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ADVANCEMENTS IN DETECTION OF PERFORMANCE ENHANCING DRUGS IN DRIED BLOOD SPOTS – FOCUSING ON ERYTHROPOIETIN

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Advancements in detection of performance enhancing drugs in dried blood spots – focusing on erythropoietin

Thesis for Doctoral Degree (Ph.D.)

By

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”And if also any one contend in the games, he is not crowned unless he contend lawfully.”

2 Timothy 2:5 (The Bible)

Popular science summary of the thesis

The use of drugs to improve performance in sports is an age-old controversy, but as the health of the athlete and demands for clean sport are pushed in society, some athletes continue to push back. As various drugs are developed, the anti-doping world tries to keep up with efficient collection programs, sensitive drug detection methods, and clear consequences for doping athletes. While urine has been the standard sample collected for doping control, dried blood spots (DBS) have emerged as another potential matrix for doping analysis. Many doping substances have been detected in DBS, but little has been published about the detection of the hormone erythropoietin (EPO) and its analogues (which are used as doping agents for endurance). It was determined that the extent of EPO detection in DBS and its detection in relation to other hormones (steroids and growth hormone) required better understanding. It was seen in my studies that low concentrations of EPO (both the body's natural hormone and pharmaceutical products) are well-detected in DBS and that growth hormone markers may be more sensitive to storage time and temperatures than EPO in DBS. In addition, the effect of steroids on EPO detection in DBS may not be so distinct because of the natural variation of one's EPO levels. EPO detection in DBS has become a realistic scenario for doping controls, but there is still room for improvement in detection methods and sample collection strategies.

Abstract

The use of erythropoietin (EPO) for performance enhancing purposes is detected mainly in urine and serum samples in anti-doping laboratories. However, dried blood spots (DBS) have emerged as a potential additional sample for EPO and other various doping substances. The aims of these studies were to develop a method for EPO analysis from DBS (study I) and use this method to sensitively detect endogenous EPO, micro-doses of exogenous EPO, and the *EPO* c.577del protein VAR-EPO (studies I-III). I also observed the stability of EPO and insulin-like growth factor 1 (IGF-I) in DBS (study I and V), along with investigations into the relationship between EPO and testosterone (study IV). **Sample collection:** Capillary blood (for DBS), venous blood (for DBS and serum) and urine were collected from healthy volunteers. The DBS devices used were Capitainer®B 50, Mitra® VAMS, Tasso-M20, and Whatman 903 filter paper cards for EPO and IGF-I detection. Urine and serum were also collected from 30 self-reported anabolic androgenic steroid (AAS) users to examine the presence of EPO in such samples. **Methods:** The primary method I used for detecting EPO and VAR-EPO in the collected samples was immunopurification with the EPO Purification Gel Kit, SAR-PAGE, and Western blot. EPO concentrations were measured in serum using a commercial immunoassay kit. IGF-I was analyzed by LC-MS/MS and with the automated immunoassay system IDS-iSYS. Serum AAS was quantified using an immunoassay. **Results:** Endogenous EPO was sensitively detected on both polymer and paper blood supports (studies I, II, III), providing stable results (study I), while urinary EPO fluctuated more (study I) and occasionally showed degradation or undetectable bands (studies I and III). The instability of IGF-I in DBS at room temperature (study V) and the slight variations of EPO detection indicate that currently DBS is not suited for longitudinally monitoring these markers. In addition, micro-doses of various recombinant EPOs (rEPO) and VAR-EPO were well-detected in 4 DBS devices from healthy volunteers (studies II, III). When rEPO micro-doses were administered with testosterone to healthy volunteers, as expected, there was no increase in EPO signal intensity in DBS, but in AAS users who were positive for testosterone, serum EPO concentrations were slightly elevated, along with some hematological parameters (study IV). **Conclusion:** The detection and knowledge of EPO in DBS has made progress, but DBS detection methods, sample collection, and storage strategies still require further discussion and investigation.

List of scientific papers

I. **Heiland CE**, Ericsson M, Pohanka A, Ekström L, Marchand A. Optimizing detection of erythropoietin receptor agonists from dried blood spots for anti-doping application. Drug Testing and Analysis. 2022;14(8): 1377-1386. Doi: 10.002/dta.3260.

II. **Heiland CE**, Martin L, Zhou X, Zhang L, Ericsson M, Marchand A. Dried blood spots for erythropoietin analysis: Detection of micro-doses, *EPO* c.577del variant and comparison with in-competition matching urine samples. Drug Testing and Analysis. 2023;1-5. doi: 10.1002/dta.3596

III. **Heiland CE**, Lehtihet M, Börjesson A, Ekström L. Evaluation of a single Eporatio® micro-dose in urine and dried blood spots. Drug Testing and Analysis. 2024;1-4. doi: 10.1002/dta.3651

IV. **Heiland CE**, Schickel Y, Lehtihet M, Börjesson A, Ekström L. Supra-physiological doses of anabolic androgenic steroids impact erythropoietin and blood parameters. Drug Testing and Analysis. 2023;15(6):599-604. doi: 10.1002/dta.3452

V. **Heiland CE**, Mongongu C, Semence F, Pohanka A, Ericsson M, Marchand A, Ekström L. IGF-I intra-individual variation in serum and DBS using immunoassay and LC-HRMS methods. Manuscript.

Scientific papers not included in the thesis:

Heiland CE, Masquelier M, Bhuiyan H, Ericsson M. A simple method to immunopurify erythropoiesis stimulating agents from urine, aiming to optimize erythropoietin screening by SAR-PAGE. Drug Testing and Analysis. 2019; 11(11-12):1666-1674. doi: 10.1002/dta.2730.

Bergström H, **Heiland C**, Björkhem-Bergman L, Ekström E. MiRNA and erythropoietin profiles during the menstrual cycle in relation to hematological and lipid biomarkers. 2021. bioRxiv; 2021. DOI: 10.1101/2021.11.05.467468.

Contents

1 Literature review ... 3
1.1 Erythropoietin ... 3
1.1.1 Endogenous EPO hormone ... 3
1.1.2 Exogenous EPO hormone ... 4
1.1.3 Adverse effects of EPO use ... 5
1.2 EPO in sports ... 5
1.2.1 Why is EPO prohibited in sports? ... 5
1.2.2 Prevalence of EPO use in sports ... 6
1.2.3 EPO micro-dosing ... 7
1.3 EPO samples ... 8
1.3.1 Traditional matrices: Urine and serum/plasma ... 8
1.3.2 Alternative matrix: Dried blood spots (DBS) ... 8
1.4 Direct detection of EPO ... 9
1.4.1 Immunopurification methods ... 9
1.4.2 Polyacrylamide gel electrophoresis (PAGE) and Western blot ... 10
1.4.3 *EPO* gene variant ... 11
1.5 Co-use of EPO and growth hormone (GH)/ anabolic androgenic steroids (AAS) ... 11
1.6 Indirect detection of doping by longitudinal monitoring ... 12
1.6.1 The hematological passport module ... 12
1.6.2 The endocrine passport module ... 13
2 Research aims ... 15
3 Methods ... 17
3.1 Sample collection ... 17
3.1.1 Ethical approval ... 17
3.1.2 Inclusion criteria ... 18
3.1.3 Venous whole blood: DBS ex vivo (studies I and II) ... 18
3.1.4 Venous whole blood: serum (studies I and V) ... 18
3.1.5 Capillary whole blood: DBS in vivo (studies I, II, III and V) ... 18
3.1.6 Urine (studies I and IV) ... 19
3.2 Immunopurification ... 19
3.3 SAR-PAGE and Western blot ... 19
3.4 Quantification of EPO ... 19
3.5 Growth hormone biomarker analyses (IGF-I and P-III-NP) ... 20

3.6 Statistical analyses 20
3.7 Ethical considerations 20
4 Results and discussion 23
4.1 Detection of endogenous EPO in DBS 23
4.2 Detection of rEPO in DBS 25
4.3 Detection of EPO in relation to AAS intake 26
4.4 Longitudinal intra-individual variation: EPO and IGF-I 28
4.5 Challenges of DBS collection 30
5 Conclusions 31
6 Points of perspective 33
7 Acknowledgements 35
8 References 37

