an S/N above 4, and so it was accepted as the LOD.

Although characterisation of the developed method was achieved using full-scan mode only, a PRM selective MS/MS mode was tested in order to provide a confident tramadol identification and a potential quantitation tool for further developments. Tramadol spiked in blood

at either 10 or 100 ng/ml was fragmented using the $[M + H]^+$ precursor ion (264.1958 m/z) and following the main product ion (58.06513 m/z) observed and known from literature. Energy collisions ranging from 10 to 60 eV were tested, and the best compromise to simultaneously observe the precursor and product ions was obtained with a collision energy value of 10 eV.

## 3.2 | Haematocrit influence and HemaXis DB10 repeatability

The impact of haematocrit (HCT) on tramadol concentration was first investigated, as it is recognised that haematocrit may induce a change in viscosity of blood and in quantitative performance of DBS.[17] A variety of samples representative of normal haematocrit range spiked with a mix solution of tramadol and its two metabolites at a fixed concentration were studied. The results demonstrated a homogenous response factor across the different haematocrit values without any apparent association between HCT values and peak area ratio (Figure 1). The observed peak area ratio of spiked tramadol and its metabolites in blood at different HCT values presented an average CV of 22% (22.5% for tramadol, 21.4% for *O*-desmethyl-tramadol and 22.1% for *N*-desmethyl-tramadol) suggesting that haematocrit does not show a significant influence on the detection of tramadol and its two main metabolites. Because the whole DBS with fixed blood volume was used for the analysis, no significant influence of haematocrit is expected. Because HemaXis DB10 device was selected as on-field collection method for tramadol use detection in cycling competition, the repeatability from sample collection to analysis was assessed by extracting 16 times a fresh blood sample spiked at 100 ng/ml and spotted onto filter cards using the device. The analysis of the 16 different spots exhibited a variability lower than 6% for all analytes (i.e., of 5.8% for tramadol, 5.7% for *O*-desmethyl tramadol and 5.3% for *N*-desmethyl tramadol). These results indicate that the whole procedure, from sample collection to DBS extraction and analysis, presents limited variability. The stability of the analytes in DBS was also assessed by transporting the samples at room temperature before analysis. The results were similar to freshly prepared or refrigerated spiked samples.

## 3.3 | In vivo intake

To further evaluate the sensitivity of the developed methodology, an exploratory kinetics study on a healthy volunteer was performed. After oral intake of a single 50-mg dose, blood levels of tramadol and its two metabolites were monitored at regular intervals and the concentration of tramadol and its metabolites using the DBS workflow were estimated (Figure 2). Tramadol rose to a peak concentration between 400 and 500 ng/ml 2 h after intake. Here, tramadol was detectable up to 30 h and both metabolites up to 8 h after the intake, respectively. Ratios between tramadol and its metabolites (tramadol/*O*-desmethyl tramadol and tramadol/*N*-desmethyl tramadol) were around 1 to 20. Reported usual ratios are around 1 to 10 for a normal metabolizer of the CYP2D6 and CYP3A4.[1] The obtained results were in agreement with previous pharmacokinetics study using liquid–liquid extraction at pH 11 and reverse phase LC-MS/MS.[16] The performance was similar and supports the use of DBS workflow as an alternative to liquid–liquid extraction of blood for tramadol intake detection. While this proof-of-concept provides insights regarding the analytical method performance, it also gives confidence about what should be expected in terms of performance for actual routine study

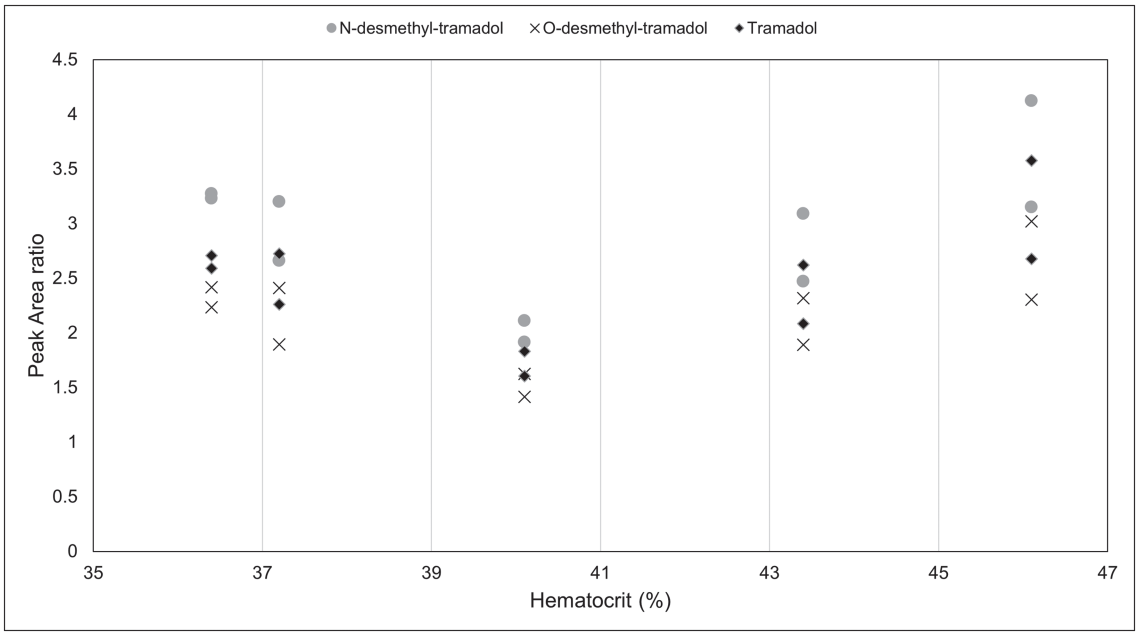


**FIGURE 1** Ratios of tramadol, its *O*- and *N*-desmethyl metabolites and $^{13}C$ tramadol in dried blood spot (DBS) with different haematocrit values analysed by LC-HRMS. Two replicates of injection for each value of haematocrit represented were evaluated

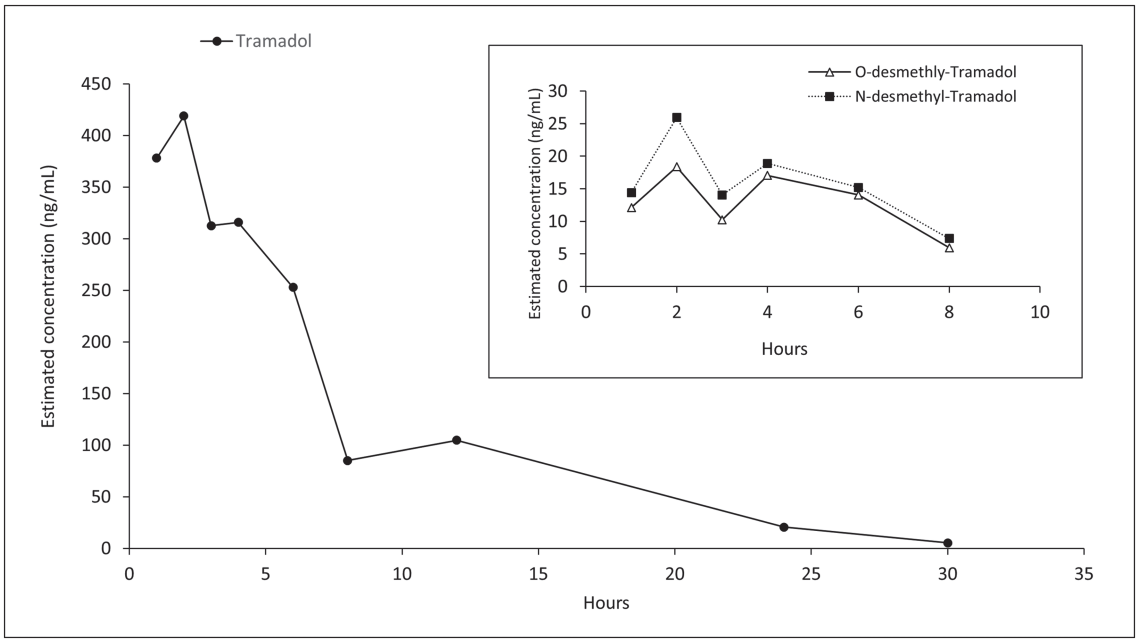


**FIGURE 2** Example of pharmacokinetics of an intake of 50 mg of tramadol collected by dried blood spot (DBS) and analysed by liquid chromatography linked to high resolution mass spectrometry (LC-HRMS) monitored in one male individual ($n$ = 1). Only concentrations above the limit of detection (LOD) are included

with analysis of samples with no a priori information about metabolising characteristics of the tested athletes. It implies that the parent compound should be detected for about 30 h after intake of a 50-mg dose of tramadol, whereas both metabolites could be detected above the LOD approximately 8 h after Tramal® administration. This approximate detection window seemed in accordance with a blood sample collection next to the time of racing (before or after).

## 3.4 | Transfer of methodology to UPLC-MS/MS

To increase detection sensibility, a UPLC-MS/MS system (QTrap 6500 MS) was used. MRM transitions for each analyte were first optimised by direct infusion of standard solutions in positive ionisation mode. For tramadol (264.1 m/z) and *O*-desmethyl tramadol (250.1 m/z), the most intense fragment detected (i.e., m/z 57.8) was selected for quantitative purposes. Such fragment has been reported in the literature and was also previously characterised using HRMS in the present study. The next abundant fragment (m/z 42.3, tenfold less intense) was used as qualifier. For *N*-desmethyl tramadol, the transition 250.1 > 43.8 was selected as quantifier, whereas the 250.1 > 231.7 transition served as qualitative confirmation (Table S1). After transfer of all operational parameters and confirmation of proper separation of all analytes, further evaluation was carried out. The response function was similarly assessed and considered as linear for each analyte over the same concentration range as the UHPLC-HRMS method. Calibration curves for each analyte (ranging from 5 to 500 ng/ml) were also built with a weighting factor of 1/$x$. Using lower concentrations in spiked blood, the LOD was assessed and was determined at 2 ng/ml with a S/N ratio above 3. The transfer of methodology also allowed to determine the positivity criteria (limit of identification) for a DBS sample. It was defined as the presence of tramadol, *O*- and *N*-desmethyl tramadol in the DBS sample above a concentration of 5 ng/ml.

## 3.5 | Recovery, matrix effects, cross contamination and carry over

Matrix and recovery effects were calculated following the recommended methodologies and the results summarised in Table 1. For each concentration, two replicates of extraction were analysed for matrix and recovery effects. Matrix effects were found to increase the signals of the studied analytes by 10% to 40% according to the tested analytes. Tramadol and its *O*-desmethyl metabolite were found to be affected, and these matrix effects were found to be consistent over the essayed range of concentrations. Recoveries showed a decrease of the signal of 30–50% due to the extraction process itself depending on the molecule. *O*-desmethyl tramadol presented the poorest recovery, whereas *N*-desmethyl tramadol was better recovered compared with the other metabolite and the parent compound. Recoveries were consistent for all the tested concentrations, suggesting that it does not influence the extraction of the analyte from DBS.

Furthermore, the risk of cross-contamination during DBS handling was evaluated to address one of the main concerns in antidoping analyses: reporting results that are reliable and with the minimal false positive rate. First, the possibility to transfer a detectable amount of analytes from one DBS to another by bearing them on a contaminated puncher, pliers or punching surface was evaluated. To this end, a DBS spot spiked at 500 ng/ml with tramadol and its metabolites was

**TABLE 1** Matrix effects and recovery in DBS for the three analytes at four concentration levels

| | Matrix effect ($n$ = 2) | Recovery ($n$ = 2) |
|---|---|---|
| Tramadol (ng/ml) | | |
| 5 | 143% | 63.1% |
| 50 | 142% | 60.8% |
| 100 | 126% | 65.1% |
| 500 | 137% | 64.6% |
| *O*-desmethyl tramadol (ng/ml) | | |
| 5 | 122% | 50.4% |
| 50 | 123% | 48.9% |
| 100 | 109% | 53.8% |
| 500 | 115% | 53.0% |
| *N*-desmethyl tramadol (ng/ml) | | |
| 5 | 139% | 61.5% |
| 50 | 139% | 64.4% |
| 100 | 122% | 68.9% |
| 500 | 130% | 68.4% |

deliberately punched throughout the blood spot on the punching surface, folded and processed. Then, a second, blank DBS was processed with the same tools without applying any cleaning process. Second, carryover was assessed by injecting a blank sample after the most concentrated QC (500 ng/ml of tramadol and both metabolites). In both cases, negligible carryover effect was found with values under 0.15% for all the studied molecules: 0.07% for tramadol, 0.11% for *N*-desmethyl tramadol and 0.06% for *O*-desmethyl tramadol. Our results show that cross-contamination is not a limitation of the developed methodology for concentrations up to 500 ng/ml, even if the same handling tools and surfaces are used. As expected, the intrinsic nature of DBS minimises the risk of analyte transfer to the tools and other samples.

