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List of papers

I. **Bejder J**, Hoffmann M, Ashenden M, Nordsborg NB, Karstoft K., Mørkeberg J. Acute hyperhydration reduces Athlete Biological Passport OFF-hr score. *Scand J Med Sci Sports* 2016;26(3):338-47.

II. **Bejder J**, Aachmann-Andersen NJ, Bonne TC, Olsen NV, Nordsborg NB. Detection of erythropoietin misuse by the Athlete Biological Passport combined with reticulocyte percentage. *Drug Test Anal* 2016;8(10):1049-1055.

III. **Bejder J**, Andersen AB, Goetze JP, Aachmann-Andersen NJ, Nordsborg NB. Plasma volume reduction and hematological fluctuations in high-level athletes after an increased training load. *Scand J Med Sci Sports* 2017;27(12):1605-1615.

IV. **Bejder J**, Andersen AB, Solheim SA, Gybel-Brask M, Secher NH, Johansson PI, Nordsborg NB. Time trial performance is sensitive to low-volume autologous blood transfusion. *Med Sci Sport Exerc* 2018 Accepted [Epub ahead of print]

V. **Bejder J**, Gürdinez G, Hall F, Gybel-Brask M, Andersen AB, Dragsted LO, Secher NH, Johansson PI, Nordsborg NB. Detection of autologous blood transfusion by metabolomics – a double-blind placebo-controlled cross over study. 2019 Draft.

List of papers not included

VI. Martin L, Ashenden M, **Bejder J**, Hoffmann M, Nordsborg N, Karstoft K, Mørkeberg J, Sharpe K, Lasne F, Marchand A. New insights for identification of doping with recombinant human erythropoietin micro-doses after high hydration. *Drug Test Anal* 2016;8(11-12):1119-1130.

VII. **Bejder J**, Andersen AB, Buchardt R, Larsson TH, Olsen NV, Nordsborg NB. Endurance, aerobic high-intensity and repeated sprint cycling performance is unaffected by normobaric "Live High-Train Low" – a double-blind placebo-controlled cross over study. *Eur J Appl Physiol* 2017;117(5):979-988.

VIII. **Bejder J**, Nordsborg NB. Specificity of "Live High-Train Low" altitude training on endurance performance. *Exerc Sci Sport Rev* 2018;46(2):129-136.

Abstract

The present thesis aims at developing knowledge and detection strategies related to blood manipulation aimed at illegal human performance enhancement, as it appear to be a continuous problem. A current need is to establish and improve the sensitivity of current test methodologies, identify confounding factors for test sensitivity and specificity as well as to being able to describe the importance of possible confounders. In addition, it is important to establish the physiological effects of novel doping regimens to determine the lower limit of relevance to anti-doping.

Specifically, the present thesis aimed to establish the natural fluctuations in plasma volume during a high water ingestion as well as during and after a period of increased endurance exercise load. It was hypothesized that the natural fluctuations of plasma volume are important for the sensitivity and specificity of the Athlete Biological Passport. It was further hypothesized that plasma albumin, soluble transferrin receptor and pro-atrial natriuretic peptide are sensitive markers for the plasma volume fluctuations and that adding the plasma volume independent reticulocyte percentage increase the sensitivity of the passport. Finally, the hypothesis that an autologous blood transfusion of half a blood bag (~135 ml red blood cells) improve cycling time-trial performance and that an autologous blood transfusion of two blood bags (~500 ml red blood cells) is detectable by an untargeted metabolomics analysis of urine was investigated.

Paper I and II of the present thesis confirm the hypothesis that natural fluctuations of plasma volume is of high importance for interpretation of the blood profile in the Athlete Biological Passport due the influence on the concentration-based variables hemoglobin concentration and OFF-hr score, which cause a reduction in sensitivity and specificity. Contrary to the hypothesis, paper I and II also demonstrate that plasma albumin, soluble transferrin receptor or pro-atrial natriuretic peptide all

are weak ($R^2 < 0.25$) predictors of the rapid natural shifts in plasma volume of 4-10 %. In paper III it is demonstrated that addition of the plasma volume independent reticulocyte percentage to the Athlete Biological Passport significantly improved the sensitivity.

Paper IV of the present thesis demonstrate that in a randomized double-blind placebo-controlled cross over design, an autologous blood transfusion of only ~135 ml red blood cells is sufficient to improve a 650-kcal time trial by ~5 %. Unfortunately, autologous blood transfusion is difficult detect. Paper V demonstrate that an untargeted metabolomics analysis of urine samples does not reveal novel and sensitive biomarkers for detection of autologous blood transfusion, but confirm that the presence of plasticizers in urine as a potential marker.

In conclusion, the present thesis demonstrate that natural shifts in plasma volume is a major confounder to the Athlete Biological Passport. However, addition of the plasma volume independent reticulocyte percentage yield promising results. Furthermore, the hitherto lowest volume autologous blood transfusion was sufficient to improve time trial performance, highlighting that even small transfusion volumes are relevant in the context of anti-doping efforts. Unfortunately, untargeted metabolomics of urine provides no novel sensitive biomarkers for detection of autologous blood transfusion. Future research should aim to identify and validate a method or biomarker that is independent of or able to account for changes in plasma volume and to develop a highly sensitive detection method for autologous blood transfusion. Furthermore, an untargeted metabolomics of human plasma or red blood cells could be of value.