List of abbreviations

AAF	Adverse analytical finding
AAS	Anabolic androgenic steroids
ABP	Athlete biological passport
BRP	Biological reference protein
CERA	Continuous erythropoietin receptor activator, also known as MIRCERA
DBS	Dried blood spot(s)
ELISA	Enzyme-linked immunosorbent assay
EPGK	Erythropoietin Purification Gel Kit
EPO	Erythropoietin
EPO-Fc	Erythropoietin fused with human immunoglobulin heavy chain Fc
ERA	Erythropoietin-receptor agonist
ESA	Erythropoiesis stimulating agent
GH	Growth hormone
HCT	Hematocrit
hEPO	Human erythropoietin, refers to endogenous EPO
HGB	Hemoglobin
IGF-I	Insulin-like growth factor 1
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
NESP	Novel erythropoiesis stimulating protein
P-III-NP	Procollagen III amino-terminal propeptide
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
RBC	Red blood cell
rEPO	Recombinant erythropoietin

RET%	Reticulocyte percentage
SAR	N-lauryl-sarcosine sodium salt
T	Testosterone
VAMS	Volumetric absorbtive microsampling
VAR-EPO	EPO protein produced from the *EPO* gene variant c.577del
WADA	World Anti-Doping Agency

Introduction

The use of drugs for enhancing athletes' performance has been reported over decades for various sport disciplines, from amateurs to elites. While drug doses used in professional sports for performance enhancement are not necessarily toxic, chronic use (particularly when combining multiple doping substances) can be harmful, and their use is unfair to athletes who compete cleanly. However, issues with doping and drug abuse are not only in the sports arena, but also in the general public, especially since many doping substances are easily available online for purchase. In 1991, the Swedish law (Lag (1991:1969)) against some doping substances (like anabolic androgenic steroids (AAS)) was established,[1] and to assist in combatting these problems, the Anti-Doping Hot-Line in Sweden has endeavoured to provide information and advice to those seeking help on their own behalf, for a loved one, or to healthcare professionals and law officers.[2]

Overall, the goals of the anti-doping community surround the health of the athletes participating in sports at various levels and creating and maintaining a fair playing field for all athletes. To do this, the World Anti-Doping Agency (WADA, created in 1999) seeks to globally harmonize the fight against doping in sports by dictating rules, developing technical documents, and listing the prohibited methods and substances that must be evaluated in athletes' samples by high-quality WADA-accredited laboratories in their analytical processes. In this way, prohibited hormones, like erythropoietin (EPO), AAS, and growth hormone (GH) are well-detected and monitored in doping control urine and serum samples. This project endeavours to present the results from the alternative anti-doping matrix of dried blood spots (DBS) for (mainly) EPO, to demonstrate the sensitivity of the method, and to encourage its use as a deterrent for cheating athletes.

1 Literature review

1.1 Erythropoietin

1.1.1 Endogenous EPO hormone

EPO is a hormone that regulates oxygen transport in the body by stimulating the production of red blood cells (RBCs) (Figure 1). This protein has 165 amino acids, with 4 glycosylation sites (three N- and one O-linked).[3-5] Its production is tightly regulated by the kidney via a negative feedback loop that ensures a stable range of RBC production. Upon oxygen deprivation, in anemic or hypoxic conditions, (caused by, for example, renal dysfunction, cancer, or high altitudes),[4] hypoxia-inducing factor is activated in the kidneys to stimulate *EPO* gene transcription, thus up-regulating EPO protein release in the blood circulation.[6] EPO binds to the membrane-bound EPO receptor on precursors of RBCs in the bone marrow.[7] This activates a signal cascade that leads to the transcription of genes, promoting survival and proliferation of immature erythroid cells, eventually differentiating them into erythroblasts, reticulocytes, and erythrocytes.[7] Consequently, the RBCs in circulation are increased, thus increasing oxygen transport to body tissues and muscles.[8]

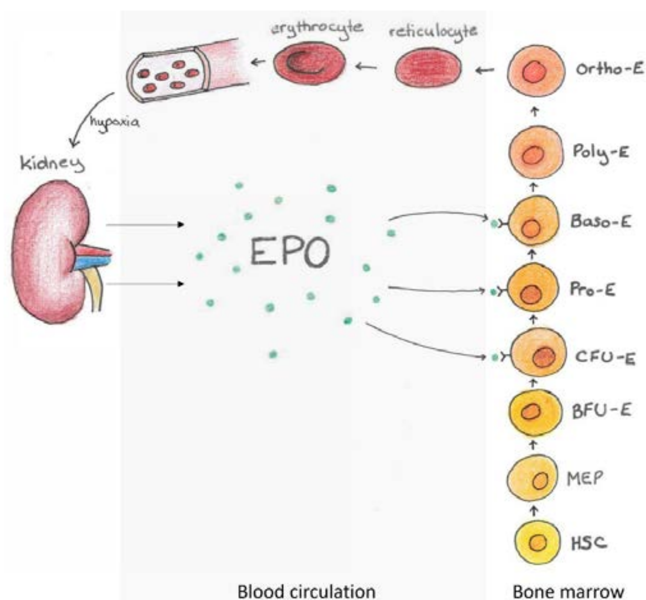

Figure 1. Erythropoietin (EPO) production cycle. Ortho-E = orthochromatic erythroblast; Poly-E = polychromatic erythroblast; Baso-E = basophilic erythroblast; Pro-E = proerythroblast; CFU-E = colony-forming unit-erythroid; BFU-E = burst-forming unit-erythroid; MEP = megakaryocyte erythroid progenitor; HSC = hematopoietic stem cell. ©Carmel E. Heiland

1.1.2 Exogenous EPO hormone

To treat anemia, production of erythropoiesis stimulating agents (ESAs) of various recombinant, biosimilar, and analogue forms of endogenous EPO has taken off since the 1980s and continues today. These products are also known as erythropoietin-receptor agonists (ERAs). The first recombinant forms of EPO (rEPO) were developed in the 1980s[9] using recombinant DNA technology to produce EPO in Chinese hamster ovary cells and baby hamster kidney cells using the human gene.[7] These first-generation rEPOs (also known as, epoetins, e.g., epoetin α, ß, ω, and θ), have identical amino acid sequences to that of the human EPO protein, but with changes in the glycan structure of the EPO polypeptide chain due to its production in cultured mammalian (but non-human) cells,[10] thus slightly affecting the biological activity and half-life of the molecule.[5] For rEPO, subcutaneous or intravenous injections two to three times per week are needed for therapeutic use due to the rapid elimination of the drug (half-life of approximately four to eight hours[9]).

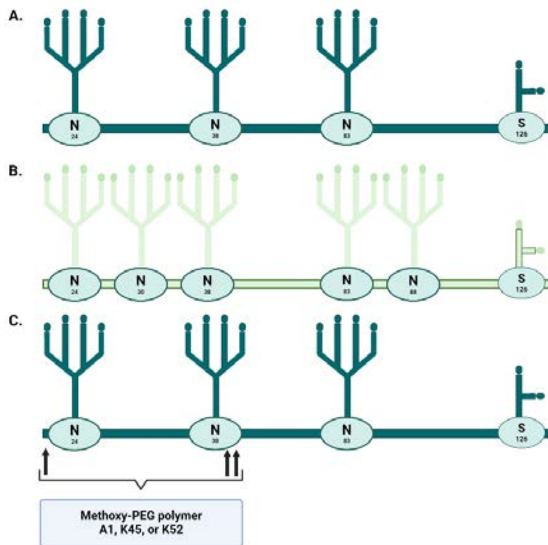

Figure 2. Difference in glycosylation patterns between (A) rEPO, (B) NESP, and (C) CERA. Amino acids: N = asparagine; S = serine; A = alanine; K=lysine. Figure created in Biorender.com and modified from Jelkmann (2007).[10]

The second generation of exogenous EPO, darbepoetin alfa (also known as Novel Erythropoiesis Stimulating Protein, NESP) has two additional N-glycosylation sites and consequently more glycans compared to endogenous EPO.[10] With this modification of the molecule, the half-life increased about two- to three-fold[9] (25 – 48 hours[11]) compared to rEPO, thus reducing the dosing frequency (about once/week or once every two weeks) while still being as effective as rEPO for anemia treatment.[11]

In 2007, MIRCERA (Continuous Erythropoietin Receptor Activator; CERA) was produced, consisting of epoetin beta attached to methoxy polyethylene glycol (PEG).[9] The addition of PEG increases the molecular weight (to about 60 kDa[10])

and limits glomerular filtration and elimination in urine.[12] PEG consequently increases the molecule's half-life even more than that of the second generation (almost one week), thus requiring a lower dosing frequency of every two to three weeks.[10,13] A summary of the molecular differences between epoetin, darbepoetin, and CERA is shown in Figure 2.