### 3.6 | Interplatform comparison of pharmacokinetics samples

DBS samples from the in vivo intake experience were also analysed on the QTrap system. It could be observed that the concentrations estimated for tramadol were consistent across the two MS platforms with a significant correlation ($r$ = 0.97) (Figure 3A). For both metabolites, the results were also similar ($r$ = 0.82 *O*-desmethyl tramadol; $r$ = 0.97 *N*-desmethyl tramadol), and while the estimated concentrations were slightly elevated compared with those estimated with HRMS, the LC-MS/MS platform also demonstrated excellent suitability for qualitative analysis and detection. Consequently, the detection window could be extended to at least 12 h for both metabolites with a strong MS/MS signal and estimated concentrations above LOD (Figure 3B). The identification capability, also referred as the limit of identification, would correspond to the detection of tramadol and its two metabolites in DBS above a concentration of 5 ng/ml for each compound. It suggests that using this platform, a DBS sample can be considered as positive (detection of parent, *O*- and *N*-desmethyl metabolites) up to half a day after tramadol ingestion. These results reinforce the idea that tandem MS operating in MRM mode is preferred to assess the best sensitivity for the detection of drug abuse in the context of cycling.

### 3.7 | Implementation in the testing of professional cyclists

Although cyclists are routinely subject to doping controls, the implementation of a new collection method (i.e., DBS) might have been perceived as a source of stress. The experience and professionalism of the doping control officers in combination with well-designed kits perceived by athletes as 'safe and clean', as reported by the doping control officers, resulted in 100% of the sampling occasions producing a valid sample for analysis. The main drawback reported by tested athletes was the longer duration of the control. After the validation of the overall workflow, a total of 711 samples were collected from 361 different cyclists between March and November 2019 and were analysed. They were mainly road professional athletes in UCI World Teams and UCI Pro Teams, and samples were also collected from 23 female professional riders, nine BMX competitors and 17 mountain-bikers during World Championships. DBS sampling occurred during regular doping controls performed by trained blood control officers from the Cycling Anti-Doping Foundation and collected in parallel to urine samples sent to WADA accredited laboratories for standard antidoping testing. In all samples collected in cyclists, no positive case was observed. One blinded positive control sample obtained from DBS sampling in a healthy individual 4 h after the ingestion of a single dose of 50 mg of tramadol was additionally sent to the laboratory. Both tramadol and its metabolites were detected and confirmed by a second analysis. Tramadol is a substance, which is part of the WADA Monitoring Program since many years. The substances listed in the Monitoring Program are analysed by accredited laboratories in urine samples collected in competition for antidoping purposes. The statistics of the Monitoring Program are provided each year by WADA to all antidoping organisations as support to detect potential pattern of misuse in their sport. The results of tramadol findings in cycling (tramadol >50 ng/ml in urine collected In Competition [IC]) for the last 3 years are presented in Table 2. These statistics demonstrate a significant decrease of the prevalence of tramadol in cycling in 2019 (1.1%) compared with previous years. The lack of correlation between the numbers of tramadol findings in urine samples (144) and DBS samples (0) can be explained simply by the number of samples collected for DBS, corresponding to 5.5% of total IC urine samples collected in 2019, decreasing the likelihood of detecting a positive sample. Moreover, DBS samples were collected in UCI-elite cycling competitions while the WADA Monitoring Program also includes results of urine samples collected outside of UCI-registered competitions. Nevertheless, this remarkable decrease of prevalence can reasonably be attributed to the huge deterrent effect of the

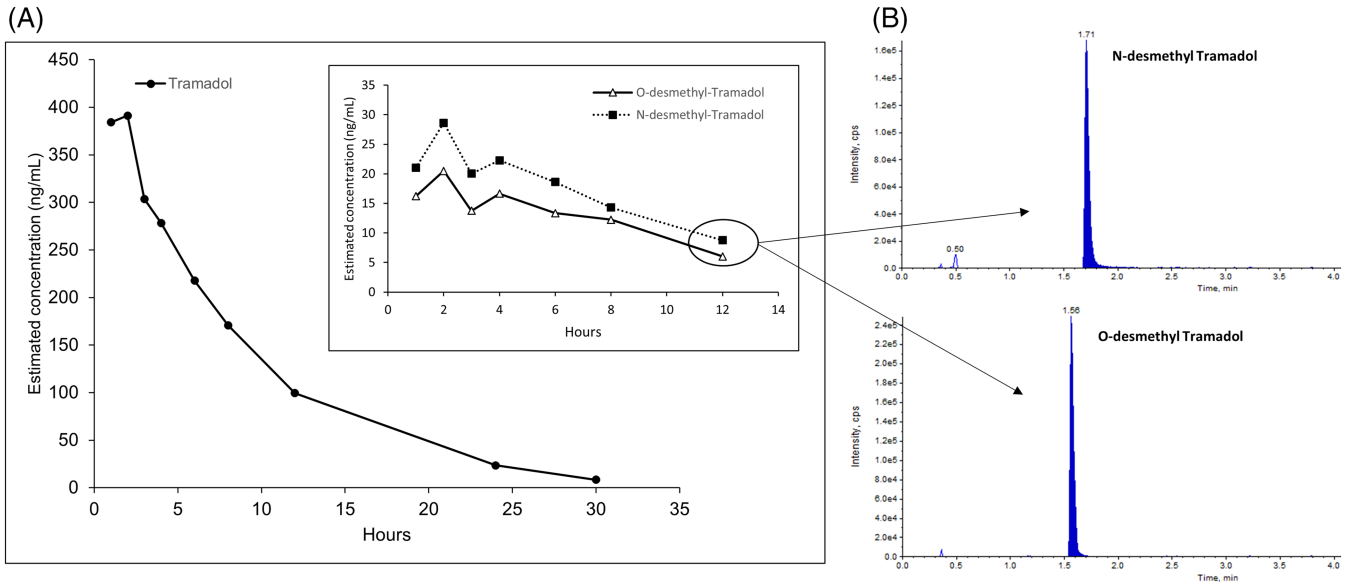


**FIGURE 3** (A) Estimated concentrations of tramadol and its metabolites after tramadol ingestion in DBS analysed by liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) monitored in one male individual (n = 1). Only concentrations above the limit of detection (LOD) are included. (B) Chromatogram of *N*-desmethyl and *O*-desmethyl tramadol in DBS sample collected 12 h after tramadol administration

**TABLE 2** Summary of tramadol findings in cycling as part of the WADA monitoring program for the years 2017, 2018 and 2019

| Year | IC samples | Tramadol (>50 ng/ml) | Prevalence |
|---|---|---|---|
| 2017 | 12,554 | 548 | 4.3% |
| 2018 | 13,381 | 574 | 4.3% |
| 2019 | 12,806 | 144 | 1.1% |

implementation of the new medical rule for tramadol by UCI in the World Tour professional races. The penalties associated with this rule (see above) did not include any possible margin of error on the part of the competing athletes representing therefore a strong disincentive to misusing tramadol during cycling competitions.

## 4 | CONCLUSION

To fight the misuse of potentially dangerous substances like tramadol in cyclist competitions, herein, we present the development of a method to monitor tramadol intake with DBS as the sample collection procedure. The proposed method includes a simple and convenient collection of blood from fingertip with a DBS microsampling device and a fast UHPLC-MS analysis to detect tramadol and two of its metabolites.

We successfully demonstrated that spiked whole blood extracted with the workflow allows to detect in a linear manner tramadol, *O*-desmethyl tramadol and *N*-desmethyl tramadol in concentration ranging from 5 to 500 ng/ml. The method allows to distinguish tramadol from venlafaxine, a nonbanned substance that could represent a potential interference in the analysis. Repeatability of the method and influence of haematocrit were also assessed with satisfactory results. Cross-contamination and carryover effects were shown to be negligible.

Administration of commercially available Tramal® to a healthy volunteer served as a case study of how the proposed workflow allows to track the in vivo pharmacokinetics of tramadol and its metabolites for up to 8 h after the intake of a single dose of 50 mg. As expected from the literature, the in vivo ratio of tramadol and its metabolites was comprised between 1 to 5 and 1 to 20, with a tramadol concentration reaching its highest point 1 h after drug administration.

The method has been successfully transferred to a MS/MS platform with improved sensitivity compared with HRMS enabling a positive detection (presence of tramadol, *N*-desmethyl and *O*-desmethyl tramadol) of tramadol up to 12 h after intake of a therapeutic dose. This fits the purpose of the method to identify whether a cyclist was under the influence of the drug during the competition from DBS samples collected just before or right after a race. The absence of positive cases in all analyses performed may support the deterrent effect for the preliminary communication of the related medical ruling and the subsequent penalties in case of positive controls imposed by the UCI.

Sampling of blood spotted and dried on a matrix was for instance first described more than a century ago.[18] Recent implementation of contemporary mass spectrometry analyses has considerably widened the range of analytes measured with a high sensitivity using DBS and would allow a rapid application not only for tramadol.[19] In the specific context of the fight against doping, WADA has recently announced collaborations to 'further research the development and implementation of this exciting method for drug testing in sport'.[20] The International Olympic Committee has tried to drive home this point stating that 'DBS method could very well revolutionize the anti-doping fight,

since it will allow for fast, simple and cost-effective collection of samples that can be easily and cheaply transported and stored'.[21] In conclusion, the first worldwide application of DBS in a "real-life" context is here to be credited to the UCI with the successful testing for tramadol misuse in cycling starting in March 2019 at the Paris-Nice stage race.

## ACKNOWLEDGEMENTS

We are grateful to Union Cycliste Internationale for their financial support and we thank the CADF staff for the technical assistance.

## CONFLICT OF INTEREST

Xavier Bigard is the medical director of UCI and was involved in the prohibition of tramadol in cycling competitions.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Salamin O, Garcia A, González-Ruiz V, et al. Is pain temporary and glory forever? Detection of tramadol using dried blood spot in cycling competitions. *Drug Test Anal*. 2020;12:1649–1657. https://doi.org/10.1002/dta.2923

# Appendix IV

Loop-mediated isothermal amplification (LAMP) as an alternative to PCR: A rapid on-site detection of gene doping

*Salamin O, Kuuranne T, Saugy M, Leuenberger N. Drug Test Anal. 2017 Nov; 9(11-12):1731-1737*

Perspective

Drug Testing and Analysis

Received: 28 July 2017 Revised: 21 September 2017 Accepted: 4 October 2017 Published online in Wiley Online Library: 23 November 2017

(www.drugtestinganalysis.com) DOI 10.1002/dta.2324

# Loop-mediated isothermal amplification (LAMP) as an alternative to PCR: A rapid on-site detection of gene doping

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**Innovation in medical research has been diverted at multiple occasions to enhance human performance. The predicted great progress in gene therapy has raised some concerns regarding its misuse in the world of sports (gene doping) for several years now. Even though there is no evidence that gene doping has ever been used in sports, the continuous improvement of gene therapy techniques increases the likelihood of abuse. Therefore, since 2004, efforts have been invested by the anti-doping community and WADA for the development of detection methods. Several nested PCR and qPCR-based strategies exploiting the absence of introns in the transgenic DNA have been proposed for the long-term detection of transgene in blood. Despite their great sensitivity, those protocols are hampered by limitations of the techniques that can be cumbersome and costly. The purpose of this perspective is to describe a new approach based on loop-mediated isothermal amplification (LAMP) for the detection of gene doping. This protocol enables a rapid and simple method to amplify nucleic acids with a high sensitivity and specificity and with a simple visual detection of the results. LAMP is already being used in clinical application for the detection of viruses or mutations. Therefore, this technique has the potential to be further developed for the detection of foreign genetic material in elite athletes.**


**Keywords:** LAMP; PCR; gene doping

## Introduction to gene doping

During the past few years, the advances in genetic engineering allowed the development and the expansion of gene therapies. Concretely, gene therapy refers to therapeutic delivery of genetic material into an organism in attempt to treat a specific disease. It aims at compensating a defective gene, modulating the activity of a mutated gene, or enhancing gene expression as an additional gene copy.[1] Initially, gene therapy was developed to treat hereditary single-gene defects such as cystic fibrosis, hemophilia or x-linked severe combined immunodeficiency disease (SCID).[2–4] However, the current trend of gene therapy targets now diseases such as cancer, anemia, cardiovascular diseases and neurodegenerative disorders.

