This is the author accepted manuscript of:
Lima, Giscard; Shurlock, Jonathan; Wang, Guan; Karanikolou, Antonia; Sutehall, Shaun; Pitsiladis, Yannis P.; D'Alessandro, Angelo. Metabolomic Profiling of Recombinant Erythropoietin (rHuEpo) in Trained Caucasian Athletes. Clinical Journal of Sport Medicine,October 10, 2022,

The final published version is available at:
DOI: 10.1097/JSM.0000000000001074
https://journals.lww.com/cjsportsmed/Abstract/9900/Metabolomic_Profiling_of_Recombinant.47.aspx

Metabolomic profiling of recombinant erythropoietin (rHuEpo) in trained Caucasian athletes

Giscard Lima, PhD [1,2]; Jonathan Shurlock, BSc, BMBS[1]**; Guan Wang, PhD [3]; Antonia Karanikolou, MSc[1], Shaun Sutehall, BSc[1], Yannis P Pitsiladis, MMedSci, PhD[1,4,5,6*]; Angelo D'Alessandro, PhD[7*]**

[1]Centre for Stress and Age-related Disease, University of Brighton, Brighton, UK
[2]University of Rome "Foro Italico," Rome, Italy
[3]Sport and Exercise Science and Sports Medicine Research and Enterprise Group, University of Brighton, Brighton, UK.
[4]Centre for Exercise Sciences and Sports Medicine, FIMS Collaborating Centre of Sports Medicine, Rome, Italy
[5]European Federation of Sports Medicine Associations (EFSMA), Lausanne, Switzerland
[6]International Federation of Sports Medicine (FIMS), Lausanne, Switzerland
[7]Department of Biochemistry and Molecular Genetics, University of Colorado Denver – Anschutz Medical Campus, Aurora, CO, USA

***Corresponding authors:**
Yannis Pitsiladis, PhD
Centre for Stress and Age-related Disease
University of Brighton
Eastbourne
United Kingdom
E-mail: Y.Pitsiladis@brighton.ac.uk

Angelo D'Alessandro, PhD
Department of Biochemistry and Molecular Genetics
University of Colorado Anschutz Medical Campus
12801 East 17th Ave., Aurora, CO 80045
Phone # 303-724-0096

E-mail: angelo.dalessandro@ucdenver.edu

Acknowledgments The authors would like to thank all volunteers for their participation and cooperation; Dr. Pierre-Edouard Sottas (WADA) and Dr. Jingli Wang (University of Glasgow, Scotland) for their invaluable contribution to the metabolomic analysis and discussion. This research was supported by a research grant from the World Anti-Doping Agency (12C05YP). ADA was supported by funds from the RM1GM131968 from the National Institute of General and Medical Sciences, and R01HL146442, R01HL149714, R01HL148151, R21HL150032 from the National Heart, Lung, and Blood Institute.

Disclosure of Conflict of interest The authors declare that ADA is a founder of Omix Technologies Inc. and Altis Biosciences LLC. ADA is a consultant for Rubius Therapeutics and an advisory board member for Hemanext Inc and FORMA Therapeutics Inc. All the other authors disclose no conflicts of interest relevant to this study.

Abstract

Objective: Recombinant human erythropoietin (rHuEpo) is prohibited by the World Anti-Doping Agency (WADA) but remains the drug of choice for many cheating athletes wishing to evade detection using current methods. The aim of this study was to identify a robust metabolomics signature of rHuEpo using an untargeted approach in blood (plasma and serum) and urine.

Design: Longitudinal study

Setting: University of Glasgow

Participants: Eighteen male participants regularly engaged in predominantly endurance-based activities such as running, cycling, swimming, triathlon and team sports were recruited.

Interventions: Each participant received 50 $IU \cdot kg^{-1}$ body mass of rHuEpo subcutaneously every 2 days for 4 weeks. Samples were collected at baseline, during rHuEpo administration (over four weeks) and after rHuEpo administration (week 7-10). The samples were analyzed using hydrophilic interaction liquid chromatography-mass spectrometry.

Main Outcome Measures Significant metabolic signatures of rHuEpo administration were identified in all biofluids tested in this study.

Results Regarding metabolomics data, 488 plasma metabolites, 694 serum metabolites and 1628 urinary metabolites were identified. Reproducible signatures of rHuEpo administration across all biofluids included alterations of pyrimidine metabolism (orotate and Dihydroorotate) and acyl-carnitines (Palmitoyl-carnitine and Elaidic carnitine), metabolic pathways that are associated with erythropoiesis or erythrocyte membrane function, respectively.

Conclusion Preliminary metabolic signatures of rHuEpo administration were identified. Future studies will be required to validate these encouraging results in independent cohorts and with orthogonal techniques, such as integration of our data with signatures derived from other "omics" analyses of rHuEpo administration (e.g., transcriptomics).

Keywords: rHuEpo; metabolomics; mass spectrometry; anti-doping; metabolic signatures; serum; plasma; urine

Introduction

Recombinant human erythropoietin (rHuEpo) is a synthetic analogue of the endogenous hormone erythropoietin (EPO) and is been used by endurance athletes to enhance sporting performance by stimulating red blood cell production leading to increased oxygen delivery to the exercising muscles [1,2]. Indirect methods of rHuEpo detection using haematological markers of altered erythropoiesis are being used with moderate success due to limited specificity and sensitivity [3,4]. As such, novel strategies to detect rHuEPO doping in an unbiased and systematic fashion have become the focus of recent research with the development of techniques allowing for both targeted and untargeted analyses for various endogenous and exogenous substances. As such, "omics" technologies such as metabolomics have become the focus of anti-doping research and leading anti-doping agencies such as the World Anti-Doping Agency (WADA) with the goal to maximize the potential of these technologies, improving the existing longitudinal monitoring of athletes such as the Athlete Biological Passport (ABP) [5,6].