Resumé

Denne afhandling har til formål at skabe viden og nye sporings metoder relateret til bloddoping der har til formål at ulovligt fremme præstationsevnen. Der er behov for at etablere og forbedre følsomheden af nuværende eller potentielt nye testmetoder, identificere faktorer der kan påvirke følsomheden eller specificiteten af testmetoderne og kunne beskrive vigtigheden af disse faktorer. Desuden er det vigtigt at kende den fysiologiske effekt af nye dopingmetoder for at finde den nedre grænse for hvornår det er relevant i en anti-doping sammenhæng.

Et af målene for afhandlingen er at skabe viden om den naturlige variation i plasma volumen ved indtag af en stor mængde vand såvel som under og efter en periode med øget træningsbelastning. Den fremsatte hypotese var at den naturlige variation i plasma volumen er vigtig for følsomheden og specificiteten af Atletens Biologiske Pas. Yderligere var hypotesen at plasma albumin, opløselige transferrin receptorer og pro-atrialt natriuretisk peptid er følsomme markører for ændringer i plasma volumen og at tilføjelsen af den plasma volumen uafhængige markør retikulocyt procenten øger følsomheden af det biologiske pas. Endeligt undersøgtes hypotesen at en autolog blodtransfusion af en halv blodpose (~135 ml røde blodceller) forbedrer præstationsevnen i en tidskørsel på cykel og at en autolog blodtransfusion af to poser blod (~500 ml røde blodceller) kan spores med en ikke-målrettet metabolomics analyse i urin.

Afhandlingens studie I og II bekræfter hypotesen at naturlige variationer i plasma volumen har stor betydning for fortolkningen af blodprofilen i Atletens Biologiske Pas, forårsaget af plasma volumens effekt på de koncentrationsbaserede variable, hæmoglobin koncentrationen og OFF-hr scoren, hvilket medfører en nedsat følsomhed og specificitet. Modsat forkaster studie I og II hypotesen at plasma albumin, opløselige tranferrin receptorer og pro-atrialt natriuretisk peptid er følsomme

markører for ændring i plasma volumen, da de alle var svage prædiktorer ($R^2 < 0.25$) for ændringer i plasma volumen på 4-10 %. Studie III bekræfter at tilføjelsen af den plasma volumen uafhængige markør retikulocyt procenten til Atletens Biologiske Pas kan medføre en signifikant forbedret følsomhed.

I et randomiseret dobbelt-blindet placebo-kontrolleret overkrydsnings design viser studie IV at en autolog blodtransfusion af kun ~135 ml røde blodceller er tilstrækkeligt til at forbedre en 650-kcal tidskørsel med ~5 %. Desværre er autolog blodtransfusion svært at spore. Studie V viser at en ikke-målrettet metabolomics analyse af urinprøver ikke identificerer nye og følsomme markører for sporing af autolog blodtransfusion, men bekræfter at tilstedeværelsen af plastificeringsmidler i urin er en potential markør.

Denne afhandling demonstrerer at naturlige variationer i plasma volume kan påvirke følsomheden og specificiteten af Atletens Biologiske Pas, men at tilføjelsen af den plasma volumen uafhængige markører retikulocyt procenten imidlertid giver lovende resultater. Derudover vises det at den hidtil lavest anvendte autolog blodtransfusions volumen er tilstrækkelig til at forbedre præstationsevnen i en tidskørsel, og derfor er transfusioner af små volumener relevante at kunne spore i anti-doping sammenhæng. Endeligt identificerer en ikke-målrettede metabolomics analyse i urin ikke nye følsomme markører til sporing af autolog blodtransfusion. Fremtidig forsknings bør fokusere på identificering og validering af metoder eller biomarkører der er uafhængige af plasma volumen eller i stand til at redegøre for ændringer i plasma volumen. Derudover er der behov for udvikling af en stærkt følsom metode til sporing af autolog blodtransfusion, hvorunder analyse af plasma og røde blodceller med en ikke-målrettet metabolomics kan være værdifuldt.

Introduction

For more than 5,000 years, humans have used substances or methods with the purpose of enhancing exercise performance[1]. However, in 1967 the International Olympic Committee introduced the first List of Prohibited Substances and implemented anti-doping testing at the Summer Olympic Games in Mexico in 1968[1]. Today, the World Anti-Doping Agency (WADA) is coordinating the anti-doping movement and developing anti-doping policies with the aim of providing equal opportunities, fair competition and protecting the health of athletes. However, the anti-doping system is not perfect, and when the development of tests for detection of current prohibited substances or methods is successful, athletes wishing to cheat likely change the applied doping methods[2]. One of the most widespread doping practices in endurance sports is blood doping, defined by WADA as "*the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more oxygen to muscles and therefore increase stamina and performance.*" Blood doping has a long history within sports[2], and is likely popular as it represents a relatively easy and low-risk method to improve aerobic exercise performance substantially and can be difficult to detect[3]. Furthermore, the estimated prevalence of doping is high[4-6], up to ~45 % in the World Championships of athletics in 2011[6]. Thus, continued research within detection of blood doping is necessary to reduce the prevalence and increase the likelihood of catching cheating athletes. The present thesis will add to the continuation of anti-doping research within blood doping.