At the beginning of the 2000s, rapid progress of this technology raised some concerns about its illicit use – namely gene doping - to improve physical performance. Thereby, in 2004, the World Anti-Doping Agency integrated gene doping in the list of prohibited substances and methods. The current WADA Prohibited List defines gene doping as "the transfer of polymers of nucleic acids or nucleic acid analogues" and/or "the use of normal or genetically modified cells".[5]

Those concerns were strengthened by the publications of various studies[6,7] performed with animal models involving gene transfer and enhancement of performance, which have drawn attention from the media and the sports community. Particularly, in 2004, a group of the Howard Hughes Medical Institute genetically engineered so-called "marathon mice". By altering the gene coding for the protein peroxisome proliferator-activated receptor delta (*PPAR δ*), the muscle composition was modified and the mice were capable of continuous running of up to twice the distance of a wild-type littermate.[6] Moreover, those genetically modified mice demonstrated resistance to obesity even in absence of exercises. In-between, other potential performance-enhancing genes such as erythropoietin (*EPO*), growth hormone (*GH*), insulin-like factor-I (*IGF-I*), myostatin or vascular endothelial growth factors (*VEGF*) have been suspected as main candidates for gene doping. Some genes are intended to increase strength while others are supposed to increase endurance. Table 1 summarizes the main potential gene targets for doping, their function and the expected physiological response as well as their possible adverse effects.

Despite the fact that most gene therapies are not yet well established, it is very likely that athletes do not wait until the clinical approval before they use these new methods to improve their performance.[7] The struggle became real when a German athletics trainer was accused of providing Repoxygen to his athletes.[16] At this period, no detection method was developed and gene doping represented a real threat to high-level sports. Repoxygen was a

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**Table 1.** Potential doping genes, its associated function and expected physiological response and potential risks of adverse effects.

| Gene of interest | Function Expected physiological response | Potential adverse effects | References |
|---|---|---|---|
| *EPO* | Stimulates red blood cells production and thereby increases blood oxygenation Increased endurance | Increased blood viscosity Hypertension Heart failure Severe immune response | [8] |
| *HIF-1* | Regulates transcription at hypoxia response elements (*EPO, VEGF)* Increased endurance | Increased blood viscosity Hypertension Neoplastic disease | [9] |
| *IGF-1/GH* | Regulates cell growth and development (IGF-I) Increases lipolysis, protein synthesis and glycogenolysis (GH) Increased muscle power and mass | Acromegaly Neoplastic disease Insulin resistance and diabetes Intracranial hypertension Headache Peripheral oedema Cardiomegaly Carpal tunnel syndrome Joint and muscle pain | [10,11] |
| *Myostatin* | Negatively regulates muscle cell growth and differentiation Increased muscle mass and strength | Reduction of cardiac and respiratory functions Damage to the ligaments, tendons and bones | [12] |
| *VEGF* | Stimulates angiogenesis and vasculogenesis Increased endurance | Neoplastic disease Immune response | [13] |
| *PPAR δ* | Regulates the oxidation of fatty acids and increases mitochondrial activity and muscular glucose uptake Enhances slow twitch muscle fibers and decrease fast twitch muscle fibers Increased speed and endurance | Overexpression of sex hormones | [6,14] |
| *ACE* | Regulates blood pressure by adjusting Angiotensin II levels and increases the proportion of slow-twitch muscle fibers Increased endurance | Angioedema | [15] |
| *Endorphin, enkephalin* | Reduces pain and fatigue threshold Increased endurance | Risk of overload Sudden death | [13] |
| *PEPCK-C* | Regulates gluconeogenesis and is involved in Krebs cycle Increased endurance | Hyperglycaemia | [7] |

Abbreviations: *EPO*, erythropoietin; *HIF*, hypoxia-inducible factor; *IGF-I*, insulin-like growth factor I; *GH*, growth hormone; *VEGF*, vascular endothelial growth factor; *PPAR δ*, peroxisome proliferator-activated receptor; *ACE*, angiotensin-converting enzyme; *PEPCK-C*, phosphoenolpyruvate carboxykinase.

virus-based drug containing the human *EPO* gene used to treat anemia. However, the progress of production was discontinued by the company after preclinical development. Several other studies demonstrated that *EPO* gene transfer can induce a sustainable and safe delivery of *EPO* with a long-term sensitive regulation of hemoglobin and hematocrit using mice and primate models.[8,17–19] Those examples emphasize the potential of *EPO* as a primary candidate for gene doping. Indeed, a single administration of the *EPO* gene would ensure a long-term delivery of the resulting protein and a huge improvement of the aerobic capacity. On the contrary, this might also result in a large production of EPO protein and an increase of red blood cell number which could be harmful and irreversible for the athlete.

## Methods of gene transfer

The delivery of DNA into the organism can be accomplished with two distinct approaches: *in vivo* or *ex vivo* transfer. The *in vivo* gene therapy implies the direct injection of DNA into the target tissue. The introduction of the transgene can be achieved either via biological (viral vectors, plasmids), chemical (cationic liposomes) or physical methods (direct injection).[7]

Up to now, the different studies demonstrated that viral gene transfer was the most efficient method for gene therapy.[9] The injection of viral vector can be either intravascularly for a systemic expression, either directly into the target tissue for a gene expression limited to the site of injection.[13] The most common viruses for gene transfer are Adenoviruses, Adeno-associated viruses (AAV), Herpes viruses (HSV), Lentiviruses and Retroviruses. Using genetic engineering, the therapeutic gene is inserted into the viral vector and the viral genes are removed to hinder replication and to become less immunogenic. Thereby, the transgene is released in the target tissue/cells and expressed using the cell replication machinery.[9] Almost all studies investigating gene therapy/doping used rAAV vector due to its high efficiency in transducing muscle tissue and its low immunogenicity.

The other non-viral gene delivery involves the direct injection of plasmid DNA (pDNA) into the target tissue. The gene of interest is produced in bacterial plasmids, purified and then injected. This strategy offer some advantages over viral vectors as the pDNA can be easily produced in large quantities at reduced costs with

reduced immunogenicity.[1] However, the success of this approach is limited by the poor transfection efficiency and short duration of successful transgene expression.

The *ex vivo* approach consists of the collection of cells from the patient and the transfection of the cultured cells *in vitro* with the therapeutic gene by different methods, including electroporation or gene gun.[7] The genetically modified cells are then re-introduced into the patient. This method corresponds to the second category of gene doping by the WADA namely "the use of normal or genetically modified cells". Nevertheless, this kind of a gene transfer is limited by its low efficiency, significant costs and the poor survival of the transduced cells. This approach was described earlier by Lippin et al. in the study on patients with anemia of chronic renal failure.[20] In their study, an adenovector designed to express human EPO under the control of the cytomegalovirus (CMV) promoter was transfected into dermal cores (Biopump). Those dermal cores were harvested, transduced *ex vivo*, and implanted back into chronic kidney disease (CKD) patients. It resulted in a significant increase of serum EPO levels to therapeutic levels for up to 14 days. However, the increase was transient and the decline of *EPO* expression correlated with the immune response against the dermal cores.

To avoid potential adverse effects resulting from transgene overexpression, different regulatory systems have been developed. One approach is to use a regulated promoter that can switch gene expression on and off. Rapamycin, doxycycline and tetracycline regulatory systems have been widely applied in large animal studies.[21–23] However, this extrinsic regulatory system can be costly and cumbersome. Therefore, Binley et al. developed a homeostatic system of gene therapy called "Oxford Biomedical Hypoxia Response Element" (OBHRE) based on sensing and correcting tissue hypoxia given the normal regulation of the erythropoietin gene.[19] They demonstrated that anemic mice treated with AAV containing the murine *EPO* gene under the control of OBHRE showed correction of the hematocrit to a physiologically level that was stabilized for 7 months.

## Detection of gene doping

Another challenge regarding gene doping concerns its detection. Indeed, this method is considered undetectable because the introduced gene and its corresponding protein are almost identical to endogenous counterpart. Nevertheless, novel detection methods need to be developed and validated to deter athletes from using gene transfer technology. Some indirect methods aiming at detecting responses of the body (metabolic, transcriptomic, proteomic changes) as a consequence of the delivery and expression of the transgene have been proposed.[24] However, this strategy can be subject to confounding factors such as age, gender, ethnic background which may complicate the results interpretation. Immune reaction resulting from viral and plasmid delivery has also been suggested as an indirect method of detection. However, this approach is hampered by the risk of natural viral infection of the athlete increasing the risk of false positive result. Moreover, from a legal point-of-view, direct methods are always preferred over indirect ones for the support of disciplinary processes.

Most studies investigating gene therapy/doping demonstrated that intramuscular injection is the most likely delivery system for gene doping application owing to physical accessibility, mass of the tissue and access to the vasculature.[25] However, due to its high invasiveness, muscle biopsy would be impossible for sample harvesting. Direct injection into a target tissue has been considered as an approach which leads to entry of minutes amounts of transgenic DNA into the blood stream.[26] Hence, several studies explored the possibility to detect trace amounts of transgenic DNA in blood samples after intramuscular gene transfer.

Lasne et al. were the first to describe an approach for the detection of *EPO* gene doping in blood.[27] They demonstrated that EPO proteins derived from transgenic DNA in muscle cells and proteins encoded by genomic DNA differ in their glycosylation pattern due to different post-translational modifications in various tissue. These differences can thus be easily exploited using conventional EPO test consisting of isoelectric focusing and double-blotting. However, this method is specific to *EPO* gene and cannot be extrapolated to other potential doping genes.[1]

Various direct detection methods based on nested and real-time polymerase chain reaction (PCR) have been proposed for the detection of gene doping practices.[24–26,28–30] Those approaches are based on subtle differences between the sequences of a doping transgene and its endogenous counterpart in genomic DNA (gDNA). Indeed, the gene sequences of transgenic DNA (tDNA) do not contain intron sequence parts of the gDNA which allows the detection of trace amounts of a transgene in a large background of gDNA. Beiter et al. were the first to develop a method based on single-copy primer-internal intron-spanning PCR (spiPCR) enabling detection of tDNA on a single molecule level within ordinary blood samples.[28] Using two-round nested PCR and analysis of the final PCR product by agarose gel, the established spiPCR protocol was tested for *EPO* and *VEGF-d*. The method was able to detect down to one copy of *EPO* and *VEGF-d* tDNA in the presence of 300 ng of gDNA. Using the same protocol, and outer and inner intron-spanning primer pairs, the investigators developed a multiplex PCR protocol allowing simultaneous detection of multiple prime candidate genes (*EPO, IGF-I, VEGF-A, VEGF-D, GH-I, FST*) in a mouse model receiving intramuscular AAV-mediated gene transfer.[26] The sample preparation and PCR setup procedures were established to ensure straightforward, cost effective and minimally invasive clinical testing with high specificity and sensitivity. The applicability and reliability of the method was validated by a screening approach including 327 blood samples collected from professional and recreational athletes. While the nested PCR based approach demonstrated high sensitivity, the two-step PCR encompasses risks of cross-contamination associated with false-positive results. Thus, a one-tube nested PCR protocol containing both primer pairs was developed for the detection of *EPO* transgene.[30] This system minimizes the workflow and the potential for cross-contamination, while maintaining high specificity and sensitivity.