The use of metabolomics-based analytical techniques are rapidly growing within the fields of cancer cell metabolism and treatment [7], nutrition [8,9], as well as disease prognosis [10,11]. Metabolomics allows for thousands of metabolites to be investigated within a biological sample at any given time frame, creating a "snapshot" of the biological state of an organism [12], with the biological (phenotypic) state inferred by profiling different biofluids. For example, in recent years, our group leveraged metabolomics approaches to determine the impact of high-altitude hypoxia-stimulated erythropoiesis on systems metabolism, including signatures in blood [13] and muscles [14]. Since then, we have also captured some of these signatures in the context of exercise-induced [15,16] or pathological hypoxia, such as in sickle cell [17] or chronic kidney disease [18]. While this full potential for identify biological responses remains to be realized, anti-doping applications should be explored with particular focus on understanding the metabolic changes mediated by drugs such as rHuEpo.

As a first step towards bridging this gap, in the present study we sought out to identify small molecule metabolic markers of rHuEpo doping in humans. This project represents a continuation of a project sponsored by WADA in 2008 entitled "A gene-microarray based approach to the detection of rHuEpo doping in endurance athletes (08C19YP)", which aimed to provide the basis

for the development of new improved methods to detect rHuEpo doping based on gene expression profiles[19]. Given the initial successes of this project and recent advances in metabolomic profiling, the aim of this study was to investigate the metabolomic response to rHuEpo administration in the previous rHuEpo intervention study involving European endurance-trained healthy male volunteers.

Methods

Subjects and experimental design

Sample characteristics and experimental design have previously been described in detail in Durussel et. Al [20]. Briefly, 18 endurance-trained males (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7 kg, height: 179.5 ± 5.4 cm; VO_2max: runners 60.3 ± 5 ml•kg•min^{-1} and other activities 51.6 ± 3.5 ml•kg•min^{-1}) not involved in sporting competition during the study period were recruited in Glasgow, Scotland. All subjects received 50 IU.kg^{-1} body mass subcutaneous injections of rHuEpo (Neorecormon-Epo beta, Hoffmann-La Roche Ltd, UK) every 2 days over 4 weeks. Plasma, serum and urine were sampled twice at baseline, i.e. two weeks before the intervention (B1 and B2; days -14, -7, respectively), during (EPO 3-5; days 2, 14 and 28 from rHuEpo administration) and post (Post 6-8; weeks 7-10) rHuEpo administration with an overview of the study design provided in Figure 1.A.

Sample preparation

Plasma, serum and urinary samples were collected using the K_3EDTA Tube (Greiner Bio-One Ltd, Stonehouse, UK), the BD Vacutainer® SSTTM II Advance Serum Tube (BD, Plymouth, UK) and the SterilinTM Polypropylene 30 mL Universal Container (Thermo Scientific, Wilmington, DE, USA), respectively. Plasma and serum samples were immediately isolated by centrifugation at 1500 x g for 15 min at 4˚C, before storage at -80°C for further processing. Three 1 mL urine aliquots per sample were stored at -80°C prior to downstream analysis. A volume of 100 µL isolated plasma, serum or urine was each mixed vigorously with 100 µL chloroform and 300 µL methanol on a cooled shaker at 4˚C for 1 hr, followed by centrifugation at 13,000 x g at 4°C for 3 min. The supernatant was transferred to fresh screw capped tubes stored at -80°C until transportation packed with dry ice to the Polyomics Facility, University of

Glasgow, for the hydrophilic interaction liquid chromatography (HILIC)-mass spectrometry (MS).

Data processing and analysis

Samples were analyzed by HILIC-MS (UltiMate 3000 RSLC, Thermo Fisher, San Jose, California, USA) using a 150 x 4.6 mm ZIC-pHILIC column (Merck SeQuant, Umea, Sweden) running at 300 µL.min-1 and Exactive Orbitrap (Thermo Fisher, San Jose, California, USA) detection. Buffers consisted of A: 20 mM ammonium carbonate in H_2O and B: Merck SeQuant: acetonitrile. The gradient ran from 20% A: 80% B to 80% A: 20% B in 15 min, followed by a wash at 95% A: 5% B for 3 min and equilibration at 20% A: 80% B for 5 min.

Data processing and statistical analysis

Raw mass spectrometry data was processed using the standard pipeline, consisting of XCMS (for peak picking) [21], MzMatch (for filtering and grouping) [22] and IDEOM (for further filtering, post-processing and identification) [23]. Core metabolite identifications were validated against a panel of unambiguous standards using accurate mass and retention time. Additional putative identifications were assigned by accurate mass along with a retention time prediction algorithm. Relative intensity ratios were calculated and independent t-tests were carried out in IDEOM [23]. Additional statistical analyses, including time series ANOVA, hierarchical clustering analyses and partial least square-discriminant analyses (PLS-DA), correlation to hematological parameters (Spearman) and calculation of univariate and multivariate Receiver Operating Characteristic (ROC) curve analyses were performed through MetaboAnalyst 5.0 [24]. To ease visualization in the main body of the manuscript, data is presented as either mean ± standard deviation or medians ± ranges, while individual replicates are shown in Supplementary Materials where indicated.