The latest development of EPO-derived therapeutics is the addition of the dimeric Fc region to the original EPO sequence, which increases the molecular weight of the molecule and its half-life in the blood circulation.[14] Due to its size (~60 kDa), glomerular filtration is limited.[15] Since the expiry of the protected patent, first- and second-generation rEPO drugs, called EPO biosimilars, are now produced in many countries around the world[16] and easily purchased on the black market. There are also EPO analogues that are for laboratory and research use only (e.g., biological reference protein (BRP), a combination of two first-generation rEPOs).

1.1.3 Adverse effects of EPO use

The most common adverse effects reported during ESA treatment have been hypertension, headaches, thrombosis, injection site pain,[17] and there have been some reports of immune reactions.[18,19] Sudden death in an animal study was observed[20] and there have been deaths suspected in connection with EPO use in athletes.[21] This was particularly problematic in the early 1990s, when high rEPO doses led to athletes having high hematocrit (HCT) levels (> 50%), leading to a high risk of thrombosis adverse events (e.g., cardiac arrest).[22]

1.2 EPO in sports

1.2.1 Why is EPO prohibited in sports?

EPO increases RBC production, favouring oxygen distribution to muscles during physical exertion, which can improve athletes' endurance. Still, there are possible health risks to taking EPO. For this reason, the use of ESAs has been prohibited from the sports scene by the International Olympic Committee since the 1990s.[23] The WADA Prohibited List places EPO (group S2: Peptide hormones, growth factors, related substances and mimetics) among the substances that are prohibited in- and out-of-competition.[24]

1.2.2 Prevalence of EPO use in sports

Although efficient detection methods are regularly evolving since the first detection method published in 2000,[25] some athletes are not deterred from using ESAs in endurance sports, possibly because of the timing of the doping control session or the low doses used. In 2022, most adverse analytical findings (AAFs, i.e., positive cases) were reported in athletics (40 AAFs in urine, 4 in blood) and cycling (20 AAFs in urine, 3 in blood).[26] Table 1 shows the number of samples collected for ERA analysis from 2015 to 2022.[26]

Table 1. Number of urine and blood erythropoietin-receptor agonist (ERA) samples collected and analyzed worldwide 2015-2022.[26] The percentage of adverse analytical findings (AAFs) for urine and blood are also indicated along with the total ERA samples for each year.

Year	Urine samples	Urine AAFs	% urine AAFs	Blood samples	Blood AAFs	% blood AAFs	Total samples
2022	51 678	66	0.13	3776	7	0.19	55 454
2021	50 940	52	0.10	4 953	14	0.28	55 893
2020	35 963	29	0.08	1 845	3	0.16	37 808
2019	51 929	78	0.15	3 757	14	0.37	55 686
2018	47 955	61	0.13	4 792	16	0.33	52 747
2017	44 322	56	0.13	4 531	29	0.64	48 853
2016	43 246	44	0.10	3 464	22	0.64	46 710
2015	32 999	45	0.14	3 219	1	0.03	36 218

Between 2016 and 2022, 0.2-0.6% of blood samples and about 0.1% of urine samples were positive. This higher incidence of positive cases in blood samples should be noted for sample collection programs. From 2016 to 2021 (excluding the first Covid epidemic year in 2020), about 15-30% of AAFs were from blood samples despite representing only 10% of the doping control samples analyzed for EPO[26] (Figure 3).

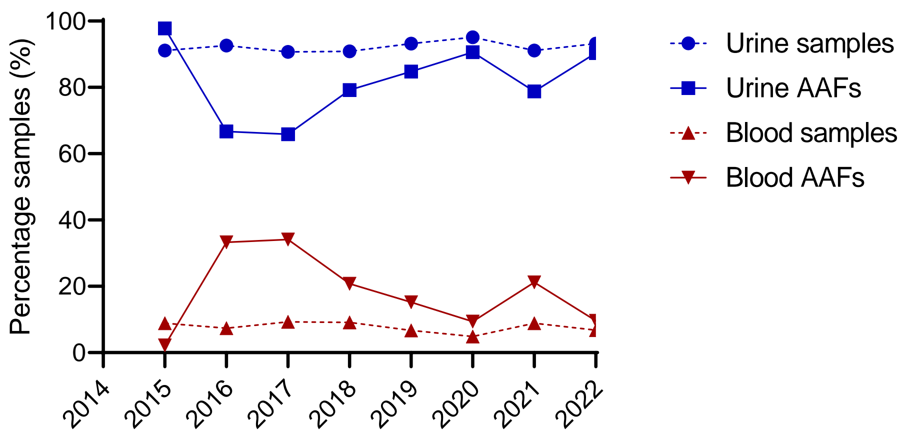

Figure 3. World Anti-Doping Agency testing figures for urine and blood samples collected for EPO analysis worldwide 2015-2022. AAF = adverse analytical finding. Figure created from WADA Testing Figures 2022.[26]

Although urine is the primary way of elimination of EPO, it is known that EPO will be more stable in blood samples (less risk of proteolytic/bacterial degradation), and even some ERAs (e.g., CERA and EPO-Fc) are not easily filtered into urine and are therefore more easily detected in blood.[27] This highlights the importance of increasing blood sample collection and that the lack of more frequent doping control blood samples can hinder the detection of ERA abuse in sports. A solution to the current difficulties in collecting blood samples (e.g., high costs, need of a phlebotomist, refrigerated shipments) would be an alternative blood sample collection method, e.g., a microvolume of capillary blood collected as dried blood spots (DBS).

1.2.3 EPO micro-dosing

To increase RBC count and oxygen levels by other means than several days at high altitudes, some endurance athletes have done one or a combination of the following: use of nitrogen tents or hypoxia chambers (not prohibited), have an homologous or autologous blood transfusion (prohibited), or use of an ERA (prohibited)[28]. The examination of variations of the hematological parameters that can indirectly lead to identification of blood doping is complicated by the natural hematological variations that can mask a real doping case. It has been suggested that, to evade detection and still obtain a performance enhancing effect,[29] EPO micro-doses (e.g., <20 IU/kg[30-33]) are used by athletes. By taking micro-doses,

athletes may also decrease the risks for health complications. Detection of micro-doses has been investigated in studies II and III.

1.3 EPO samples

1.3.1 Traditional matrices: Urine and serum/plasma

As discussed above, urine is the most common sample collected for doping control, making up over 90% of samples taken for EPO analysis since 2015.[34] This is because it is a less invasive method than venous blood collection and the high volumes can easily be collected (usually >100 mL).[35] Venous whole blood is collected in serum gel tubes for analyzing EPO and GH, and in EDTA tubes for analyzing the hematological parameters for the ABP (see section 1.4), homologous blood transfusion, or, if needed, ESAs from plasma. For analysis, high sample volumes (10-15 mL urine, 500 µL serum) are often needed to obtain maximal sensitivity for identification of the prohibited ERA.

1.3.2 Alternative matrix: Dried blood spots (DBS)

Alternative matrices are tested for forensic and doping control analyses (e.g., hair, saliva, exhaled breath, sweat)[35] and DBS from capillary blood is one that is progressing rapidly in the anti-doping community. DBS was introduced by Ivar Christian Bang to measure blood glucose from dried blood on filter paper,[36] then famously, it was implemented in the 1960s for detecting phenylketonuria in new-borns.[37] For over 20 years now, it has been investigated as a doping control matrix for various substances.[38-45] DBS for doping control can be collected from a finger prick or from the upper arm (Figure 4). If these collection sites are for some reason unavailable, samples can be taken from the ear lobe, abdomen, or leg.[46] The collection devices have different supports for the capillary blood collection (filter paper, polymers, or blood reservoirs with anticoagulant) and collect about 10-100 µL of blood, depending on the device. The advantages attached to DBS for anti-doping are simple, phlebotomist-free collection compared to venous blood, and room temperature shipping, and DBS requires less storage space in the fridge/freezer.[35,47] It is less intrusive than urine collection, less invasive than venous blood collection, and therefore appreciated by athletes.[48] During the 2020-2022 pandemic, challenges in venous blood collection forced the hand of the doping control community to examine more closely the possibility of DBS collection, with many National Anti-Doping Organizations already testing and implementing this method,[49,50] particularly since DBS became an official doping control matrix in 2021.[51] WADA has stipulated some collection and storage requirements to

harmonize anti-doping laboratories in their handling of these samples.[52] However, DBS comes with challenges in sensitivity due to the low sample volumes (assessed in study I), logistics performing multiple analyses (i.e., screening and confirmation), preserving the integrity of the samples, and sufficient sample volumes for the diverse analytical methods. Observations of the integrity of EPO and insulin-like growth factor I (IGF-I) in DBS over time are discussed in studies III and V.