Another approach is based on real-time PCR (RT-PCR) specific detection of intronless transgene after DNA extraction from whole blood. Baoutina et al. developed a RT-PCR that target sequences within the transgene DNA corresponding to exon/exon junctions in human blood spiked with cDNA.[24] Using RT-PCR allows a one-step analysis in a closed system that prevents any possible contamination of a sample and avoids both the electrophoresis detection from conventional PCR analysis and the multiple amplification steps of nested PCR. The method was further improved and validated three years later with the incorporation of plasmid linearization step to the PCR protocol.[29] Using the same approach, it was demonstrated that detecting doping transgene in blood of nonhuman primates was possible.[25] Indeed, intramuscular injection of a conventional plasmid and rAAV vectors resulted in the presence of DNA that could be detected at high levels in blood and that rAAV genomes can persist for several months in WBCs.

More recently, to reduce the technical limitations associated with nested PCR and RT-PCR-based strategies, a new protocol based on digital droplet PCR (ddPCR) was developed for the detection of *IGF-I*.[31] The method allowed the detection of *IGF-I* transgenic elements for a 33-day period in DNA extracted from whole blood of 6 mice injected intramuscularly with AAV9-*IGF-I* elements. A duplex ddPCR protocol for *IGF-I* and *EPO* transgene elements was further developed to simultaneously detect two candidate genes for gene doping in a single assay. Finally, to further improve the sensitivity of the assay, a DNA extraction protocol including on-column DNA restriction enzyme digestion was optimized for ddPCR. Contrary to qPCR, ddPCR includes absolute quantification and does not rely on external standard curve which enables outstanding day to day reproducibility. Moreover, ddPCR can handle large amounts of background DNA in the reaction and is more tolerant to inhibitors and variation in amplification efficiencies.[31]

These researches highlight the great effort that has been invested on the development of direct detection method of gene doping. It also demonstrates the will of anti-doping authorities to operate in a proactive way. However, none of these methods has been implemented in accredited anti-doping laboratories yet due to a validation lack. Furthermore, as mentioned above, most of those protocols involves multiple steps increasing the risk of cross-contamination and requires considerable skill, time and expensive equipment facilities. Therefore, a new approach involving simpler and faster sample manipulation would be an interesting alternative to these developed protocols.

## Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a novel method that amplifies DNA combining high specificity, efficiency and rapidity under isothermal conditions.[32] While the logistics of LAMP is much simpler than PCR, the principle of amplification is more complex. LAMP employs only a single type of DNA polymerase with high strand displacement activity that amplifies DNA at a constant temperature.[33]

A set of four specially designed primers (two inner (FIB/BIP) and two outer primers (F3/B3)) targeting six distinct gene regions are used for amplification. The methodology of amplification relies on repetition of two types of elongation reactions occurring at the loop regions: self-elongation of templates from the stem loop structure formed at the 3′-terminal and the binding and elongation of new primers from the loop region.[32,34] In the initial steps of the LAMP reaction, all four primers are used to produce a dumbbell-shaped structure with loops at both ends (Figure 1). During the

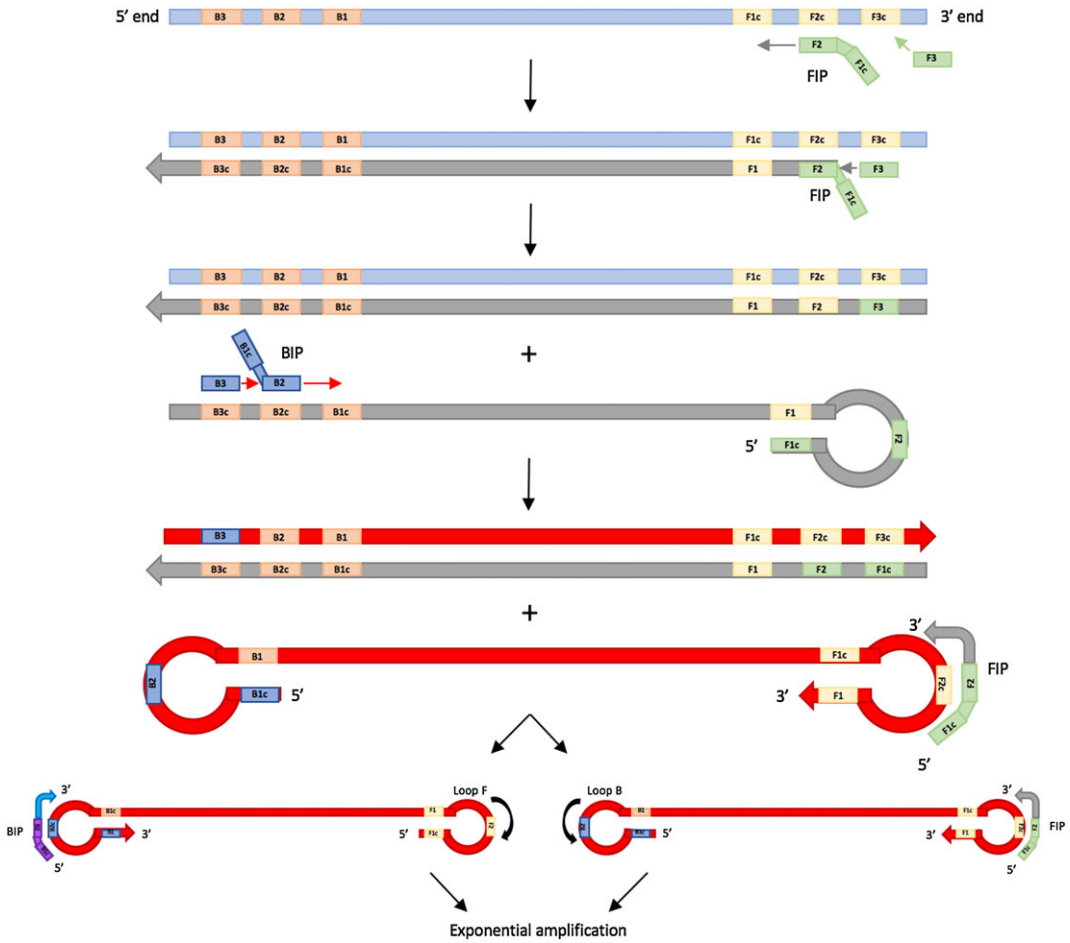


**Figure 1.** Schematic representation of the first steps of the LAMP mechanism. Four primers (FIP, F3, BIP, B3) target 6 sequences of the DNA. The LAMP method employs a single strand of DNA shaped like a dumbbell with loops at both ends as a starting template for exponential amplification. The amplification under isothermal conditions produces stem-loop DNA of different lengths and cauliflower-like structures.

cycling reaction, only the inner primers (FIB/BIP) are used for strand displacement DNA synthesis (exponential amplification). These primers are designed to produce looped structure every time a single-strand DNA is synthesized. They consist of two sets of sequences corresponding to the sense and antisense sequences of target DNA with a TTTT spacer between the sets of sequences.

The basic principle and reaction steps of LAMP amplification are illustrated in Figure 1. Initially, inner primer FIP hybridizes with its complementary region F2c of the target DNA and through the activity of DNA polymerase with strand displacement activity, a complementary DNA strand is synthesized. The outer F3 primer then slowly anneals to the F3c region on the target DNA and initiates strand displacement DNA, which releases the FIP-linked complementary strand. This released single strand forms a stem-loop structure at the 5′ end and serves as template for BIP-initiated DNA synthesis that follows the same logic leading to the production of the dumbbell-shaped structure. As mentioned above, this structure is used as the starting material for LAMP cycling. The elongation reactions are then sequentially repeated using the stem-loop region as a template with both inner primers and DNA polymerase-mediated strand-displacement synthesis.[33] The final products of the amplification are a mix of stem-loop DNA structures of different lengths and cauliflower-like structures formed by annealing between alternatively inverted repeats of the target in the same strand. This method can amplify a few copies of DNA to $10^9$ in less than an hour at constant temperature and with great specificity due to the fundamental characteristics of the inner primers.

Another key advantage of LAMP is the simple and selective detection method that can be used because of the high specificity and large output of the amplification products.[33] Different detection methods such as visual methods have therefore been developed. Because magnesium pyrophosphate is formed during DNA amplification, the products can be detected using a real-time turbidity measurement device.[35] Another LAMP amplicon detection relies on fluorescence. Fluorescence can be visualized with naked eyes or UV light by adding DNA-intercalating dyes such as SybrGreen or by adding metal-ion indicators such as hydroxynaphtol blue (HNB) or calcein.[34,36] Recently, different groups have applied the current and accessible technologies to the visualization of the amplicon. Using various strategies, smartphone-based fluorescence measurements have been developed to improve the discrimination of positive/negative signals.[37,38] LAMP can also be combined with a lateral flow dipstick (LFD) device for the direct visualization of the resulting amplicons.[39] In this protocol, LAMP reaction is performed with a biotinylated FIP primer associated with the adjacent addition of a specific FITC-labelled probe to the reaction mixture. The LFD is then inserted into the reaction mixture mixed with the detection buffer containing Rabbit anti-FITC antibodies coupled with gold. In a positive reaction, LAMP products migrate with the buffer flow and are retained at the Test Band containing a biotin ligand. The gold associated with anti-FITC antibodies bind to FITC molecule and a dark band develops over time. A second band, called Control Band, contains an anti-Rabbit antibody which retains some uncoupled gold-conjugated antibody is formed and should always be visible. In case of a negative reaction, only the second band is perceptible (explanation of the whole concept[39]).

Therefore, using these different detection methods, the diagnostic is easily and readily interpretable by a Yes/No detection, and does not require either costly specialized equipment or highly trained personnel.

The simplicity of the method maintained throughout LAMP protocol offers a wide range of possible applications such as point-of-care testing, rapid testing for food contamination, genetic testing in developing countries or ecological studies. A high throughput LAMP-based method has been developed for the detection of malaria,[40] while this amplification strategy was evaluated in food testing for bacterial pathogens and fungal contaminants.[41] Mutation responsible for cancer development can also be detected by this methodology.[42] In a different context, Centeno-Cuadros et al. developed a LAMP protocol for sex determination of raptor species.[43] In comparison with PCR-based methods, LAMP amplification demonstrates better sensitivity and specificity. Wang et al. demonstrated that this method was more sensitive and reliable than conventional PCR or multiplex for the detection of *Arcobacter* species.[44] The same conclusion was drawn for the detection of *Toxoplasma gondii* DNA from both mice obtained tachyzoites and leukemic patient's clinical samples.[45]

**Table 2.** Comparison between LAMP and PCR-based methods for the detection of gene doping. +++: very advantageous, ++: advantageous, +: disadvantageous, —: impossible

| — | LAMP | PCR-based methods |
|---|---|---|
| **Visualization of PCR products** | +++ | ++ |
| **Analysis time** | +++ | + |
| **Cost of the analysis** | ++ | + |
| **Efficiency of the amplification** | +++ | +++ |
| **Simplicity of the method** | +++ | ++ |
| **Primers design** | ++ | +++ |
| **On-site analysis** | +++ | - |

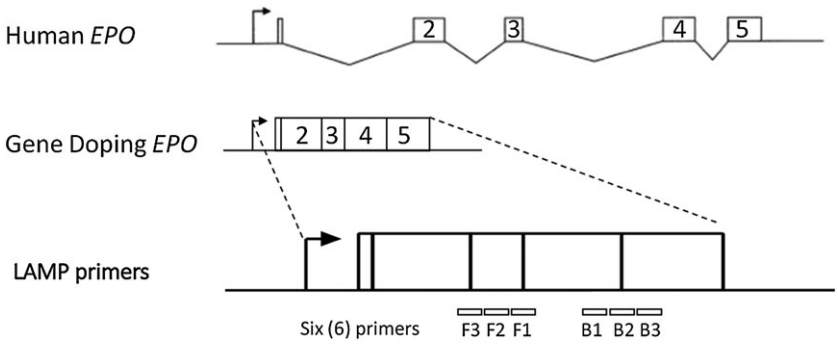


**Figure 2.** Potential design of LAMP primers for the amplification of *EPO* transgene for the detection of gene doping.