Ethical Considerations

All subjects underwent a medical assessment performed by a physician. The 18 participants that were included in this study had medical approval to take part of the study and provided written informed consent to participate. This study was approved by University of Glasgow Ethics Committee (FBLS 0617) and conformed to the Declaration of Helsinki. Subjects were requested

to maintain their normal training but abstain from official sporting competition for the duration of the research study. The participants self-declared no prior use of illicit enhancer performance drugs.

Results

Treatment with rHuEpo significantly impacts hematological parameters

To confirm the efficacy of our treatment with rHuEpo, hematological parameters were monitored in the subjects enrolled in this study (Figure 1.B), with these results indicating that rHuEpo administration resulted in early significant increases in reticulocyte counts (RET), followed by increases in hematocrit (HCT) and hemoglobin (Hb). These parameters allowed to extrapolate alterations of OFFhr scores, a doping detection algorithm based on the haemoglobin (Hb) level concentration and the percentage of reticulocytes (OFF-hr model; Hb(g/l)-60•√%ret), as approved by the World Anti-Doping Agency [25].

Overview of metabolomics results

Following metabolomics data acquisition and processing (Figure 2.A), 488 plasma metabolites, 694 serum metabolites and 1628 urinary metabolites were identified (Supplementary Table 1). Metabolomics data were thus subjected to statistical comparisons (time series ANOVA), followed by false discovery rate (FDR) correction for the total number of metabolites identified in this study in each matrix (i.e., plasma; 488, serum; 694 and urine; 1628).

Metabolic markers of rHuEpo administration in plasma

Unsupervised statistical elaborations – including time series ANOVA and t-distributed stochastic neighbor embedding (t-SNE) classification (Supplementary Figure 1.A-B) were performed to identify plasma metabolic markers of rHuEpo administration. Additional analyses included partial least square-discriminant analysis (PLS-DA – Figure 2.B) and hierarchical clustering of the top 50 metabolic signatures by time series ANOVA (Figure 2.C and Supplementary Figure 1.A). Among the top markers, statistical analyses highlighted metabolites in lipid metabolism (especially palmitoyl-carnitine, linoleaidyl-carnitine, elaidic-carnitine and cholesterol sulfate), pyrimidine metabolism (orotate and dihydroorotate) and basic amino acids (citrulline and N-

acetyl-lysine), which ranked amongst the top plasma metabolic markers of rHuEpo administration (Figure 2.D-F and Supplementary Figure 2).

Metabolic markers of rHuEpo administration in sera

The hierarchical clustering of all the significant metabolites by time series ANOVA in sera from all the 18 male well trained athletes administered rHuEpo can be seen in Supplementary Figure 3. To ease visualization of these data and highlighted the impact of time from rHuEpo administration, subjects were clustered via PLS-DA (Figure 3.A – PC1 explaining ~10% of the total variance across samples) and hierarchical clustering of significant metabolites in sera (Figure 3.B). Consistent with analyses in plasma, significant metabolites identified as serum markers of rHuEpo administration again included carnitine metabolites (palmitoyl- and elaidic-carnitine – Figure 3.C) and pyrimidine metabolites (orotate and dihydroorotate). In addition, this analysis highlighted a significant impact of rHuEpo administration in serum levels of metabolites involved in arginine/nitric oxide metabolism, including arginine, citrulline, ornithine, which are substrates and product of nitric oxide synthesis or result from the activity of competing enzymes with nitric oxide synthase (NOS), arginase (Figure 4). Early increases in citrulline upon rHuEPO stimulation are followed by late increases in ornithine, suggestive of a sequential activation of NOS followed by activation of arginase. Interestingly, this was accompanied by increases in (asymmetric) dimethyl-arginine (ADMA), an inhibitor of NOS which is synthesized through S-Adenosyl-methionine (SAM)-dependent methylation of arginine. Of note, dimethyl-arginine, SAM and choline (a methyl group donor to recharge SAM), followed overlapping trends with ornithine, suggestive that normalization of citrulline levels upon early accumulation secondary to rHuEpo treatment may be at least in part explained by a compensatory inhibition of NOS by ADMA (Figure 4). Serum levels of several metabolic markers of hypoxia were altered in response to rHuEpo treatment (Figure 5). For example, rHuEpo administration was immediately accompanied by significant decreases in the serum levels of sphingosine 1-phosphate (S1P – Figure 5), a metabolite that accumulates in the bloodstream upon exposure to high-altitude[26] or pathological hypoxia (e.g., sickle cell[17] or chronic kidney disease[18]).

On the other hand, plasma adenosine, another circulating marker of systems-wide responses to hypoxia[27,28], followed trends that overlapped with increases in RET (Figure 5). Metabolites

involved in liver and kidney function (e.g., transamination markers alpha-ketoglutarate and phosphocreatine, respectively) were characterized by late increases (EPO 6) followed by sharp decreases towards normalization (EPO 7-8) in the sera of subjects who were administered rHuEpo (Figure 5). It is worthwhile to note the role of circulating carboxylic acids – including alpha-ketoglutarate – in the stabilization and degradation of hypoxia inducible factor (HIF) by means of hydroxylation (or inhibition of) through prolyl hydroxylase activity[29,30]. Notably, iatrogenically-stimulated erythropoiesis is consistent with altered kidney function and increased RBC metabolism, as suggested by circulating bilirubin levels (Figure 5) – with trends overlapping those noted for Hb and HCT (Figure 1).