Figure 4. Dried blood spot collection devices for finger prick and upper-arm. A. Whatman 903 filter paper cards (finger). B. Capitainer®B 50 (finger). C. Tasso-M20 (upper-arm). D. Mitra® VAMS (finger). ©Carmel E. Heiland

1.4 Direct detection of EPO

1.4.1 Immunopurification methods

Various methods are used in anti-doping laboratories to “clean up” urine, serum/plasma, and DBS samples before analysis begins. To do this, anti-EPO antibodies are used to specifically extract it from the sample matrix, in a plate (i.e., in enzyme-linked immunosorbent assay (ELISA)), on magnetic beads (i.e., Dynabeads, or NHS beads), or on Sepharose gel beads (i.e., EPO Purification Gel Kits).[27,53] The EPGK uses Sepharose gel beads coupled to anti-EPO antibody that extract EPO from the sample[54] (Figure 5). The kit offers simple sample handling and high sample throughput while still maintaining sensitivity.[55] The EPGK was used in studies I-IV. ELISA for DBS has been tested and/or validated by other researchers.[45,56,57]

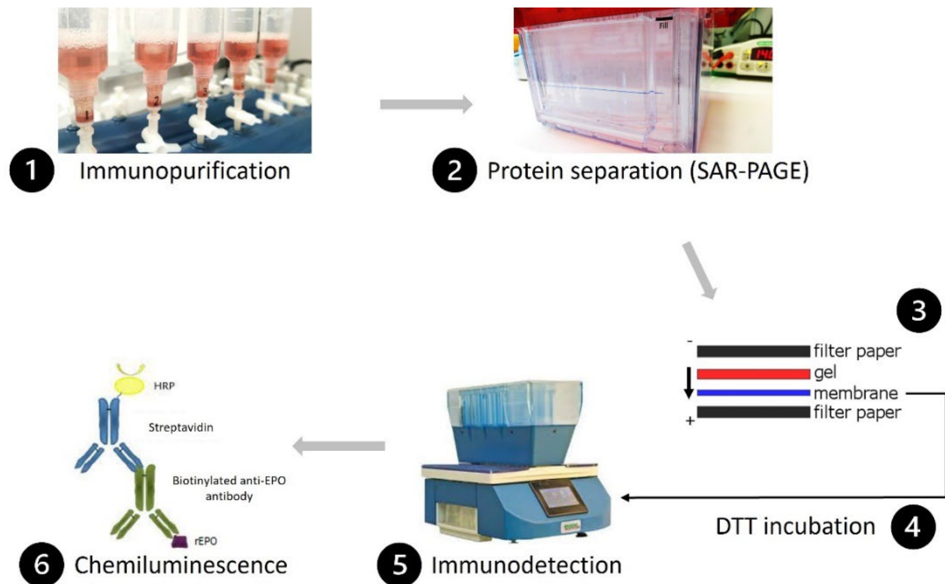

Figure 5. Overview of the EPO analytical method. (1) Samples are immunopurified using the EPO Purification Gel Kit. (2) Purified samples undergo N-lauryl sarcosine polyacrylamide gel electrophoresis (SAR-PAGE), which separates the EPO proteins based on their molecular weight. (3) The proteins are transferred from the gel to a polyvinylidene fluoride membrane, which is then (4) incubated in dithiothreitol (DTT) reducing agent and then (5) blocked with 5% low-fat milk, incubated in biotinylated anti-EPO antibody, in streptavidin coupled to an enzyme, and washed several times in between and after. These steps are done automatically in the BlotCycler, depicted here. (6) The addition of horseradish peroxidase substrate causes a chemiluminescent reaction with the streptavidin-coupled enzyme, which allows EPO in the membrane to be photographed with a CCD camera. ©Carmel E. Heiland

1.4.2 Polyacrylamide gel electrophoresis (PAGE) and Western blot

After EPO isolation, immunopurified samples are analyzed with electrophoretic methods on PAGE (Figure 5), followed by transfer of the separated proteins on a polyvinylidene fluoride membrane and incubation with a detection antibody anti-EPO to specifically reveal EPO signals. Developments in EPO Western blot detection methods since the early 2000s,[25] using a separation of the various ERAs by their molecular weight, have made sodium dodecyl sulphate- and N-lauryl-sarcosine sodium salt (SAR)-PAGE common for analysis, thus providing a simpler analytical method from the original isoelectric focusing. When evaluating results, distinct bands for CERA, EPO-Fc, and NESP may be detected and compared to a reference standard mix and a negative control sample.[58] A challenge with this method is the close molecular weights between first-generation rEPOs (~30-34 kDa) and endogenous EPO (~30 kDa). The presence of rEPO in urine or blood samples, creates either a mixed exogenous/endogenous EPO band (a smear) or a single distinct band above the endogenous[58,59] (Figure 6). Even with these

methods, artefacts (or non-specific bands) can occasionally be detected, but the specificity, sensitivity, and robustness of the method makes it appropriate for ERA analysis.

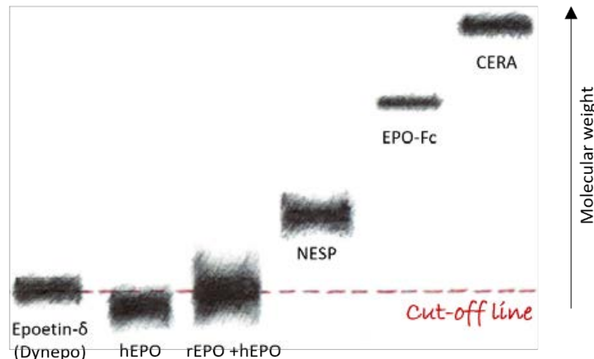

Figure 6. Evaluation of erythropoietin-receptor agonist analysis. Bands that are above or extend beyond the cut-off line are considered suspicious. hEPO = endogenous EPO; rEPO+hEPO = recombinant EPO + endogenous EPO (mixed band/smear). ©Carmel E. Heiland

1.4.3 *EPO* gene variant

The recent discovery of the *EPO* gene variant c.577del (occurring in <1% of the East Asian population) has changed the way suspicious samples are considered.[60] The mutation induces the production of EPO with additional amino acids (VAR-EPO) thus presenting a band of higher molecular weight than the wild-type endogenous EPO, close to the molecular weight of rEPO. Consequently, heterozygous expression with the presence of endogenous EPO and VAR-EPO can be confused with the presence of rEPO. Therefore, procedures have been put in place to prevent athletes from being falsely accused of rEPO use.[58] However, only urine and serum had been tested for the variant, and not in DBS until recently (assessed in study II and by Leuenberger et al.[61]).

1.5 Co-use of EPO and growth hormone (GH)/ anabolic androgenic steroids (AAS)

Athletes who are doping sometimes combine several prohibited doping substances for prime ergogenic effects. There has been evidence of EPO being co-used with GH and AAS.[22,62] An EPO/GH co-administration study reported that GH did not contribute to EPO's effects on hematological parameters, but this may be because of the low doses administered.[29] GH treatment is given to patients with GH deficiency since it contributes to building muscle tissue,[17] increasing

muscle strength, and decreasing body fat, among other effects,[63] thus explaining why GH has been detected in the sports scene.[64,65] GH is typically detected in serum samples using luminescence immunoassay kits measuring GH isoforms of recombinant and pituitary GH.[66] Due to the short detection window of GH,[65] indirect detection methods for procollagen III amino-terminal propeptide (P-III-NP) and insulin-like growth factor 1 (IGF-I) were implemented (section 1.6.2).