With this perspective, we suggest that LAMP could be extrapolated to anti-doping field for the detection of gene doping. This approach would offer several advantages over PCR-based protocol for the detection of transgenic material in athlete (Table 2). LAMP amplifies DNA with high efficiency under isothermal conditions without the need of expensive thermocyclers used in conventional PCR - only a water bath or a chemical heating pouch would serve the purpose. With the proper design of the primers, LAMP can reach high specificity, and can be easily and quickly performed with the possibility of direct and selective detection of positive results with naked eyes. Indeed, this simplified visual inspection of DNA amplification diminishes the need for gel electrophoresis which reduces the assay time. It could also operate with dried blood spot (DBS), which would facilitate the transport to the laboratory for gene doping detection. Moreover, the ease of the protocol would also allow to perform testing on competition site, which would greatly speed up the process of screening and act as a potent deterrent.

Figure 2 illustrates a possible example of primer design for the detection of *EPO* transgene. These primers would target exon/exon junctions of the *EPO* cDNA similarly to what was developed for PCR-based gene doping detection. Preliminary results demonstrated feasibility of isothermal assay in EDTA-whole blood and DBS spiked with pork DNA plasmid. The limit of detection (naked eyes) with lateral flow dipstick was determined as 50 copies of DNA with 20 μl of fresh whole blood and on DBS support. Those preliminary results demonstrate that LAMP-based method can be applied for gene doping detection and this strategy could be easily extrapolated to other potential doping genes such as *IGF-I* or *VEGF*.

In summary, in this perspective, we propose to study in deeper details the applicability of a new, cost-effective and on-site method to detect gene doping. LAMP-method is simple and fast, and it does not require expensive laboratory equipment or experienced personnel. Furthermore, the results can be directly visualized with naked eyes and finally the testing can be directly performed directly on the site of the competition.

#### Acknowledgments

We are very grateful to Dr. Gianpaolo Rando from SwissDecode Company for sharing the Figure 2 and his expertise regarding the LAMP amplification.

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# Appendix V

Hepcidin as a potential biomarker for blood doping

*Leuenberger N, Bulla E, Salamin O, Nicoli R, Robinson N, Baume N, Saugy M. Drug Test Anal. 2017 Jul;9(7):1093-1097*

Short communication

Drug Testing and Analysis

Received: 12 July 2016 Revised: 13 October 2016 Accepted: 16 October 2016 Published online in Wiley Online Library: 18 November 2016

(www.drugtestinganalysis.com) DOI 10.1002/dta.2122

# Hepcidin as a potential biomarker for blood doping

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**The concentration of hepcidin, a key regulator of iron metabolism, is suppressed during periods of increased erythropoietic activity. The present study obtained blood samples from 109 elite athletes and examined the correlations between hepcidin and markers of erythropoiesis and iron metabolism (i.e., haemoglobin, erythropoietin (EPO), ferritin, erythroferrone (ERFE), and iron concentration). Furthermore, an administration study was undertaken to examine the effect of recombinant human EPO (rhEPO) delta (Dynepo™) on hepcidin concentrations in healthy male volunteers. The effects on hepcidin were then compared with those on reticulocyte percentage (Ret%) and ferritin concentration. There was a significant positive correlation between hepcidin and ferritin, iron, and haemoglobin levels in athletes, whereas hepcidin showed an inverse correlation with ERFE. Administration of rhEPO delta reduced hepcidin levels, suggesting that monitoring hepcidin may increase the sensitivity of the Athlete Biological Passport (ABP) for detecting rhEPO abuse.** 

*Additional supporting information may be found in the online version of this article at the publisher's web site.*

**Keywords:** hepcidin; blood doping; EPO

## Introduction

The greatest deterrent to blood doping using erythropoiesis-stimulating agents (ESAs) is the haematological module of the Athlete Biological Passport (ABP).[1] The haematological module focuses on long-term monitoring of specific blood parameters, such as haemoglobin concentration (Hb) and the reticulocyte percentage (Ret%), to detect abnormal absolute and/or relative changes in individual profiles that may indicate doping with ESAs; however, the shift towards blood transfusions and 'micro-dose' injections of ESAs means that new markers, which can be integrated into the ABP haematological module to increase its sensitivity, are required to detect ESA abuse.[2,3]

Previous studies show that proteins involved in erythropoiesis and iron metabolism have potential utility as biomarkers to detect blood doping.[4–6] Ferritin is an iron storage protein in the liver. It is detectable in serum and is a potential biomarker for ESA abuse and blood transfusions; in particular, it is indicative of blood withdrawal.[5,6] Leuenberger *et al.* suggested that measuring iron levels in ethylenediaminetetraacetic acid (EDTA)-plasma may be a cost-effective method of screening for blood transfusions,[4,7] thereby providing evidence of blood manipulation. In 2014, erythroferrone (ERFE) was identified as a novel erythroid regulator of iron metabolism in a mouse model.[8] ERFE is thought to act by suppressing hepcidin levels during periods of increased erythropoietic activity.[8] Hepcidin is a key player in iron metabolism (Figure 1).[9] This recently discovered peptide hormone is produced by hepatocytes and induces internalization and degradation of ferroportin.[10] By inhibiting ferroportin, hepcidin prevents gut cells from allowing iron into the hepatic portal system, thereby reducing the absorption of dietary iron. Degradation of ferroportin also reduces iron release from macrophages. Recent reports suggest that hepcidin levels are altered by blood transfusions. For example, the mean hepcidin concentration in the blood increases significantly (by 7-fold) at 12 h post-transfusion, and remains 4-fold higher at 1 day post-transfusion in a human clinical study.[5,11] Thus, hepcidin may be a novel biomarker for detecting blood transfusion in an anti-doping context. In addition, Honda *et al.* showed that long-term administration of ESAs such as darboepoetin-α, or continuous administration of erythropoietin receptor activator, to patients on haemodialysis reduces both hepcidin and ferritin levels.[12] As a follow-up to our earlier work, the aim of this study was to examine the correlation between hepcidin levels and markers of erythropoiesis in elite athletes. To simulate a doping control context, we also examined the effect of recombinant human erythropoietin (rhEPO) delta (Dynepo™) administration on hepcidin levels in healthy male volunteers.

## Material and methods

### Analytical methods

Hepcidin levels were measured by liquid-chromatography high resolution mass spectrometry (LC-HRMS) as previously described.[5] EPO, ferritin, and iron levels were measured using Dimension EXL 2000 and Immulite 2000 XPi technology (Siemens Healthcare Diagnostic SA, Zürich, Switzerland), and haemoglobin (Hb) concentrations were measured using a fully automated haematology

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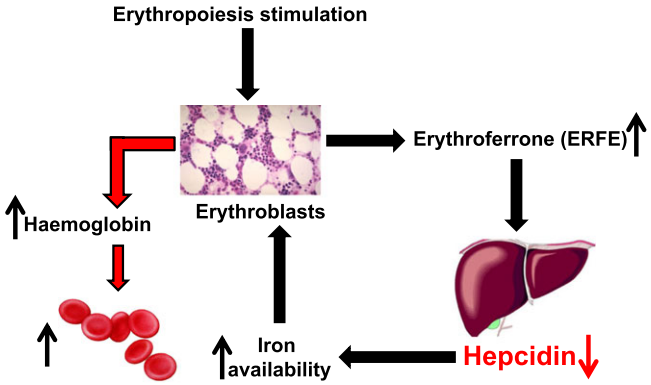


**Figure 1.** Effect of erythropoiesis on regulators of iron metabolism. After stimulation of erythropoiesis, erythroblasts increase production of erythroferrone (ERFE), which is then secreted into the circulation where it represses hepcidin production by the liver. Suppressing hepcidin increases the amount of iron available for synthesis of haemoglobin and new red blood cells. [Colour figure can be viewed at wileyonlinelibrary.com]

analyzer (Sysmex XN 2000, Sysmex, Norderstedt, Germany), as described by Leuenberger *et al.*[5] The OFF-score was calculated as follows, as previously described[6]:

$$OFF - score = [Hb] - 60\,x\,\sqrt{Ret\%} \quad (1)$$

ERFE concentrations were measured using commercial human ELISA kits (Aviscera Bioscience, Santa Clara, CA, USA), according to the manufacturer's instructions. The following assay characteristics were specified by the manufacturer: limit of quantification, 5 ng/mL; antibody specificity for two different ERFE epitopes, 100%. The specificity of human antibodies for their target protein was validated by western-blotting. The concentration of the ERFE standards supplied by the manufacturer ranged from 31.25 to 2000 ng/mL. Intra-assay and inter-assay precision was 6–8% and 8–12%, respectively. Linearity and recovery were tested. A recovery of 118% was obtained using a 4-fold dilution of ERFE standards.

### Correlation between hepcidin levels and markers of erythropoiesis and iron metabolism in elite athletes

Anonymized routine doping control in-competition EDTA-blood samples (n = 109) were used to investigate the correlation between hepcidin levels and markers of erythropoiesis and iron metabolism in high-level male athletes. In all cases, the athlete provided written informed consent. After reception, whole blood was analyzed for haematological parameters and then centrifuged for 15 min at 1500 g. The resulting plasma was aliquoted and frozen at -20 °C.

### Administration of rhEPO delta

Details regarding the participants and the time of plasma sample collection are described elsewhere.[13] Briefly, six healthy Caucasian males (mean age, 27.0 years (SD 4.1); mean body mass index (BMI), 23.9 kg/m$^2$ (SD 2.66)) received a single intravenous injection of rhEPO delta (5000 UI; Dynepo™, Dynepo Shire Pharmaceuticals, Basingstoke, UK) on Days 1, 3, and 5 of the study. Samples were collected in the morning (07:00–09:00 a.m.). A complete red blood cell count was performed, and the reticulocyte cell population was measured using a Sysmex analyzer (XT-2000i analyzer, Sysmex, Norderstedt, Germany).

All subjects provided written informed consent, and the protocol was authorized by the Ethical Commission for the Clinical Research of the Faculty of Biology and Medicine (University of Lausanne, Switzerland) (Protocol no. 02/09).

### Statistical analysis

Unless specified otherwise, data are expressed as the mean ± SEM versus baseline. Statistical comparisons were performed using a two-tailed Student's t-test or a non-parametric Wilcoxon signed-rank test. The Shapiro-Wilk test was used to test the normality of the distribution. $P < 0.05$ was considered statistically significant. Conditions were compared using one-way ANOVA (the *aov* function in R), and post-hoc pairwise comparisons were performed with Tukey's Honestly Significant Difference test (the *Tukey HSD* function in R). All statistical comparisons and Spearman's correlation calculations were performed using standard software (StataIC, StataCorp).

## Results

Spearman's correlation analysis was performed to examine the correlation between hepcidin, ferritin, iron in EDTA-plasma, EPO, Hb, and ERFE (Table 1). The results revealed a significant positive correlation between hepcidin and Hb, iron and ferritin. In contrast, a significant negative correlation between ERFE and hepcidin was observed.

As expected, erythropoietic activity following injection of rhEPO led to an increase in Ret% (Figure 2). Administration of rhEPO also affected hepcidin and ferritin levels (Figure 2 and Table 2). Ferritin was decreased 2-fold at Day 7 when compared with the day before treatment. By contrast, hepcidin levels fell by 13.7-fold after three injections.

Ret%, Hb levels, and the OFF-score are all considered indirect biomarkers with respect to the ABP strategy.[1] A mean 2.3-fold increase in Ret% was observed after three rhEPO injections. By contrast, there was no change in Hb levels (Table 2). The OFF-score decreased after rhEPO injection, mainly due to the change in Ret% (Table 2).

## Discussion

We found a significant positive correlation between hepcidin levels and Hb levels and EDTA-plasma iron. Hb is a key parameter in the

**Table 1.** Correlation between erythropoiesis, iron levels, and other blood parameters.

| Correlation (n = 109) | ERFE | Hb | Hepcidin | EPO |
|---|---|---|---|---|
| **Hb** | **-0.23 | | | |
| **Hepcidin** | *-0.20 | *0.25 | | |
| **EPO** | **0.30 | -0.12 | -0.09 | |
| **Iron** | *-0.20 | **0.29 | **0.29 | 0.06 |
| **Ferritin** | -0.01 | 0.17 | **0.49 | -0.11 |

*$P < 0.05$ and

**$P < 0.01$ indicate statistically significant correlations. Significant values are highlighted in bold.

EPO, erythropoietin; ERFE, erythroferrone; Hb, haemoglobin; iron, EDTA-plasma iron.