Metabolic markers of rHuEpo administration in urine

Metabolomics data of urine analyses upon rHuEpo administration were analyzed via PLS-DA (Figure 6.A) and hierarchical clustering of significant metabolites by time series ANOVA (group averages in the heat map in Figure 6.B for the top 35 variables). Analyses confirmed again an impact of rHuEpo administration on biofluid (in this case urine) levels of amino acids (especially acetylated lysine and glutamate – Figure 6.C), pyrimidines (orotate – Figure 6.D) or other amino acids involved in one-carbon metabolism (Figure 6.D), substrates that fuel nucleobase synthesis to sustain erythropoiesis. In addition, several urine markers of rHuEpo administration included short chain peptides (e.g., Lys-Asp, Val-His, Glu-Pro, Arg-Val-Asn-His) and odd-chain/short chain fatty acids, suggestive of potential alterations of proteostasis (increased proteolysis) or altered gut microbiome metabolism.

Metabolic correlates to hematological parameters

To further delve into the potential translation relevance of our metabolomics findings, we performed correlation analyses of metabolomics data from all biofluids (plasma, sera, urine) to hematological parameters in the same subjects throughout the duration of this clinical study. Results are provided in the form of volcano plots in Figure 7, with highlighted correlates in the form of line plots in Figure 8. Expectedly, hematological parameters showed strong correlations among each other, with HCT and Hb showing strong positive correlations (Figure 7.A-C). OFFhr showed significant positive and negative correlations with Hb (but also HCT) and RET counts, respectively (Figure 7.B-D). Notably, several metabolites showed stronger correlations to some of these hematological parameters. Top correlates included plasma amino acids involved in

one carbon metabolism (methionine sulfoxide) or carboxylic acids (4-hydroxy-2-oxoglutarate) amongst top correlates to HCT (Figure 8.A), serum/plasma N-acetly-lysine, linoleidyl-carnitine and elaidic carnitine as top correlates to RET counts (Figure 8.B) and urinary indole quinone (metabolite of potential bacterial origin) and fatty acids (hexadecatetraenoic acid – FA16:4) as top correlates to Hb levels (Figure 8.C).

Finally, ROC curves were calculated to determine early (EPO 3-5) or late (post 6-8) candidate metabolic markers of rHuEpo administration as compared to baseline parameters (B1-2) in any of the biofluids tested in this study (Figure 8.D-E). Of note, RET counts performed as the best predictor of rHuEpo administration only immediately within the first four weeks of continuous EPO administration, when RET counts were highest (AUC: 0.956 – Supplementary Figure 4.A), though its capacity to discriminate between the two groups decreased significantly at the later time points when RET had declined below baseline values (Supplementary Figure 4.B). Significant decreases in urine methionine excretion was the second highest early marker of rHuEpo administration (AUC: 0.892) (Figure 8.D). Increases in urinary excretion of glucoronate and peroxidized lipids (HPETE) were instead the top late markers of rHuEpo administration, with AUC > 0.75 in both cases (Figure 8.E). Multivariate analyses of all tested metabolites across all matrices suggested a strong sensitivity and specificity of the combination of the top 10 metabolic markers in determining rHuEpo administration (Supplementary Figure 4.C).

Discussion

Blood (both plasma and serum) as well as urine are the most frequently studied samples in human metabolomic studies given the minimally or non-invasive collection procedures required as well as the many areas of metabolism involved in these biofluids[31,32]. While overall metabolomic patterns in plasma and serum are expected to be similar, some important differences may be expected given the different sample preparation methods and the cell-derived components as a result of the process of clotting in sera[31]. However, overlapping signatures between these biofluids are critical as they represent both an internal validation and lead the way for easier sampling. Previous studies have shown strong significant correlations between metabolite concentrations in matched plasma and sera (mean correlation coefficient $r = 0.81 \pm 0.10$, particularly for most acyl carnitines mean $r = 0.86 \pm 0.09$), with higher concentrations of metabolites observed in serum[33]. Despite the larger numbers of metabolites identified in urine,

overall signatures in this biofluid were more variable and less consistent across subjects than those observed in plasma/sera, suggesting that this biofluid may represent a sub-optimal matrix for the detection of markers of rHuEpo supplementation in humans.

In the present study, the concentration of dihydroorotate in both plasma, serum and urine was significantly increased 14- (EPO4) and 28- days (EPO5) following the first rHuEpo injection relative to baseline (Base1). Dihydroorotate is an intermediate in the pyrimidine metabolism[34] and a substrate for dihydroorotate dehydrogenase, which catalyses the conversion of dihydroorotate to orotate, which was also significantly correlated to rHuEpo administration, the single redox step in pyrimidine de novo synthesis[35]. This is the primary pathway for pyrimidine biosynthesis[34] and most purine and pyrimidine compounds are found in both immature erythroid cells and mature erythrocytes[36]; a reflection of the importance of pyrimidine metabolism in the development of erythroid cells. Metabolites of pyrimidine biosynthesis, including dihydroorotate, are more abundant in reticulocytes compared to mature erythrocytes in both humans and rodents[37] and in this study the levels of these metabolites are consistently correlated with RET counts.