AAS (e.g., testosterone, stanozolol, drostanolone[34,67]) are often used to increase muscle mass and strength.[68] It is known that AAS affects the regulation of erythropoiesis,[69-71] increasing RBC count and HCT levels (both of which are observed in the blood ABP) (study IV), and iron metabolism.[72] AAS is reported to increase circulating blood EPO and steroid use has presented differences between the sexes in terms of EPO urinary excretion (higher in men than women).[73] Currently, there are no studies with co-administration of testosterone and rEPO in healthy subjects on the effect of the detection of rEPO and/or testosterone (preliminary results presented below). To detect AAS, urine samples are analyzed for endogenous and exogenous androgenic steroids with gas chromatography mass spectrometry and liquid chromatography mass spectrometry (LC-MS).

1.6 Indirect detection of doping by longitudinal monitoring

The ABP is a tool to longitudinally monitor athletes' blood and urine parameters. This provides an indirect look at the consequences of doping rather than identifying the substance/method used.[9] The ABP software calculates adaptive limits that evolve with each new sample collected and extrapolates "physiological" intra-individual ranges. Overtime, if values from new samples land outside of the limits, the results can be used as proof of doping (adverse passport case) or allow identification of suspicious athletes that can be better targeted for testing.

1.6.1 The hematological passport module

The blood passport (active since 2009) monitors venous blood parameters, thus allowing detection of abnormal variations potentially indicative of blood doping[74] by rEPO use or transfusions.[30] Changes in the oxygen-carrying capacity would be reflected in aberrated blood values, especially hemoglobin (HGB), reticulocyte percentage (RET%), and OFF-score, which are the primary markers of blood doping.[9] The ABP then expanded to include a urinary steroidal module in 2014,[74] and is now extended to a serum steroid and endocrine module[75] (mentioned in study V).

1.6.2 The endocrine passport module

This module of the ABP involves the measurement of GH biomarkers P-III-NP and IGF-I, both of which increase in serum after a GH administration.[65] IGF-I is quantified by electrochemiluminescent immunoassay or a with an LC-MS method.[76] An adaptation of the LC-MS method for DBS, along with longitudinal monitoring of IGF-I, and the use of DBS for ABP have recently been published[77-79] (also examined in study V). Studies into a DBS ABP are also ongoing for longitudinal genetic markers of EPO.[80,81] The strict regulations for ABP collection (time and storage temperature[74]) highlight the advantages of DBS collection: once dried (during transport), the markers are stabilized.

Table 2. Literature summary of publications regarding EPO detection in DBS.

Author	Year	DBS type	DBS volume	Analyte(s)	Methodology used	Origin of DBS
Reverter-Branchat et al.	2018	Whatman 903	25 µL	BRP, NESP, CERA, EPO-Fc	ELISA	Spiked modelled blood, clinical samples
Rocca et al.	2021	Whatman 903, Mitra® VAMS	20 µL	CERA	ELISA	Modelled blood
Heiland et al. (study I)	2022	Whatman 903, Tasso-M20	20-75 µL	BRP, NESP, CERA, EPO-Fc, hEPO	EPGK	Spiked venous blood, upper-arm capillary blood
Requena-Tutusaus et al.	2023	Qiagen QIAcard FTA DMPK-C	25 µL	BRP, NESP, CERA, EPO-Fc	ELISA, EPGK	Spiked venous blood
Heiland et al. (study II)	2023	Tasso-M20, Mitra® VAMS, Whatman 903	~40 µL	hEPO, Eprex®, *EPO* gene c.577del	EPGK	Upper-arm capillary blood, venous blood
Heiland et al. (study III)	2024	Capitainer®B 50, Mitra® VAMS	40-50 µL	Eporatio®	EPGK	Finger capillary blood
Leuenberger et al.	2024	Whatman 903, Tasso-M20	20 µL	DNA, VAR-EPO	DNA sequencing	Finger and upper-arm capillary blood

2 Research aims

The specific aims of each study in this thesis were:

Study I: to investigate the use of the EPO Purification Gel Kit for EPO detection from DBS, looking at specific analytical parameters.

Study II: to evaluate the use of DBS for in-competition EPO analysis and the *EPO* gene variant, in addition to a single rEPO (Eprex®) micro-dose.

Study III: to evaluate the detection of a single rEPO (Eporatio®) micro-dose in polymer- and filter paper-based DBS collection devices and in urine.

Study IV: to investigate urinary and serum disposition of endogenous EPO after supraphysiological doses of AAS, and blood parameters followed in the ABP.

Study V: to evaluate the longitudinal monitoring of IGF-I from DBS.

3 Methods

A summary of general information regarding the study participants is presented in Table 3.

Table 3. General information regarding the longitudinal and administration study populations. Study IV and part of study II were cross-sectional and therefore not included here.

<table>
<tr><th rowspan="2"></th><th rowspan="2">n</th><th rowspan="2">Population</th><th rowspan="2">Sex</th><th>Age</th><th>HGB (g/dL)</th><th>HCT (%)</th></tr>
<tr><th>Mean years</th><th colspan="2">Mean (range)</th></tr>
<tr><td colspan="7">Longitudinal endogenous hormone studies</td></tr>
<tr><td>Study I (EPO)</td><td>5</td><td rowspan="2">Healthy volunteers</td><td rowspan="2">M and F</td><td rowspan="2">28</td><td>14.2 (12.7-16.5)</td><td>41 (37-48)</td></tr>
<tr><td>Study V (IGF-I)</td><td>9</td><td>14.2 (11.7-15.4)</td><td>42 (36-47)</td></tr>
<tr><td colspan="7">rEPO administration studies</td></tr>
<tr><td>Study II (Eprex®)</td><td>2</td><td rowspan="2">Healthy volunteers</td><td>M and F</td><td>35</td><td>-</td><td>-</td></tr>
<tr><td>Study III (Eporatio®)</td><td>5</td><td>M</td><td>20</td><td>13.9-15.6</td><td>44.3 (40.1-47.9)</td></tr>
</table>

HGB = hemoglobin; HCT = hematocrit; M = males; F = females

3.1 Sample collection

3.1.1 Ethical approval

Urine, venous blood, and capillary blood were collected from study participants with approval from the Swedish Ethical Review Authority (studies I and V: Dnr 2020-04258; study III: Dnr 2023-0105-01; study IV: Dnr 2016/108-31), and the WADA International Standards for Laboratories for anonymized athlete samples (studies I and II).[82] Other permits were obtained from international study collaborators: the Algerian Ministry of Pharmaceutical Industry (006/CNA/INT/TECH/2021), the Beijing Tsinghua Changgung Hospital Ethics Committee (22417-5-02), and according to French research laws (Code du Sport Article R232-43) following WADA International Standards for Laboratories annex A for research on anti-doping samples collected from athletes with consent (study II).

3.1.2 Inclusion criteria

For studies I and V, the inclusion criteria were 18-45 years old, healthy, not undergoing hormone treatment and no current use or abuse of illegal substances, narcotics, steroids, or growth hormone. In study II, participants could not be undergoing EPO treatment. For the triathletes, all the participants were professional athletes claiming no use of prohibited substances and they consented to research on their samples. For study III, the inclusion criteria were persons with healthy physical condition, not undergoing hormonal treatment, and have normal blood pressure and hematological values. For study IV, the inclusion criteria were participants with current or previous abuse of performance enhancing substances according to WADA and older than 18 years of age. Informed consent was given prior to the start of each study.

3.1.3 Venous whole blood: DBS ex vivo (studies I and II)

Venous blood was collected in K2 EDTA tubes from the arms of healthy volunteers, pseudo-anonymized, and analyzed on Sysmex XN-1000 to measure HCT, RET%, and HGB. The blood was then either kept blank or spiked and then pipetted onto Whatman 903 filter paper cards (studies I and II) to obtain DBS. Samples were then dried for at least 2 hours and stored at room temperature, 4°C, or -20°C until analysis.

3.1.4 Venous whole blood: serum (studies I and V)

Venous blood was collected in serum tubes for separating the serum fraction for analysis. Serum tubes were centrifuged at 1500 rpm for 15 min and serum was transferred to a new tube. Samples were stored at -20°C until analysis.