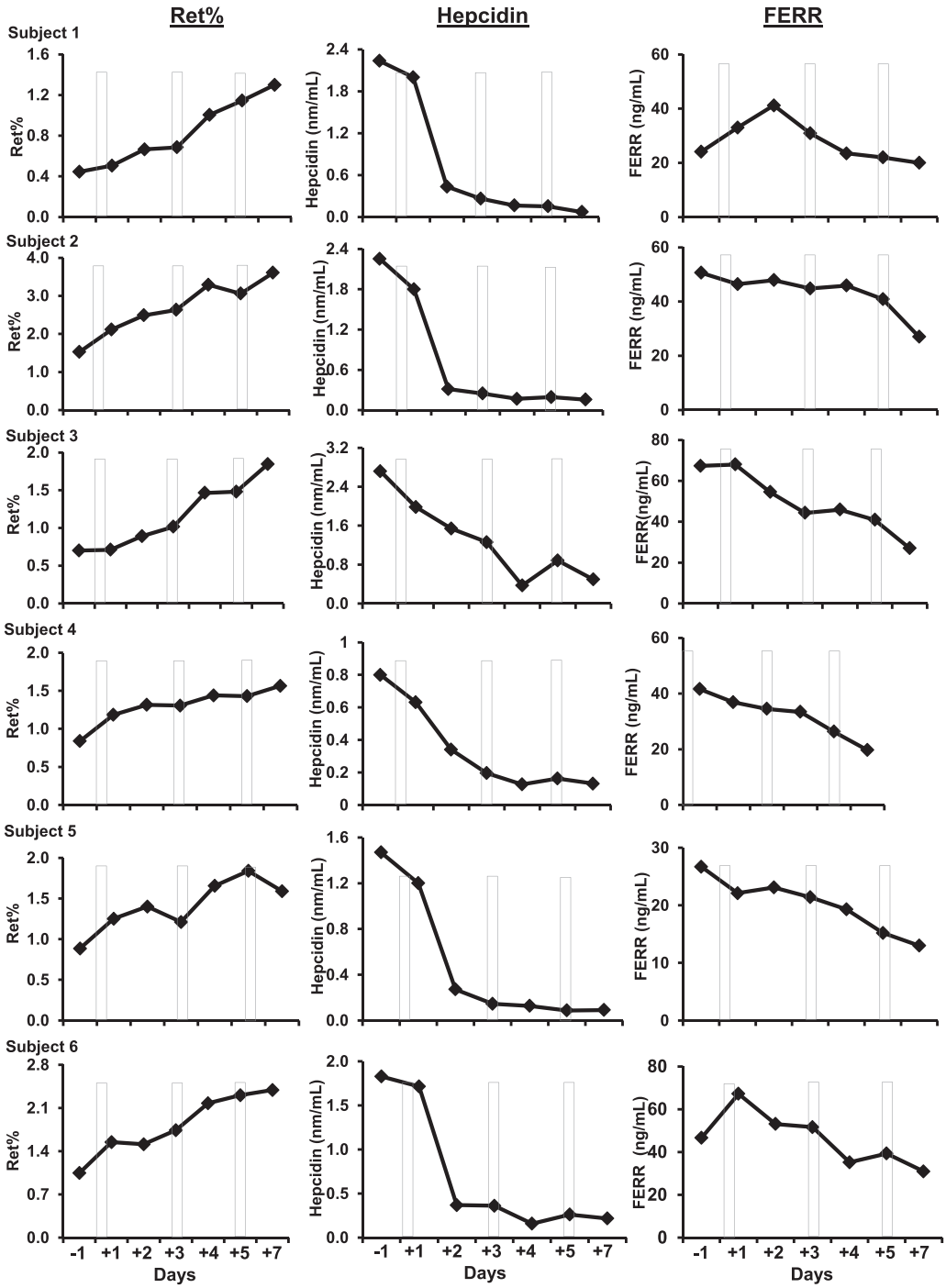


**Figure 2.** Effect of recombinant human erythropoietin (rhEPO) delta administration on the reticulocyte percentage (Ret%) and hepcidin and serum ferritin concentrations in six subjects. White rectangles indicate the times of rhEPO administration. FERR, ferritin.

ABP strategy.[1] That suggests that hepcidin could complement the follow-up of Hb level to detect ESA abuse.

Previously, both hepcidin and EDTA-plasma iron were identified as biomarkers for blood transfusion.[4,5] Measurement of iron levels in plasma samples obtained using EDTA as an anti-coagulant is not recommended due to the strong chelating effect of EDTA; however, the relationship between iron concentrations in paired serum and EDTA-plasma samples demonstrates a strong linear correlation.[7,14] The correlation between hepcidin levels and Hb and EDTA-plasma iron levels in athletes described herein provides additional information supporting the use of these parameters as potential biomarkers for blood doping.

There was also a significant positive correlation between hepcidin and ferritin. This correlation has been demonstrated in previous studies,[12,15,16] and strengthens the significant findings obtained using Spearman's correlation analysis herein. There was a significant positive correlation between ERFE and EPO, and a significant negative correlation between ERFE and Hb (Table 1); similar findings were reported in mice.[8] The correlation between erythropoiesis and factors that play a role in iron metabolism are



  

**Table 2.** Hematological variables and iron metabolism after injection of rhEPO delta (n = 6).

| | Day-1 | Day + 1 | Day + 2 | Day + 3 | Day + 4 | Day + 5 | Day + 7 |
|---|---|---|---|---|---|---|---|
| **Ret%** | **0.91 ± 0.12** | **1.22 ± 0.19** | **1.38 ± 0.21** | **1.43 ± 0.23** | **1.84 ± 0.27*** | **1.88 ± 0.23*** | **2.05 ± 0.28*** |
| **Hb** | **15.23 ± 0.11** | **15.62 ± 0.25** | **15.80 ± 0.19** | **15.60 ± 0.22** | **15.53 ± 0.23** | **15.43 ± 0.22** | **15.47 ± 0.19** |
| **Off-score** | **125.0 ± 0.3.18** | **119.52 ± 3.84** | **116.6 ± 4.53** | **113.0 ± 5.79** | **100.08 ± 7.0*** | **97.98 ± 5.87*** | **93.12 ± 5.87*** |
| **He pci din** | **1.88 ± 0.23** | **1.55 ± 0.18** | **0.54 ± 0.16**** | **0.41 ± 0.14**** | **0.19 ± 0.03**** | **0.29 ± 0.10**** | **0.20 ± 0.05**** |
| **Ferritin** | **43.06 ± 6.00** | **46.38 ± 6.13** | **42.78 ± 3.93** | **37.92 ± 3.68** | **33.03 ± 3.37** | **28.8 ± 3.37** | **21.5 ± 3.37*** |

***P ≤ 0.05 and**

****P ≤ 0.01**; **compared with the day before injection of rhEPO delta. significant values are highlighted in bold.**

in line with the demonstrated link between ERFE, EPO, ferritin, and hepcidin (Figure 1).

A previous study revealed that administration of long-term ESAs to patients on haemodialysis reduces hepcidin and ferritin levels.[12] Our study examined the regulation of hepcidin, ferritin, and Ret% by rhEPO in healthy human subjects following administration of EPO delta, a human recombinant form of EPO that is very similar to endogenous EPO.[13] The data presented herein suggest that hepcidin is more sensitive than Ret%, and ferritin as potential indirect marker of rhEPO abuse. Hepcidin is a peptide hormone comprising 25 amino acids and is easily measured in human plasma or serum by LC-HRMS, a technique available in most accredited laboratories. Thus, hepcidin could be the first biomarker of ESA abuse that can be routinely detected by LC-HRMS.

Athletes show inter-individual variations in hepcidin levels.[5] Therefore, individual follow-up (as occurs in the ABP strategy) is appropriate for detecting this biomarker. As is the case for many variables in an anti-doping context, hepcidin levels are influenced by inflammation.[17] For example, interleukin-6 (IL-6) is both necessary and sufficient for hepcidin induction. Therefore, measuring inflammatory markers such as IL-6 could easily be incorporated into a test to rule out the effects of inflammation (as described by Leuenberger *et al.*[5]).

By contrast, we found that ERFE responses following rEPO administration were highly variable (Supplemental Figure). Induction of high levels of ERFE following administration of rEPO was observed in a mouse model of β thalassemia, which is associated with a chronic inflammatory state.[18] Recently, Honda *et al.* observed clear induction of ERFE in patients on haemodialysis.[12] The healthy subjects included in the present study did not have thalassemia, were not on haemodialysis, and had normal Hb levels (Table 2). Therefore, our data suggest that ERFE is not a reliable indirect biomarker that can be used to identify ESA abuse in healthy athletes.

This study has some limitations. First, the influence of confounding factors such as physical exercise on the proposed biomarkers was not explored. The effects of iron administration (injection or oral absorption) on the levels of hepcidin and other markers of iron metabolism are currently under investigation. Moreover, it would be interesting to examine the impact of micro-doses of rhEPO delta on hepcidin levels over a longer period.

In conclusion, the data presented herein suggest that rhEPO delta, a first-generation recombinant EPO, regulates hepcidin levels. In 2014, World Anti-Doping Agency statistics revealed that more than 82% of adverse analytical findings in rEPO cases were first generation.[19] The data presented herein suggest that hepcidin has utility as a potential indirect biomarker for detecting first generation rEPO with previously observed with blood transfusion. Thus, hepcidin is of general interest in the field of blood doping.

**Acknowledgements**

We thank Dr Séverine Lamon for managing the clinical study of rEPO delta administration. We are also grateful to Dr Tiia Kuuranne for critical reading of the manuscript. This research was supported by the Département Universitaire de Médecine et Santé Communautaire (grant: 06/2015).

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## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

# Appendix VI

RNA stabilized blood tubes to measure haemoglobin concentration

*Leuenberger N, Schobinger C, Salamin O, Saugy M, Kuuranne T. Drug Test Anal. 2017 Jun;9(6):942-943*

Application note

Drug Testing and Analysis

Received: 13 January 2017 Revised: 24 February 2017 Accepted: 24 February 2017 Published online in Wiley Online Library: 17 April 2017

(www.drugtestinganalysis.com) DOI 10.1002/dta.2179

# RNA stabilized blood tubes to measure haemoglobin concentration

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## Introduction

Previously, Salamin *et al.* suggested that measurements of gene transcripts from reticulocytes in RNA stabilized blood tubes could be biomarkers of blood transfusion.[1] It was also demonstrated that the same strategy was applicable for the detection of erythropoietin stimulating agents in micro-doses.[2] RNA stabilized blood tubes such as Tempus™ blood RNA tubes are mainly used for multi-site clinical studies in order to facilitate the transport and storage of samples.[3,4] Indeed, Tempus™ tubes are stable at room temperature for up to five days, unlike EDTA tubes that have to be kept at 4°C and analysed within 48 hours after collection. Theoretically, reticulocyte messenger RNA (mRNA) biomarkers could complement the already existing athlete biological passport (ABP) variables and provide additional evidence for blood manipulation. Because haemoglobin concentration (Hb) is a key parameter of the ABP for detecting blood doping, and was shown to increase rapidly after a blood transfusion or erythropoiesis-stimulating agent abuse, the possibility of reliable measurement of Hb in RNA stabilized blood tubes was investigated.[5] Correlation of Hb between paired RNA stabilized blood tubes and EDTA blood samples and storage stability were tested.

## Material and methods

RNA stabilized blood samples (Tempus™ blood RNA tubes, Applied Biosystems, Foster City, CA) and EDTA samples (K2EDTA tubes, 7.2 mg, 4 mL, BD Vacutainer®, Plymouth, UK) were obtained from 54 male volunteers of mean age of 27.6 years (SD 6.4) and mean body mass index (BMI) of 23.6 kg/m$^2$ (SD 3.6). All subjects gave signed consent and the protocol was authorized by the Ethical Commission for Clinical Research on the Human Being (Canton de Vaud, Switzerland; Protocol no. 2016–00324).