Another signature of rHuEpo administration that was consistently observed across all the biofluids investigated relates to the alteration of carnitine metabolism which is of clinical importance as Palmitoylcarnitine is thought to be involved in the pathogenesis of myocardial ischemia[38] through alterations in the fluidity of erythrocyte membranes[39]. In a recent study on testosterone replacement therapy in hypogonadic subjects or testosterone supplementation in longitudinal samples from subjects undergoing gender reassignment therapy[40], acyl-carnitines represented the most significant pathway. Elaidic-carnitine levels in serum were also significantly elevated at 28 days (EPO5) vs. baseline (Figure 5 & 11 and Supplementary Table 1). Elaidic-carnitine is an acyl carnitine; a fatty acyl ester of L-carnitine. There is some indirect evidence from studies investigating hemodialysis patients linking the concentration of elaidic-carnitine to erythropoiesis and erythrocyte membrane function. For example, reduced free carnitine (i.e. L-carnitine) and elevated medium- and long-chain acyl carnitine levels have been reported in both serum[41] and plasma[42] samples obtained from hemodialysis patients. Increasing plasma and intracellular carnitine and carnitine esters in this patient group, using high doses of

L-carnitine[42], seem to enhance erythrocyte survival[43]. However, not all studies support the use of carnitine therapy in hemodialysis patients[44].

Although numerous functions of Dihydroorotate/orotate, Palmitoyl-R-carnitine and Elaidic carnitine are described in the literature and the human serum metabolome database (www.serummetabolome.ca[45]), there is no direct link between these metabolites and EPO; a consequence of adopting the hypothesis-free approach that could lead to the discovery of potentially new biomarkers and underlying biology.

In another study involving two healthy male subjects and using a targeted urinary metabolomics approach, Appolonova et al.[46] investigated the effects of a single intravenous 2000 IU rHuEpo dose on ADMA, symmetrical dimethylarginine, arginine and citrulline levels. These authors reported an increase in the concentration of the targeted metabolites after a single injection without specifying a detection window. The present study replicated these preliminary findings in sera, suggestive of an early activation of NOS following rHuEpo stimulation and a secondary inhibition of NOS activity by increased synthesis of ADMA and late accumulation of ornithine. These observations are relevant in that they would help to disentangle the current findings, namely the impact of exogenous administration of rHuEpo, from the metabolic impact of erythropoiesis stimulated by high-altitude hypoxia, as previously described[13]. Similarly, previous studies investigating the metabolic response to high-altitude hypoxia reported concomitant increases in the levels of S1P and adenosine within hours from exposure to hypobaric hypoxia[13,26,27]. Of note, comparable trends for adenosine, a purine metabolite that positively correlated to increases in RET counts, were reported but opposite trends for S1P, consistent with the former being the by-product of endothelial cell responses to stimulation of erythropoiesis[27] and the latter being a by-product of hypoxic sensing by RBCs as a function of erythrocyte-specific sphingosine kinase activity[26]. Moreover, since both ADMA synthesis and purine synthesis are dependent on methyl-group donors, it is interesting to note that SAM and choline followed overlapping trends to rHuEpo stimulation. Even more interesting, significant decreases in urinary excretion of methionine, a SAM precursor and main methyl group donor in the bloodstream, performed almost as well as RET counts as an early marker of rHuEpo administration.

In summary, the present study identified several signatures of blood (plasma and serum) and urine metabolites associated with rHuEpo, including pyrimidine and carnitine metabolites. These metabolites had been previously associated to erythrocyte membrane function or erythropoiesis in the context of testosterone-induced erythropoiesis and may serve as candidate markers of rHuEpo for future prospective validation. Further studies are required to address the impact of potential confounding factors such age, sex, ethnicity, diet and exercise on these findings. Another limitation of the study was the dose regimen of rHuEpo used. It has been reported that one of the most common methods of evading doping detection is a constantly use of rHuEpo microdoses[47]. It has been shown that rHuEpo microdose use can lead to hematological changes that are not detected by the ABP[4]. However, the main finding of the present study is that metabolic markers performed better than RET counts as a late marker of rHuEpo administration. In addition, a combination of multiple metabolomic markers across multiple biological matrices (e.g., acyl-carnitines, pyrimidines, amino acids, carboxylic acids, sphingolipids, purines) may be more indicative than any single independent marker. Validation of such markers will provide a more detailed and thorough understanding of the perturbed system(s), thereby aiding ABP experts identify and differentiate numerous doping substances and methods when reviewing passports. Given this unique potential, the present findings should encourage further metabolomics studies and the integrated reviewing of all "omics" data generated by other WADA anti-doping studies.

References

1. Pascual JA, Belalcazar V, de Bolos C, Gutiérrez R, Llop E, Segura J. Recombinant Erythropoietin and Analogues. *Ther Drug Monit*. 2004;26(2):175-179. doi:10.1097/00007691-200404000-00016
2. Jelkmann W, Lundby C. Blood doping and its detection. *Blood*. 2011;118(9):2395-2404. doi:10.1182/blood-2011-02-303271
3. Bonne TC, Lundby C, Lundby AK, Sander M, Bejder J, Nordsborg NB. Altitude training causes haematological fluctuations with relevance for the Athlete Biological Passport. *Drug Test Anal*. 2015;7(8):655-662. doi:10.1002/dta.1757
4. Ashenden M, Gough CE, Garnham A, Gore CJ, Sharpe K. Current markers of the Athlete Blood Passport do not flag microdose EPO doping. *Eur J Appl Physiol*.