3.1.5 Capillary whole blood: DBS in vivo (studies I, II, III and V)

Capillary blood was collected from healthy volunteers and pseudo-anonymized. This was done from either a finger prick with a high-flow lancet and collected on Mitra® VAMS (studies III and V) or Capitainer®B 50 (study III), or from the upper arm skin using Tasso-M20 (studies I and II). Samples were stored at room temperature or -20°C until analysis.

3.1.6 Urine (studies I and IV)

Urine was collected from 5 healthy volunteers (study I) and from 30 male, self-reported AAS users (study IV) and stored at -20°C until analysis.

3.2 Immunopurification

Urine (10 mL), serum (500 µL), and DBS (1-3 spots, ~20-75 µL) samples were mixed with sample buffer and the Sepharose gel beads in the immunopurification column (provided in the kit; Figure 5) and incubated end-over-end for 90 min (urine), 60 min (serum), or 90 min to 2 h (DBS, depending on the study). After washing the column and discarding extra wash buffer from the column, EPO was eluted with 50 µL (urine), 35 µL (serum), or 200 µL (DBS) of elution buffer. Immunopurified urine and serum samples were then stored at -20°C until analysis. For DBS samples, the eluate was transferred to a molecular weight cut-off filter to concentrate the sample down to 15 µL. This was then stored at -20°C until analysis.

3.3 SAR-PAGE and Western blot

Immunopurified urine and serum (8-10 µL) and DBS (15 µL) samples underwent electrophoretic protein separation, and the proteins were transferred to a polyvinylidene fluoride membrane using semi-dry transfer (Figure 5). The membrane was incubated in blocking solution and probed with biotinylated anti-EPO antibody and streptavidin-horseradish peroxidase. The membrane was then subjected to a chemiluminescent reaction, by the addition of an enhanced chemiluminescence substrate, which was visualized and photographed using a CCD camera. To evaluate the results, a cut-off line was created in the analytical software, based on the reference standards included in the analysis. In this way, each sample was assessed as negative or suspicious.

3.4 Quantification of EPO

To determine the concentrations of EPO in serum samples, an ELISA method was used according to the manufacturer's instructions.[83] To evaluate the Western blot results of a sample, Image Lab and GASepo softwares were used to examine EPO

signal intensities and band profiles. Statistics were performed on the absolute volumes calculated by the GASepo software.

3.5 Growth hormone biomarker analyses (IGF-I and P-III-NP)

Peptides and proteins were extracted from DBS samples (20 µL Tasso-M20 spots) with saline solution and 2 hours of sonication. Protein precipitation was performed, and samples were transferred to a 96-well plate for automated IGF-I extraction using a strong anion cartridge on an Extrahera™ Low Volume microSPE instrument. The extract was analyzed with a Zorbax 300SB C18 column (injection volume: 25 µL; flow rate: 500 µL/min). Serum samples (20 µL) also underwent protein precipitation and identical extraction but were analyzed with a YMC-Triart C18 column and with slightly different analytical conditions (injection volume: 5 µL; flow rate: 400 µL/min). IGF-I was also measured using the automated system IDS-iSYS. In serum, the second GH biomarker, P-III-NP, was measured using Advia Centaur CP Immunoassay System as described previously by Marchand et al.[29]

3.6 Statistical analyses

Normality tests were performed to determine the distributions of the data. Mann Whitney U tests were performed to compare EPO signal intensities in studies I, III and IV and to compare stability data in study I. Spearman correlations revealed the relationships between serum EPO concentrations and urinary EPO signal intensities (study IV), serum EPO and serum testosterone concentrations (study IV), hormone concentrations and hematological parameters (study IV), and IGF-I and age (study V). Bland Altman plots were made to compare IGF-I concentrations using automated immunoassay and LC methods, and serum and DBS matrices (study V).

3.7 Ethical considerations

Urine and venous blood are considered intrusive and invasive samples to collect, respectively, while capillary blood is less invasive and intrusive.[35] Capillary blood

collection is close to the skin surface and collects small blood volumes (50-100 µL), and only temporary redness and/or slight tenderness remains after collection. All sample volumes collected in the studies herein were controlled and participants were trained to self-sample for DBS. Any complications (health-wise or logistically) were documented by the participant or the project leaders. The data were pseudo-anonymized. Capillary blood and serum samples were completely used up during analysis, and leftover urine samples from longitudinal studies I, IV, and V were securely stored in a biobank (Region Stockholm 914-Bbk2615).

4 Results and discussion

4.1 Detection of endogenous EPO in DBS

Since approximately 99.5% of EPO doping control samples are negative for an ERA, analytical results mainly show only the endogenous EPO of the athlete. Sensitive detection of the endogenous EPO band as a control sample is important for interpretation of suspicious samples and a critical factor affecting DBS method sensitivity is the blood sample volume. Endogenous EPO was faintly detected in a 20-µL Whatman filter paper DBS sample (Figure 7) and 60-µL spots were suggested as an appropriate sample volume (study I).

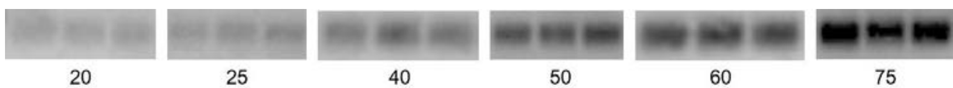

Figure 7. Endogenous EPO from venous blood prepared on Whatman 903 filter paper with different sample volumes, from 20-75 µL. Figure from study I.

However, available sampling devices dictate the sample volume by the number of spots that can be extracted from the device and the collection volume. Sufficient volume must be available for both a screening and a confirmation (A sample), a second confirmation upon request from the athlete (B sample), and the possibility of using spots to detect other doping substances. If the available sample volume is low, compensation must be made by employing sensitive methods.

For study I, it was efficient to use the same immunopurification method for urine that was already implemented in the Swedish Doping Control Laboratory and apply it to DBS samples. The specificity of the method was good and no interferences with ERAs or endogenous EPO bands were detected. However, occasionally, a white band was detected above the EPO-Fc region, which was suspected to be from albumin in the samples. Despite this, the method could be used for DBS collected from multiple devices (both paper and polymer, Figures 7 and 8), and detection limits were similar to the minimum required performance levels stipulated by WADA.[58] Other EPO immunopurification methods that have been applied to DBS are with magnetic Sepharose beads (sensitivity improvements are needed, unpublished data) and anti-EPO pre-coated wells from an ELISA kit (validated in another anti-doping laboratory),[56,57] which can be possible confirmation or alternative methods.

In urine, hEPO can sometimes appear degraded or be undetected in, for example, samples with low or high specific gravity.[84] This was observed in study IV, where

there was no correlation between EPO signal intensity and specific gravity. Undetectable endogenous EPO in blood samples is less common. When comparing urine and DBS from triathletes in-competition, DBS results revealed no degradation, non-specific binding, and missing endogenous EPO bands, unlike in urine (Figure 8). The DBS sample confirms that the urine sample does not contain ERAs (degraded or intact), thus providing crucial, additional information about the sample at the time of collection. Moreover, in-competition samples are more likely to have undetectable EPO than out-of-competition samples,[84] indicating what urinary EPO profiles an analyst may expect to see in such samples.

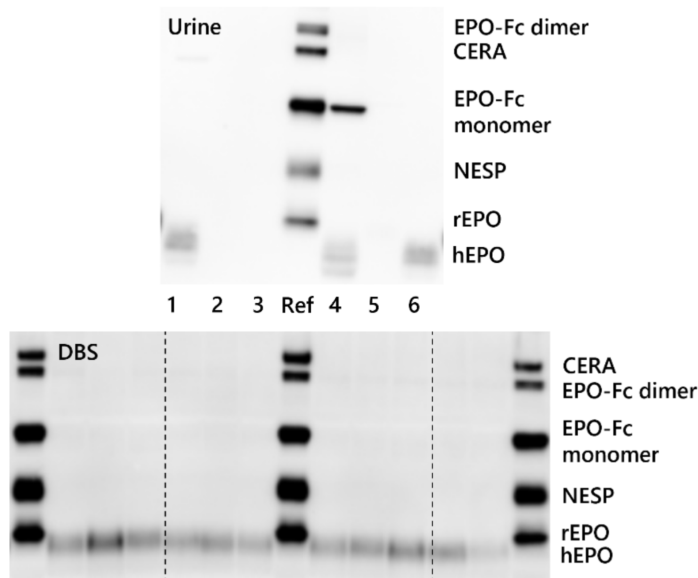

Figure 8. Urine and DBS samples collected from triathletes in-competition using Tasso-M20. Corresponding urine and DBS samples (1-6) are indicated. rEPO = recombinant EPO; hEPO = endogenous EPO. Figure from study II.