Each Tempus™ blood RNA tube contained 6 mL of stabilizing reagent. Blood (3 mL) was drawn into the tube, mixed with this reagent and red blood cell lysis occurred almost immediately. The stabilizing reagent inactivated cellular RNAses and selectively precipitated RNA whereas the proteins remained in solution. Hb was quantified using a Sysmex analyser (XT-2000i analyser, Sysmex, Norderstedt, Germany) applying the routine protocol used in routine doping control.[6].

## Results and discussion

Correlation of Hb was determined between RNA stabilized blood tubes (Tempus™ blood RNA tubes) and EDTA blood paired samples ($n = 54$). The relationship between Hb in Tempus™ tubes and paired EDTA blood samples was linear (Figure 1) and demonstrated an excellent Spearman's correlation coefficient ($r = 0.91$; $p < 0.00001$). Indeed, results within normal values of approximately 14–18 g/dL in male blood samples corresponded to 4.14–5.26 g/dL in RNA stabilized tubes.[7] The difference of range between both measurements is mainly due to the fact that blood in Tempus™ tubes is diluted, unlike the EDTA samples, but we cannot exclude other factors influencing the measurements.

Stability of Hb was also investigated after storage at room temperature (RT) for 7 days. A minor but significant decrease (mean difference of 0.08 g/L) was observed in Hb concentration (Figure 2 A). Considering the accepted technical variance (measurement in duplicates) is 0.1 g/L for Hb, the minor impact observed during storage at RT should not influence the longitudinal follow-up quantification of Hb using Tempus™ tubes as a complement to the existing ABP. However, one freezing/thawing cycle impacted Hb level in Tempus™ tubes with a mean difference of 0.17 g/dL (Figure 2B). This observation demonstrated that storage at −20°C would not be recommended for this method. Furthermore, these data suggest that Hb is stable in Tempus™ tubes when stored at RT, which is valuable in terms of general logistics in doping control of blood samples. Indeed, transport at RT and Hb stability up to 7 days could assist in rationalizing the general workflow which may have an effect on total costs (Figure 3). Although EDTA tubes cost less than Tempus™ tubes, they require transportation and storage at 4°C, which is more expensive than that at RT. The cost comparison is based on estimation done by our laboratory and prices from the European market. From the laboratory perspective, the advantages would be mainly connected to the improvements of sample storage and flexibility to batch samples for analysis. Due to those logistical and cost-effective advantages of Tempus™ tubes, and to the good correlation obtained between the two different tubes, Tempus™ tubes could be considered suitable for Hb measurements. Reticulocyte gene transcripts could be combined with Hb measurements in Tempus™ tubes in order to complement and improve the efficiency of the existing ABP.

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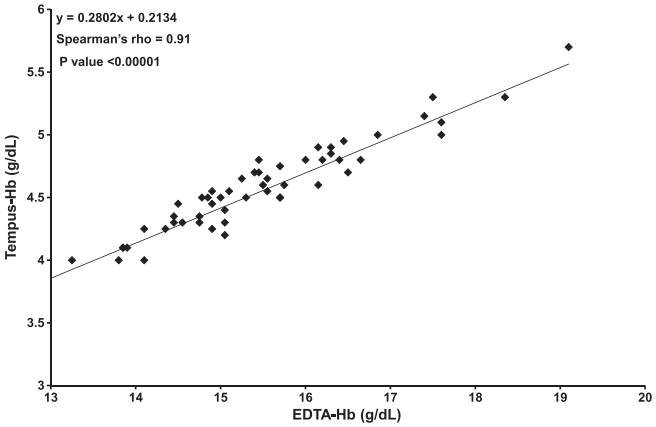


**Figure 1.** Correlation between paired EDTA and Tempus™ blood samples for Hb measurement ($n$ = 54).

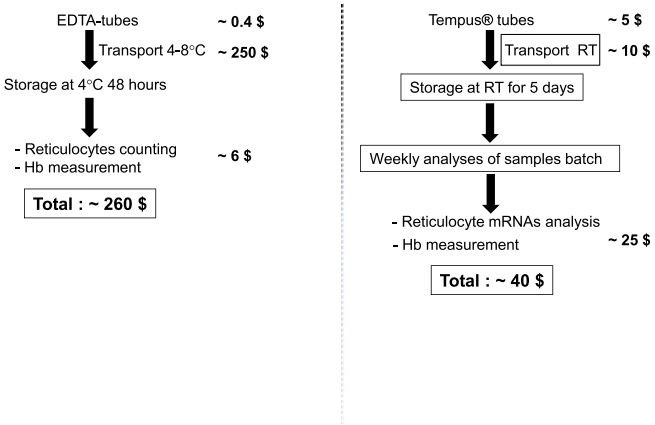


**Figure 3.** Comparison of work-flow between EDTA blood sample and Tempus™ blood RNA tubes. The cost comparison is based on estimation done by our laboratory and prices from the European market. [Colour figure can be viewed at wileyonlinelibrary.com]

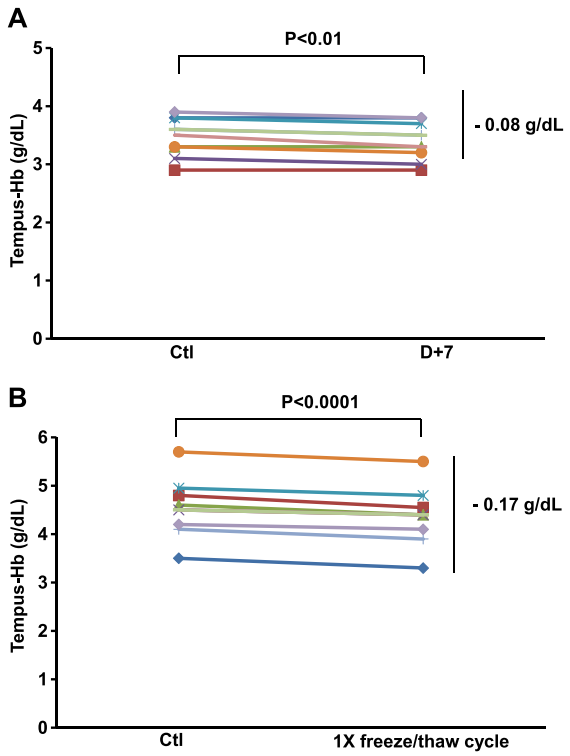


**Figure 2.** Impact of storage on Hb concentration. (A) Blood samples were stored for 7 days at room temperature. Hb quantification was measured in Tempus™ blood RNA tubes. (B) Effect of freeze–thaw cycle on Hb concentration. $N$ = 10; Ctl: control. [Colour figure can be viewed at wileyonlinelibrary.com]

## Conclusion

RNA stabilized tubes were demonstrated as a potential tool for the detection of blood doping with mRNA biomarkers. Surprisingly, RNA stabilized tubes were demonstrated to be applicable to Hb measurement as well. An excellent correlation between blood in Tempus™ tubes and EDTA-blood tubes in male volunteer samples was exhibited for Hb analysis.

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## Appendix VII

Le dialogue nécessaire entre médecine et antidopage pour l'intégrité du sport et de l'athlète [The necessary dialogue between medicine and anti-doping for the integrity of sport and the athlete]

*Faiss R, Salamin O, Astolfi T, Saugy M. Rev Med Suisse. 2018;14(613):1360-1363*

# Le dialogue nécessaire entre médecine et antidopage pour l'intégrité du sport et de l'athlète

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*Rev Med Suisse 2018; 14: 1360-3*

**En réponse aux actualités antidopage, cet article traite quelques cas particuliers et s'étend aux perspectives futures de développement de stratégies antidopage efficaces. Après avoir rappelé les principes actuels de la lutte antidopage, il aborde l'utilisation de substances tolérées à un certain seuil et évoque les autorisations à usage thérapeutique (AUT). Les substances autorisées mais pouvant présenter un risque sanitaire pour les athlètes sont discutées avant de conclure sur le développement du passeport biologique de l'athlète comme futur terrain commun pour la lutte antidopage et le suivi médical des sportifs. En conclusion, cette approche souligne le dialogue impératif entre organisations antidopage et médecine du sport afin de défendre de bonnes pratiques à même de préserver la valeur intrinsèque du sport.**

### The necessary dialogue between medicine and anti-doping for the integrity of sport and the athlete

*In the light of recurring anti-doping news, this article discusses some special cases and extends to the future prospects of developing effective anti-doping strategies. After recalling the current principles of the fight against doping, the use of substances tolerated at a certain threshold, and the therapeutic use exemptions (TUE) are discussed. Authorized substances with a health risk for athletes are discussed before concluding on the development of the athlete's biological passport as a future common ground for anti-doping and medical follow-up of athletes. In conclusion, this approach emphasizes the imperative dialogue between anti-doping organizations and sports medicine in order to defend good practices preserving the intrinsic value of sport.*

## INTRODUCTION

De grands événements sportifs habillent chaque saison le calendrier avec des performances toujours spectaculaires et souvent stupéfiantes. Les frimas des hivers olympiques cèdent la place aux championnats si populaires de football ou de hockey sur glace puis à la grand-messe du mois de juillet avec le Tour de France. Dans ce contexte, la lutte antidopage défraie souvent la chronique par des révélations sur les performances entachées de produits ou de pratiques illicites.

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L'agence mondiale antidopage (AMA) édicte, dans son Code mondial antidopage, les règles du jeu définissant le dopage non pas de façon générale mais comme la violation d'une ou plusieurs règles tacitement acceptées dans la pratique compétitive d'un sport.[1] Au-delà de sa portée juridique, le Code souligne le rôle de la lutte antidopage dans la préservation des valeurs intrinsèques du sport, soit de «l'esprit sportif». Toutes les dispositions du Code sont par ailleurs obligatoires et doivent être suivies et appliquées par chaque organisation antidopage, chaque sportif ou autre personne dans la mesure où elles leur sont applicables. Pour combattre le dopage en promouvant l'esprit sportif, le Code exige la participation des sportifs mais aussi du personnel d'encadrement du sportif. De fait, l'éducation concernant les règles en vigueur mais aussi les risques du dopage représente une clé dans le maintien d'une éthique et d'une équité sportive.

L'athlète est ainsi au centre des préoccupations de la lutte antidopage avec des partenaires sciemment informés dans sa quête d'une performance intègre. Si l'excellence dans la performance est un but, l'AMA exhorte à jouer franc jeu avec la santé comme fondement essentiel des valeurs liées au sport (p. 14).[1] Les exigences du code définissent de fait clairement le rôle des médecins car les substances ou méthodes interdites reposent sur toute «preuve médicale ou scientifique, effet pharmacologique ou expérience démontrant que l'usage de la substance ou de la méthode présente un risque avéré ou potentiel pour la santé du sportif» (Paragraphe 4.3.1.1, p. 29).[1]

D'autre part, force est de constater la professionnalisation constante dans les pratiques sportives en lien avec notamment la médiatisation qui génère des retombées lucratives tant pour les sportifs que pour leur encadrement. On ne peut alors que difficilement reprocher à un athlète, dans ce contexte d'esprit sportif, de viser l'excellence sportive en palliant douleurs ou pathologies par des traitements médicaux adéquats. Il relève cependant de l'athlète et de son encadrement (médical dans ce cas) de vérifier si un traitement adéquat demeure autorisé. Consciente qu'elle ne peut (ni ne doit) empêcher un athlète de traiter une pathologie aiguë ou chronique, l'AMA a ainsi défini le concept d'autorisation à usage thérapeutique (AUT) pour certaines substances ou méthodes interdites dans la mesure où le sportif subirait un préjudice de santé significatif si le traitement en question n'était pas administré. Le Code précise explicitement qu'une AUT ne peut être délivrée que s'il est hautement improbable que l'usage thérapeutique de la substance ou de la méthode interdite produise une amélioration de la performance au-delà de celle attribuable au retour à l'état de santé normal du sportif

après le traitement de la pathologie aiguë ou chronique. De plus, il ne doit pas exister d'alternative thérapeutique autorisée pouvant se substituer à la substance ou à la méthode interdite. Le sportif doit ainsi pouvoir compter sur un soutien avéré de son médecin traitant pour évaluer soit un éventuel risque de vouloir participer à son sport en présence d'une pathologie, soit la possibilité effective d'une AUT. Dans une étude récente, 58% (de 775) athlètes craignaient les effets secondaires de l'usage de produits prohibés même si 18% d'entre eux restaient intéressés à leur usage au détriment de leur santé.[2] Ceci étant donc contraire à toute éthique sportive et avant tout médicale.