2011;111(9):2307-2314. doi:10.1007/s00421-011-1867-6

5. Reichel C. OMICS-strategies and methods in the fight against doping. *Forensic Sci Int*. 2011;213(1-3):20-34. doi:10.1016/j.forsciint.2011.07.031

6. Pitsiladis YP, Durussel J, Rabin O. An integrative "Omics" solution to the detection of recombinant human erythropoietin and blood doping. *Br J Sports Med*. 2014;48(10):856-861. doi:10.1136/bjsports-2014-093529

7. Lee SJ, Woo S, Ahn SH, et al. Functional interpretation of metabolomics data as a new method for predicting long-term side effects: treatment of atopic dermatitis in infants. *Sci Rep*. 2015;4(1):7408. doi:10.1038/srep07408

8. Manach C, Hubert J, Llorach R, Scalbert A. The complex links between dietary phytochemicals and human health deciphered by metabolomics. *Mol Nutr Food Res*. 2009;53(10):1303-1315. doi:10.1002/mnfr.200800516

9. Scalbert A, Brennan L, Fiehn O, et al. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics*. 2009;5(4):435-458. doi:10.1007/s11306-009-0168-0

10. Beger R. A Review of Applications of Metabolomics in Cancer. *Metabolites*. 2013;3(3):552-574. doi:10.3390/metabo3030552

11. Dumas M-E, Davidovic L. Metabolic Profiling and Phenotyping of Central Nervous System Diseases: Metabolites Bring Insights into Brain Dysfunctions. *J Neuroimmune Pharmacol*. 2015;10(3):402-424. doi:10.1007/s11481-014-9578-5

12. Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R. Metabolic fingerprinting as a diagnostic tool. *Pharmacogenomics*. 2007;8(9):1243-1266. doi:10.2217/14622416.8.9.1243

13. D'Alessandro A, Nemkov T, Sun K, et al. AltitudeOmics: Red Blood Cell Metabolic Adaptation to High Altitude Hypoxia. *J Proteome Res*. 2016;15(10):3883-3895. doi:10.1021/acs.jproteome.6b00733

14. Chicco AJ, Le CH, Gnaiger E, et al. Adaptive remodeling of skeletal muscle energy metabolism in high-altitude hypoxia: Lessons from AltitudeOmics. *J Biol Chem*. 2018;293(18):6659-6671. doi:10.1074/jbc.RA117.000470

15. Nemkov T, Skinner SC, Nader E, et al. Acute Cycling Exercise Induces Changes in Red Blood Cell Deformability and Membrane Lipid Remodeling. *Int J Mol Sci*.

2021;22(2):896. doi:10.3390/ijms22020896

16. San-Millán I, Stefanoni D, Martinez JL, Hansen KC, D'Alessandro A, Nemkov T. Metabolomics of Endurance Capacity in World Tour Professional Cyclists. *Front Physiol*. 2020;11. doi:10.3389/fphys.2020.00578

17. Sun K, D'Alessandro A, Ahmed MH, et al. Structural and Functional Insight of Sphingosine 1-Phosphate-Mediated Pathogenic Metabolic Reprogramming in Sickle Cell Disease. *Sci Rep*. 2017;7(1):15281. doi:10.1038/s41598-017-13667-8

18. Xie T, Chen C, Peng Z, et al. Erythrocyte Metabolic Reprogramming by Sphingosine 1-Phosphate in Chronic Kidney Disease and Therapies. *Circ Res*. 2020;127(3):360-375. doi:10.1161/CIRCRESAHA.119.316298

19. Durussel J, McClure JD, McBride MW, et al. Validation of Blood Gene Expression Profiles Post Recombinant Human Erythropoietin Administration. *Med Sci Sport Exerc*. 2014;46:597. doi:10.1249/01.mss.0000495264.89562.4f

20. Durussel J. A novel transcriptomic based approach to the detection of recombinant human erythropoietin doping. Published online 2014. doi:glathesis:2014-4962

21. Tautenhahn R, Patti GJ, Rinehart D, Siuzdak G. XCMS Online: A Web-Based Platform to Process Untargeted Metabolomic Data. *Anal Chem*. 2012;84(11):5035-5039. doi:10.1021/ac300698c

22. Scheltema RA, Jankevics A, Jansen RC, Swertz MA, Breitling R. PeakML/mzMatch: A File Format, Java Library, R Library, and Tool-Chain for Mass Spectrometry Data Analysis. *Anal Chem*. 2011;83(7):2786-2793. doi:10.1021/ac2000994

23. Creek DJ, Jankevics A, Burgess KE V., Breitling R, Barrett MP. IDEOM: an Excel interface for analysis of LC–MS-based metabolomics data. *Bioinformatics*. 2012;28(7):1048-1049. doi:10.1093/bioinformatics/bts069

24. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Curr Protoc Bioinforma*. 2019;68(1). doi:10.1002/cpbi.86

25. Delanghe JR, Maenhout TM, Speeckaert MM, De Buyzere ML. Detecting doping use: more than an analytical problem. *Acta Clin Belg*. 2014;69(1):25-29. doi:10.1179/0001551213Z.0000000009

26. Sun K, Zhang Y, D'Alessandro A, et al. Sphingosine-1-phosphate promotes erythrocyte glycolysis and oxygen release for adaptation to high-altitude hypoxia. *Nat Commun*.