One might say that analyzing in-competition samples for EPO is futile. For an athlete to have the performance-enhancing effects but not the presence of the drug in the circulatory system, they would need to take EPO days before a competition. However, history and observation have shown that EPO may still be detected during multiday competitions (e.g., ultramarathons and Tour de France), particularly if the last dose is not eliminated from the system before the in-competition interval begins. In addition, with the improvement of analytical sensitivity, occasionally the elimination of the last dose taken before a competition can be detected in-competition, even if the dose was taken several days before.[85] This testifies to the importance of the athlete testing program that

considers, among other things, the competitions where EPO doping might occur. Distinguishing endogenous and exogenous EPO is more challenging when considering the *EPO* gene c.577del variant. DBS can be used for detection of the gene variant from Mitra® VAMS (study II), and Whatman filter paper and Tasso-M20.[61] To avoid extra collection of a venous sample and re-testing an athlete if no blood sample was initially collected, DBS presents an alternative that could be part of each collection session, to be used in cases of suspicious rEPO detection in urine.[58]

4.2 Detection of rEPO in DBS

Spiking venous blood at low concentrations of ERAs and concentrations close to the WADA minimum required performance levels gave promising detection results with 40-60 µL spots (in study I and unpublished data from study II; Figure 9). This reflected the detection abilities in samples collected during rEPO micro-dose administration studies (studies II, III, and unpublished data) using different DBS collection devices (Capitainer®B 50, Mitra® VAMS, and Tasso-M20, Figure 10). In study II, the single, subcutaneous Eprex® micro-dose could be detected in Mitra® VAMS despite clear band profile differences between the male and female subjects (study II). It cannot be concluded here that the differences were sex related. The difference may be attributed to factors like genetics and exercise regimens. The distribution and elimination are also different according to each person's body composition.

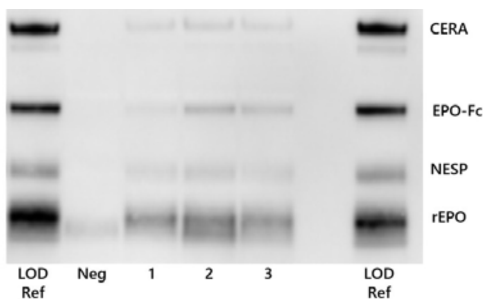

Figure 9. Tasso-M20 detection limit experiment in 3 venous blood samples (1-3). Reference standard mixes and a negative control are indicated. Spiked concentrations: CERA 250 pg/mL, EPO-Fc 100 pg/mL, NESP 10 pg/mL, rEPO 10 IU/L. Unpublished data.

The Eporatio® study (study III) showed the detection of a single, subcutaneous rEPO micro-dose in males for a 72h collection period using Capitainer®B 50 and Mitra® VAMs and compared to detection capabilities in urine. One hour after drug administration, none of the urine samples were suspicious, unlike 40% of the DBS samples (Figure 10). This reflects the initial circulation of the drug in the blood before its elimination in urine. Afterwards, the detection of EPO in urine and DBS

was similar for the micro-dose (rEPO still detected after 72h in DBS and urine). Additionally, there was no significant difference between the 2 blood supports (polymer and paper, p>0.05).

	Capitainer®B 50						Mitra®VAMS						Tasso-M20					
Hours after administration	1	10	24	36	48	72	1	10	24	36	48	72	1	10	24	36	48	72
Subjects																		
1																		
2																--		
3																		
4													x	x	x	x	x	X
5																		

Figure 10. Summary of suspicious rEPO samples from study III (Capitainer®B 50 and Mitra® VAMS) and preliminary results from a rEPO-testosterone administration study (Tasso-M20, unpublished data). Note that it was not the same 5 subjects for both studies. Blue = negative for rEPO; white = suspicious for rEPO; dash = no DBS collected in the device; x = sample results were not able to be evaluated.

Micro-doses of rEPO increase RET% and other blood parameters.[86] A limitation in our administration studies was that the ABP indirect blood doping method was not examined in our participants.

These studies show the consistent detection of rEPO micro-doses and attest to the suitability of DBS for EPO analysis. Of course, administration studies with other ERAs will be useful, but rEPO is a common finding in doping cases. Since all DBS devices can be used for rEPO detection, it comes down to sample volume and the devices preferred by the National Anti-Doping Organizations and authorized by WADA.

4.3 Detection of EPO in relation to AAS intake

Serum and urinary EPO concentrations in 30 male, self-reported AAS users were measured to determine if supra-physiological doses of AAS affect EPO concentrations (study IV). AAS users that tested positive for testosterone had higher serum EPO concentrations (11 ± 4.0 mIU/mL) than AAS users who were not positive for testosterone (5.9 ± 3.0 mIU/mL). A weak positive correlation

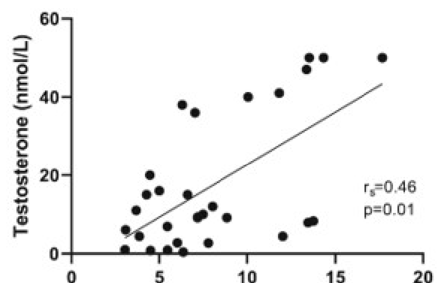

Figure 11. EPO vs. testosterone in serum in 30 male, self-reported AAS users. Figure from study IV.

between serum EPO and serum testosterone was found in this study population ($r = 0.46$, $p = 0.01$, Figure 11), corresponding to previous research showing that a supra-physiological testosterone dose in non-AAS users increases EPO concentrations.[87] However, EPO levels were still within the normal ranges (3.9-17.9 mIU/mL according to Karolinska University Hospital reference values).

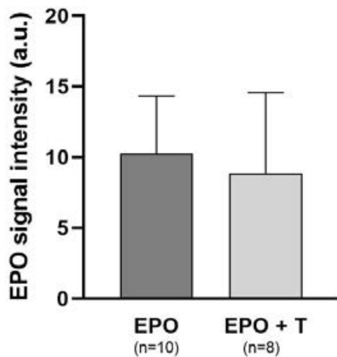

Figure 12. Mean EPO signal intensities and 95% confidence intervals in DBS samples from study participants who received EPO (15 IU/kg) or EPO (15 IU/kg) + 100 mg testosterone gel. The EPO signal intensities from two collection time points (24h and 48h) were pooled for both groups. T=testosterone. Unpublished

When comparing the EPO band signal intensities from the Eporatio® (rEPO) administration study (study III, pooled 24h and 48h data) to an unpublished study where subjects were administered one 100 mg dose of testosterone gel and one 15 IU/kg bodyweight dose of Eporatio®, preliminary results indicated that the DBS EPO signal intensities did not increase when testosterone was used (Figure 12, $p = 0.6$). This was expected since EPO signal intensities are variable within individuals and it is known that endogenous EPO is affected by some confounding factors (e.g., diet and exercise), and therefore may not be recognized in the EPO signal as affected by AAS use. Moreover, even individuals with higher AAS levels had no increased EPO signal intensity (study IV, data not shown), however, a few samples had suspicious EPO screening band profiles. Perhaps prolonged co-use of AAS and rEPO would influence the blood EPO signal intensities in the direct analysis.

Although study IV used serum for testosterone quantification, testosterone and other AAS can be detected in DBS with good sensitivity[44,88,89] and are stable in DBS even up to 1 year.[88] Future studies will continue to investigate the detection of testosterone in relation to EPO intake.

4.4 Longitudinal intra-individual variation: EPO and IGF-I

Intra-individual variations of endogenous EPO in DBS, serum, and urine were followed for 6 weeks in 5 volunteers (Figure 13). Less intra-individual variation was seen in DBS and serum than in urine (study I) and we saw that urinary EPO signal intensities and serum EPO concentrations were not significantly related (p =0.09; study IV). This is in line with results from study II, where urinary EPO may be influenced by other factors (e.g., dehydration that may cause high specific gravity measurements, sample transport/handling, etc.). Normal EPO concentration variations may also be the cause of weaker EPO detection.