Il reste important de souligner qu'un comité d'au moins trois médecins expérimentés en matière de soins et de traitements sportifs doit valider toute demande d'AUT au nom d'une organisation nationale antidopage, fédération internationale ou organisation responsable de grandes manifestations. De solides connaissances de la médecine clinique et sportive sont requises au même titre que l'indépendance des médecins impliqués (par exemple, aucune responsabilité politique dans l'organisation antidopage qui les a nommés) (Paragraphe 5.2, p. 38).[1]

En définitive, le Code mondial antidopage édicte des règles nombreuses et strictes à même de proposer une lutte antidopage efficace, dans la mesure où chacun en respecte sa teneur et ses responsabilités. A la lueur de la récurrence des actualités antidopage, nous proposons d'évoquer dans cet article quelques cas particuliers représentatifs. Nous étendrons le discours aux perspectives futures de développement de stratégies antidopage efficaces.

## PRODUITS INTERDITS OU DOSES INTERDITES

Pour aborder la situation particulière de l'utilisation de produits dans un contexte de pathologie (aiguë ou chronique), et toujours dans une réflexion antidopage, il est utile de traiter le cas du salbutamol. Agoniste des récepteurs β2-adrénergiques, son indication première est le traitement de l'asthme, notamment par inhalation.[3] Dans la liste en vigueur des produits et méthodes interdits, l'AMA inclut tous les bêta-2 agonistes sélectifs et non sélectifs (catégorie S3 de la liste des interdictions de l'AMA), y compris tous leurs isomères optiques.[4] Cependant, le salbutamol inhalé est autorisé pour un maximum de 1600 microgrammes par 24 heures répartis en doses individuelles, sans excéder 800 microgrammes par 12 heures à partir de n'importe quelle prise. Dans la mesure où il n'est pas possible d'évaluer ou de documenter avec certitudes les doses inhalées, un seuil de concentration de salbutamol dans l'urine a été fixé. La présence dans l'urine de salbutamol à une concentration supérieure à 1000 ng/ml n'est pas cohérente avec une utilisation thérapeutique et sera considérée comme un résultat d'analyse anormal (RAA), à moins que le sportif ne prouve par une étude de pharmacocinétique contrôlée que ce résultat anormal est bien la conséquence d'une dose thérapeutique (par inhalation) jusqu'à la dose maximale indiquée ci-dessus. Dans le cas du salbutamol, ce n'est donc pas directement la substance qui est interdite, mais la dose. Aucune demande d'AUT pour une dose normale n'est requise. Sachant que certains facteurs confondants (exercice physique et déshydratation, par exemple) peuvent influencer la cinétique d'élimination de cette substance et sa concentration dans l'urine, un athlète doit considérer son métabolisme individuel pour éviter un dépassement du seuil autorisé.[5]

Notablement, on constate que le salbutamol représente une médication très largement utilisée parmi les athlètes olympiques (6,1% de tous les participants aux Jeux Olympiques de Pékin en 2008).[6] Après de nombreuses modifications concernant le salbutamol inhalé dans sa liste de substances interdites, l'AMA a choisi de l'exclure de la liste en prenant soin de fixer un seuil urinaire à ne pas dépasser sur la base de connaissances scientifiques existantes en considération des recommandations thérapeutiques valides. Une étude récente a mesuré la concentration de salbutamol urinaire après un effort intense et une dose proche de la recommandation clinique dans la gestion de l'asthme (600 microgrammes).[7] La concentration urinaire moyenne de 510 ng/ml de salbutamol leur fait suggérer l'application d'un seuil limite plus bas que le seuil en vigueur. Ce résultat contraste ostensiblement avec les récentes études soulignant que, dans certains cas, l'administration de la dose maximale autorisée peut résulter dans un dépassement de la valeur seuil.[5,8] De façon très intéressante, il convient de souligner que ces deux études récentes mettent en garde les athlètes quant au risque d'excéder le seuil réglementaire en les guidant clairement vers un conseil de dosage adéquat afin de considérer ce risque.

Par ailleurs, on peut encore préciser que l'AMA a récemment intégré la notion d'effet confondant par une éventuelle déshydratation en spécifiant une correction de la limite de décision pour les substances à seuil tenant compte de la gravité spécifique de l'urine.[9] Dans ce sens, une récente étude a investigué chez 18 sujets (asthmatiques et non) la pharmacocinétique du salbutamol dans les 4 heures après administration de 800 microgrammes de salbutamol par voie inhalée ou orale.[10] Dans le cas de l'inhalation, aucune valeur n'a dépassé le seuil autorisé par l'AMA après correction avec la gravité spécifique. A l'inverse, l'administration orale résultait dans des concentrations moyennes au-delà de 2000 ng/ml. En prenant en compte les règles en vigueur et la littérature scientifique, la «futilité» du contrôle des concentrations urinaires de salbutamol évoquée récemment[11] doit absolument être mise en perspective. Les rôles respectifs de l'athlète dans sa connaissance des règles et de son médecin dans la proposition d'un traitement cliniquement adéquat représentent alors une clé de compréhension à intégrer fondamentalement.

## PRODUITS INTERDITS AVEC CERTAINES VOIES D'ADMINISTRATION AUTORISÉES

Dans l'appréhension de la relation athlète-médecin, d'autres traitements constituent un défi pour la lutte antidopage et certainement pour la santé des athlètes. En l'occurrence, l'usage des glucocorticoïdes est largement répandu pour leurs puissantes propriétés anti-inflammatoires et antiallergiques. Cependant, en compétition, tous les glucocorticoïdes sont interdits s'ils sont administrés par voies orale, intraveineuse, intramusculaire ou rectale. De fait, cela signifie que les glucocorticoïdes administrés par injection intra-articulaire, par

voie topique ou par inhalation sont autorisés. Depuis de nombreuses années, cette problématique mine le dialogue entre les médecins du sport et les autorités antidopage. En effet, les laboratoires antidopage, qui doivent rapporter toute concentration de glucocorticoïdes > 30 ng/ml, mettent très souvent les autorités antidopage dans l'embarras. En effet, il est impossible analytiquement parlant de discriminer une injection intra-articulaire, d'une injection intramusculaire ou d'une prise orale. Par l'interdiction de cette famille de substances en compétition, les organisations antidopage doivent systématiquement demander si le résultat analytique est dû à une administration récente de corticoïdes (période de compétition pouvant varier selon les sports, par exemple, 24 heures avant un match ou dès l'ouverture du village olympique).

La nécessité de dialoguer avec les médecins du sport pour établir des règles d'administration et de délai avant la reprise de la compétition devient alors impérative. En parallèle, il existe une vraie volonté des laboratoires d'établir des seuils-limites pour chaque corticostéroïde (par exemple, bétaméthasone, prednisone, triamcinolone, etc.) à l'aide d'une décision consensuelle entre médecins du sport concernant le délai entre la dernière injection et la reprise de la compétition. Face au problème, certaines organisations antidopage ou fédérations (par exemple, le Mouvement pour un cyclisme crédible (MPCC) ou l'Union cycliste internationale (UCI) par son règlement médical) imposent un repos obligatoire de 8 jours après toute injection de corticostéroïdes. L'UCI ajoute une règle de non-utilisation pure et simple d'injections (*no needle policy*). Ce principe d'événement «sans aiguille» a d'ailleurs été repris par le Comité international olympique lors des derniers Jeux Olympiques d'hiver.[12] A noter que la mesure du MPCC imposant 8 jours de repos après une injection de corticostéroïdes est prise pour l'intégrité de la santé du coureur et non à des fins antidopage. Cette réflexion prend en compte la santé des athlètes plutôt que la nécessité d'être à tout prix présent lors d'une compétition. Cette situation complexe s'inscrit également dans le contexte d'une mercantilisation de sportifs dont les transferts couvriraient parfois largement le budget annuel de l'AMA, soit dit en passant. Nous sommes cependant loin de l'idée inepte que toute prise de corticostéroïdes vise à améliorer les performances. Le manque d'information et d'éducation sur les risques d'effets secondaires délétères oriente certainement le choix d'un traitement adéquat en présence d'une pathologie dans un sens peu compatible tant avec la santé du sportif qu'avec l'éthique sportive.[13,14] Définitivement, la limite entre usage et abus est ténue[15] et il est impératif que le sportif soit informé et conscient des risques sanitaires encourus afin de préserver sa santé.

Par ailleurs, il existe également certaines substances autorisées pour lesquelles le corps médical devrait prendre position pour protéger la santé des athlètes. Par exemple, le tramadol, analgésique très puissant, utilisé comme alternative aux opiacés, ne figure actuellement pas sur la liste des substances interdites en raison de la spécificité des disciplines où on retrouve son usage abusif. Les résultats du monitoring dans certains sports, notamment dans le cyclisme, ont montré un abus significatif de l'utilisation de ce produit[16] pour un effet ergogénique discutable en plus d'un effet néfaste sur la lucidité.[17] Il en va de même pour les anti-inflammatoires non stéroïdiens. La Fédération internationale de football association (FIFA) a montré que ces produits sont utilisés de manière abusive et souvent à titre préventif dans les grands tournois.[18,19] Ces résultats remettent en évidence le rôle central du médecin sportif afin de promouvoir une meilleure compréhension dans ces pratiques potentiellement désastreuses dans les sports professionnels.[20]

## CONCLUSION

L'éducation, la prévention et la dissuasion, en amont de la détection constituent les piliers de la lutte antidopage actuelle.[21] Les efforts fournis dans la détection par les laboratoires sont considérables avec des résultats probants.[22-26] Si un nombre étendu de tests peut effectivement augurer d'un meilleur effet dissuasif,[2] la prévention repose certainement sur une éducation opportune et adéquate tant des sportifs que de leur encadrement immédiat, où le médecin sportif possède un rôle privilégié.[27] Dans cette prise en charge globale de l'athlète dans son contexte sportif, la généralisation du passeport biologique de l'athlète (ABP) indique une voie future pour la lutte antidopage en parallèle à un suivi médical efficace. L'ABP a été créé au sein du Laboratoire suisse d'analyses du dopage (LAD). Il permet de pallier les courtes fenêtres de détection de substances interdites lors de tests ponctuels en offrant un outil de sanction indirecte qui utilise un suivi longitudinal individuel de biomarqueurs pertinents.[28,29] Introduit d'abord dans le cyclisme en 2009,[30] l'ABP a déjà permis de sanctionner plus de 700 athlètes depuis son introduction malgré une utilisation initialement peu étendue.[31] Des indicateurs biologiques variant subitement (un taux de réticulocytes qui chute brusquement dans un intervalle de temps très court, par exemple) constituent autant d'anomalies potentiellement liées au dopage mais qui peuvent aussi résulter d'une pathologie sous-jacente. Pour la santé des athlètes, ces éventualités concomitantes doivent être considérées globalement. Le suivi médical pour la santé du sportif et son suivi biologique à des fins de détection de dopage se chevauchent. En conclusion, il nous semble opportun que les organisations antidopage tendent la main pour travailler de concert avec le corps médical afin de défendre avec humilité et pertinence de bonnes pratiques à même de préserver la valeur intrinsèque du sport et la santé de l'athlète.

Conflit d'intérêts: Les auteurs n'ont déclaré aucun conflit d'intérêts en relation avec cet article.

### IMPLICATIONS PRATIQUES

- Les exemples de substances autorisées à un certain seuil ou nécessitant une autorisation à usage thérapeutique (AUT) soulignent la limite floue entre amélioration des performances et traitements de pathologie sous-jacente
- L'éducation des athlètes et de leur encadrement au sujet des traitements néfastes pour leur santé est une clé pour la prévention et la dissuasion
- Agir en amont de la détection des substances ou méthodes interdites représente l'un des piliers de la lutte antidopage

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