2016;7(1):12086. doi:10.1038/ncomms12086

27. Liu H, Zhang Y, Wu H, et al. Beneficial Role of Erythrocyte Adenosine A2B Receptor–Mediated AMP-Activated Protein Kinase Activation in High-Altitude Hypoxia. *Circulation*. 2016;134(5):405-421. doi:10.1161/CIRCULATIONAHA.116.021311

28. D'Alessandro A, Xia Y. Erythrocyte adaptive metabolic reprogramming under physiological and pathological hypoxia. *Curr Opin Hematol*. 2020;27(3):155-162. doi:10.1097/MOH.0000000000000574

29. Hirota K, Semenza GL. Regulation of hypoxia-inducible factor 1 by prolyl and asparaginyl hydroxylases. *Biochem Biophys Res Commun*. 2005;338(1):610-616. doi:10.1016/j.bbrc.2005.08.193

30. Lorenzo FR, Huff C, Myllymäki M, et al. A genetic mechanism for Tibetan high-altitude adaptation. *Nat Genet*. 2014;46(9):951-956. doi:10.1038/ng.3067

31. Dunn WB, Broadhurst D, Begley P, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc*. 2011;6(7):1060-1083. doi:10.1038/nprot.2011.335

32. Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. Metabolic footprinting and systems biology: the medium is the message. *Nat Rev Microbiol*. 2005;3(7):557-565. doi:10.1038/nrmicro1177

33. Yu Z, Kastenmüller G, He Y, et al. Differences between Human Plasma and Serum Metabolite Profiles. Oresic M, ed. *PLoS One*. 2011;6(7):e21230. doi:10.1371/journal.pone.0021230

34. Garavito MF, Narváez-Ortiz HY, Zimmermann BH. Pyrimidine Metabolism: Dynamic and Versatile Pathways in Pathogens and Cellular Development. *J Genet Genomics*. 2015;42(5):195-205. doi:10.1016/j.jgg.2015.04.004

35. Almeida SS, Barros CC, Moraes MR, et al. Plasma Kallikrein and Angiotensin I-converting enzyme N- and C-terminal domain activities are modulated by the insertion/deletion polymorphism. *Neuropeptides*. 2010;44(2):139-143. doi:10.1016/j.npep.2009.12.003

36. Kim HD, Tsai YS, Lee SJ, Im JH, Koury MJ, Sawyer ST. Metabolic development in erythropoietin-dependent maturation of erythroid cells. *Prog Clin Biol Res*. 1989;319:491-502; discussion 503-4. http://www.ncbi.nlm.nih.gov/pubmed/2622926

37. Srivastava A, Creek DJ, Evans KJ, et al. Host reticulocytes provide metabolic reservoirs that can be exploited by malaria parasites. *PLoS Pathog*. 2015;11(6):e1004882. doi:10.1371/journal.ppat.1004882

38. Goñi FM, Requero MA, Alonso A. Palmitoylcarnitine, a surface-active metabolite. *FEBS Lett*. 1996;390(1):1-5. doi:10.1016/0014-5793(96)00603-5

39. Kobayashi A, Watanabe H, Fujisawa S, Yamamoto T, Yamazaki N. Effects of l-carnitine and palmitoylcarnitine on membrane fluidity of human erythrocytes. *Biochim Biophys Acta - Biomembr*. 1989;986(1):83-88. doi:10.1016/0005-2736(89)90275-7

40. Alexander K, Hazegh K, Fang F, et al. Testosterone replacement therapy in blood donors modulates erythrocyte metabolism and susceptibility to hemolysis in cold storage. *Transfusion*. 2021;61(1):108-123. doi:10.1111/trf.16141

41. Bommer J. Saving erythropoietin by administering l-carnitine? *Nephrol Dial Transplant*. 1999;14(12):2819-2821. doi:10.1093/ndt/14.12.2819

42. Wanner C, Wäckerle B, Boeckle H, Schollmeyer P, Hörl WH. Plasma and red blood cell carnitine and carnitine esters during L-carnitine therapy in hemodialysis patients. *Am J Clin Nutr*. 1990;51(3):407-410. doi:10.1093/ajcn/51.3.407

43. Nikolaos S, George A, Telemachos T, Maria S, Yannis M, Konstantinos M. EFFECT OF L-CARNITINE SUPPLEMENTATION ON RED BLOOD CELLS DEFORMABILITY IN HEMODIALYSIS PATIENTS. *Ren Fail*. 2000;22(1):73-80. doi:10.1081/JDI-100100853

44. Marcovina SM, Sirtori C, Peracino A, et al. Translating the basic knowledge of mitochondrial functions to metabolic therapy: role of L-carnitine. *Transl Res*. 2013;161(2):73-84. doi:10.1016/j.trsl.2012.10.006

45. Psychogios N, Hau DD, Peng J, et al. The Human Serum Metabolome. Flower D, ed. *PLoS One*. 2011;6(2):e16957. doi:10.1371/journal.pone.0016957