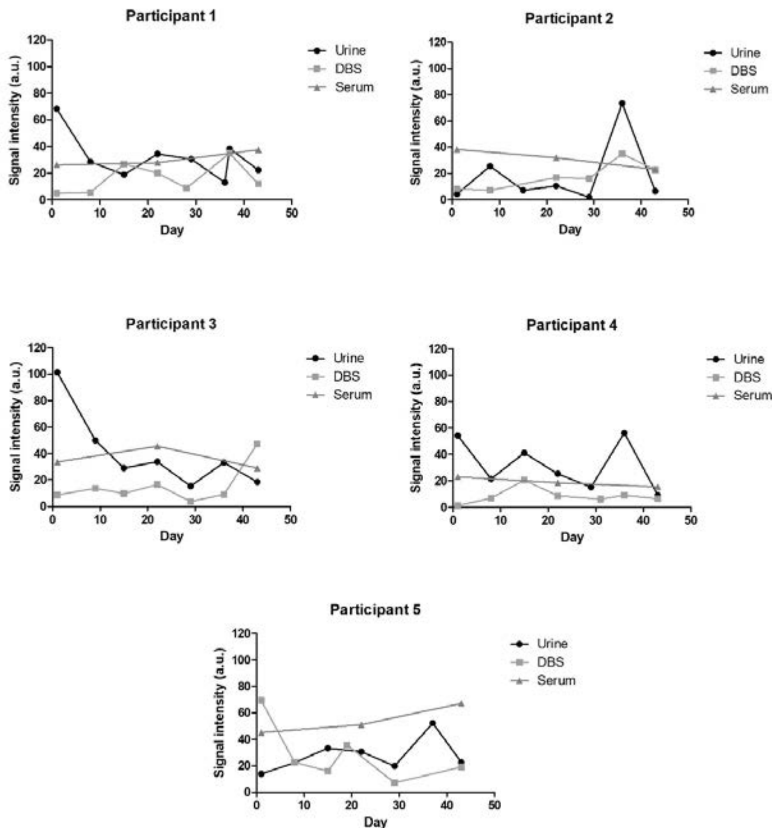

Figure 13. Intra-individual variation of EPO signal intensities from urine, serum, and DBS samples taken from 5 participants over a 6-week collection period. Figure from study I.

Endogenous EPO levels are normally not monitored in athletes, but other endogenous hormones are included in the ABP modules. Currently, IGF-I is measured in serum, and the potential to use DBS instead or in addition, still poses

challenges. Cox et al. showed stable IGF-I values over a 7-day storage period at 25°C and 37°C,[90] while Miller et al. detected GH isoforms from Tasso-M20 DBS after 1-4 weeks stored at room temperature.[91] For Marchand et al., IGF-I was stable in Mitra® VAMS for up to 1 month at -20°C, 4°C, and at room temperature.[92] However, data indicate that DBS samples stored over 6 months at room temperature had lower and more variable IGF-I concentrations (CV 15.3-37.3%) than serum (stored at -20°C) that was analyzed with the automated immunoassay (CV 1.7-24.6%) and the LC-MS/MS (CV 1.9-12.1%) methods (study V, Figure 14). The difference in IGF-I concentration between serum and DBS may have been influenced by the sample volume, inefficient desorption of IGF-I from the DBS polymer or degradation due to storage conditions (study V). This is different from EPO, which showed similar signal intensity variations in DBS and serum when stored at room temperature, although absolute EPO concentrations were not determined (study I). Thus, different doping substances are stable on DBS at different temperatures and on different blood supports,[93-95] but at present, WADA requires all DBS samples to be stored long-term at -20°C.[52] Since IGF-I and EPO show concentration variability, DBS may not be a reliable sample for longitudinal monitoring of endogenous hormones. DBS is currently more suited to detecting direct intake of doping substances.

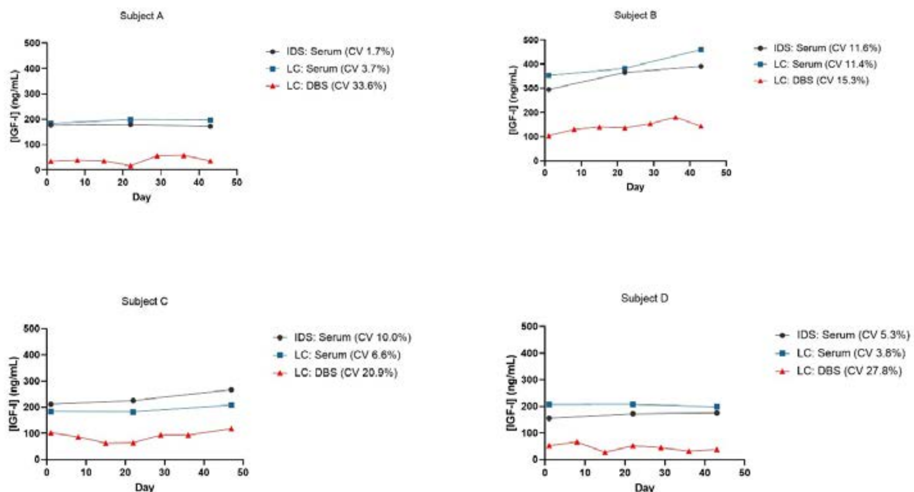

Figure 14. Intra-individual variation of IGF-I in 4 subjects over a 6-week period. Serum was analyzed with IDS-iSYS automated system and LC-MS/MS and DBS was analyzed with LC-MS/MS. Unpublished data.

4.5 Challenges of DBS collection

Using DBS for doping control gives the opportunity for athletes to self-collect their blood samples, making collection simplicity a necessity. Athletes and doping control officers prefer upper-arm sample collection over finger prick collection because of less complications and pain during sample collection.[48] Although some Tasso users (upper-arm collection in this cited study) had low device failure rates (1 of 108), our studies involved several missing samples because of failed collections with this device (13% in study I, 26% in unpublished rEPO/testosterone study). If the high failure rate were device-related, this is a notable factor when considering the cost of the kits. Extra kits would have to be available to athletes, as some sample collectors have done.[96] Sample collection difficulties during a doping control session where the athlete is self-collecting DBS, may be overcome by trained doping control officers assisting the process. Study III involved finger prick sample collection and had a 6% failure rate (3/50).

Furthermore, some DBS kits are produced primarily for therapeutic drug monitoring as it is a bigger market than the anti-doping field. So, devices must be adapted to the doping control setting, where consideration must be taken for maintaining sample integrity (tamper-proof seals, separation of A and B samples) and number of devices for collection. Altogether, the sum of devices, sealing kits, and transport can make DBS more expensive than expected.

Undoubtedly, there are analytical challenges with detecting substances from DBS, that adds to the complex network of considerations. A prominent analytical difficulty with DBS analysis for EPO was the lack of sample volume after immunopurification; samples could only be analyzed once, making re-analysis impossible. This poses problems since the amount of sample collected is also limited by the device's collection volume.

Despite these challenges, DBS still presents advantages of easy collection and transportation, simple long-term storage, and good detection for many prohibited substances.

5 Conclusions

The following conclusions can be made from the studies performed:

- Low concentrations and micro-doses of rEPO are well-detected in DBS of various sample volumes and from various collection devices.
- EPO signals in whole blood give more stable and invariable results compared to urine.
- IGF-I in DBS appears to be affected by storage temperature and time, more than EPO in DBS.
- Serum EPO concentrations increase, but remain witin a normal range when testosterone is used, so the co-use of testosterone and EPO microdoses may not influence endogenous EPO or rEPO detection in DBS.
- The use of DBS for doping controls must be considered in terms of ease of collection from athletes, cost of kits and transport, storage, and analytical sensitivity.

6 Points of perspective

The incorporation of DBS into doping controls will increase the possible blood testing frequency of athletes. This, together with high method sensitivity, increases the chances of detecting cheating athletes with products that are easily degraded in urine. Additional studies can be made regarding multiple micro-doses, various EPOs, and/or in combination with other doping substances to get a broader picture of DBS use in anti-doping. Here, our studies included mainly men, but studies with women, and EPO and testosterone or growth hormone administration may be of interest. In addition, continued stability studies are needed for IGF-I to maintain good analytical sensitivity.

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