46. Appolonova SA, Dikunets MA, Rodchenkov GM. Possible Indirect Detection of rHuEPO Administration in Human Urine by High-Performance Liquid Chromatography Tandem Mass Spectrometry. *Eur J Mass Spectrom*. 2008;14(3):201-209. doi:10.1255/ejms.922

47. Salamin O, Kuuranne T, Saugy M, Leuenberger N. Erythropoietin as a performance-enhancing drug: Its mechanistic basis, detection, and potential adverse effects. *Mol Cell Endocrinol*. 2018;464:75-87. doi:10.1016/j.mce.2017.01.033

Figure Legends

Figure 1 – Study design (A) and hematological parameters (B) in 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). Vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 2 – Metabolomics study design (A), partial least square-discriminant analysis (PLS-DA) (B) and hierarchical clustering of the top 50 metabolic signatures by time series ANOVA (C) in plasma from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). In D-F, highlighted metabolites in lipid metabolism, pyrimidine metabolism and basic amino acids ranked amongst the top plasma metabolic markers of rHuEpo administration. Y axes in these graphs indicate relative quantitative levels of each metabolite (arbitrary units), while x axes indicate the different time points. In panels D-F, vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 3 - Partial least square-discriminant analysis (PLS-DA) (A) and hierarchical clustering of the top 50 metabolic signatures by time series ANOVA (B) in sera from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). In C-D, highlighted metabolites in carnitine and pyrimidine metabolism, respectively. Metabolites in these pathways ranked amongst the top serum metabolic markers of rHuEpo administration. Y axes in these graphs indicate relative quantitative levels of each metabolite (arbitrary units), while x axes indicate the different time points. In panels C-D, vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 4 – Line plots of metabolites at the interface of arginine/nitric oxide metabolism in sera from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). Y axes in these graphs indicate relative quantitative levels of each metabolite (arbitrary units), while x axes indicate the different time points. Vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 5 – Line plots of metabolites with a role in responses to hypoxia (sphingosine 1-phosphate – S1P and adenosine), degradation of hypoxia inducible factor (alpha-ketoglutarate and its transamination product, glutamate), erythropoiesis and red blood cell metabolism (bilirubin), kidney function (phosphocreatine) in sera from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). Y axes in these graphs indicate relative quantitative levels of each metabolite (arbitrary units), while x axes indicate the different time points. Vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 6 - Partial least square-discriminant analysis (PLS-DA) (A) and hierarchical clustering of the top 35 metabolic signatures by time series ANOVA (B) in urine from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). In C-D, highlighted metabolites in amino acid homeostasis/proteolysis and pyrimidine metabolism, respectively. Metabolites in these pathways ranked amongst the top urine metabolic markers of rHuEpo administration. Y axes in these graphs indicate relative quantitative levels of each metabolite (arbitrary units), while x axes indicate the different time points. In panels C-D, vertical red arrows indicate the time point (x axis) when rHuEpo was administered.

Figure 7 - Metabolites in sera, plasma and urines were correlated (Spearman) to hematological parameters, including hematocrit (HCT), reticulocyte count (RET), hemoglobin concentration (Hb) and OFFhr (A-B). Results were plotted in the form of volcano plots, in which the x axes indicate correlation coefficients, while the y axes indicate the -Log10 of FDR-corrected p-values for each correlation.

Figure 8 - Metabolites in sera, plasma and urines were correlated (Spearman) to hematological parameters, including hematocrit (HCT), reticulocyte count (RET), hemoglobin concentration (Hb) (A-C). Line plots for selected metabolites are shown, with each point color coded depending on the time of sampling, consistent with the legend in the bottom right of the figure. Quadratic and linear correlation coefficients are provided for each plot, along with the relative p-value. Receiver Operating Characteristic (ROC) curves for top metabolite predictors of early (B1-2 vs E3-4-5) or late (B1-2 vs P6-7-8) administration of rHuEpo (D-E, respectively).

Supplementary Figure Legends

Supplementary Figure 1 – Overview of the experimental design for plasma metabolomics analyses and related heat map (A) from the time series ANOVA elaboration. This heat map shows each single biological replicate, while the corresponding heat map in Figure 2 shows only group averages for each time point for ease of visualization. In B, t-distributed stochastic neighbor embedding (t-SNE) classification of the plasma samples based on metabolomics data.

Supplementary Figure 2 – Line plots of top significant plasma metabolic markers of rHuEpo administration in 18 male volunteers who were administered recombinant human erythropoietin (rHuEpo). Y axes in these graphs indicate normalized relative quantitative levels (Autoscale – i.e., data are mean-centered and divided by the standard deviation of each variable) of each metabolite (arbitrary units), while x axes indicate the different time points. These plots are comparable to those reported in Figures 2-4, in which only raw values for medians + ranges are shown instead of the normalized values for single biological replicates.

Supplementary Figure 3 – Hierarchical clustering of the significant metabolites by time series ANOVA in sera from 18 male well trained athletes administered recombinant human erythropoietin (rHuEpo). This heat map shows each single biological replicate, while the corresponding heat map in Figure 3 shows only group averages for each time point for ease of visualization.

Supplementary Figure 4 – Receiver Operating Characteristic (ROC) curves of RET count as the top predictor overall (including metabolic signatures) or rHuEpo administration (A) but a poor late marker of rHuEpo administration (B). In C, a multivariate analysis showed a strong capacity (AUC: 1 and 0.997) to determine rHuEpo administration based off the top 5-10 metabolic markers in all matrices tested in this study.