AN EXPLORATION OF THE POTENTIAL OF METABOLOMICS FOR THE DETECTION OF RECOMBINANT HUMAN ERYTHROPOIETIN (r-HuEPO) ABUSE IN SPORT.

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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As I reminisce over the trials and experiences of the last 5 years, I would like to take this opportunity to thank various colleagues, mentors and of course the research participants without which this work would not be possible.

This path has been a professional and personal awakening, providing a glimpse into science and the nature of discovery. Above all, it has provided me with the opportunity to step forward into a brief moment of enlightenment, which will hopefully guide me for future years to come. As was most eloquently described by T. Lucretius Carus, *On the Nature of the Universe*, on page 37 of Book 2 is as follows:

> "…Our lives in very truth
> Are but an endless labour in the dark.
> For we, like children frightened of the dark,
> Are sometimes frightened in the light- of things
> No more to be feared than fears that in the dark
> Distress a child, thinking they may come true.
> Therefore this terror and darkness of the mind
> Not by the sun's rays, nor the bright shafts of day,
> Must be dispersed, as is most necessary,
> But by the face of nature and her laws."

The labour towards knowledge and scientific process, or as described above as "light", demands hardship and belief that one day we will indeed understand a little bit more than the previous day. I hope a piece of what has been presented in this Ph.D. thesis creates a link to our move towards this enlightenment. As such I have to express my gratitude towards my supervisor Dr. Dave Watson, who provided me with the opportunity to self-discover and as a child "frightened of the dark" guided me forward. In doing so, I have been given the necessary tools to not be afraid when asking the question 'why' and admitting when simply 'I don't know'. For one thing I know to be true, and that is the known unknown.

There are many collaborators and friends from the Strathclyde Institute of Pharmacy and Biomedical Sciences, The Beatson Cancer Research Institute, Glasgow University, The Metabolomics Society (EMN), my family in Oslo and Athens, that inspired me, guided me or simply just listened to me. For that I thank you and dedicate one of my personal published poems to you. It very simply speaks true to my beliefs and likewise my fears:

Controversies

Just behind the willow tree
a little bit beyond the rabbit hole
I see a child
staring up endlessly into the sky.
"Why" I ask, "are You looking up
when there is nothing there
but an unresolved puzzle?"
The child responds:
"There is more harmony in the unknown,
than where you think
you understand."

- Evangelia Daskalaki, Issue 20, *From Glasgow to Saturn*

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ABSTRACT

Recombinant human erythropoietin (r-HuEPO) administration stimulates the increase in red blood cell (RBC) mass and ultimately the oxygen carrying capacity of the blood, which results in its ergogenic benefits. EPO and its related forms are banned by the World Anti-Doping Agency. The main aim of this thesis was to apply an untargeted liquid chromatography-mass spectrometry based approach to investigate a possible phenotypic response to r-HuEPO in the plasma, urine and RBC (from whole blood and centrifuged blood residue) metabolomes of twenty physically active non-smoking males, as a potential method to detecting abuse. Participants underwent a ten week sampling protocol consisting of two weeks of baseline, four weeks of treatment ($50IU{\cdot}kg^{-1}$ every second day) followed by four weeks of wash-out. Significant metabolites (p-value < 0.05) were identified from a diverse range of metabolic pathways. Most interesting are as follows: orotate from urine, as well as linoelaidyl and elaidic carnitines, glycine, ergothioneine, thymine, 2-oxoglutarate, L-arginine, homoarginine, deoxycholic acid 3-glucuronide, D-glucuronate, D-glucosamine, 4-aminobutanoate, muramic acid and [PR] tretinoin/all-trans retinoic acid from plasma. The whole blood investigation highlighted significant metabolites which could be responsible for the generation of glutathione (namely 2-oxoglutaramate) which is an essential component to RBCs, because of its antioxidant capacity. Whereas the RBC residue obtained following removal of plasma showed significant changes in L-carnitine and acyl-carnitines (linoelaidyl, elaidic, hydroxybutyryl, heptadecanoyl, stearoyl, tetradecanoyl carnitines). As a result of the absence in a control group as well as training frequency of the participants, in the primary study, a small pilot investigation was carried out with the same untargeted methodology to investigate the phenotypic response of an hour of aerobic exercise in the urine metabolome of three physically active non-smoking males. Main significant systems affected include: purine pathway, tryptophan metabolism, carnitine metabolism, cortisol metabolism, androgen metabolism, amino acid oxidation and the gastrointestinal microbiome. It was concluded that a more robust investigation into r-HuEPO abuse would require a placebo controlled cross-over study, in order to ensure that the findings were truly related to the administration itself and not to other external stimuli, such as training.

ABBREVIATIONS

(FIA)-MS/MS	Flow injection analysis tandem mass spectrometry
(FIESI-ICR-FT)/MS	Flow injection electrospray ionization ion cyclotron resonance Fourier transform mass spectrometry
$\dot{V}O_{2max}$	Maximal oxygen uptake
^{1}H-NMR	Proton nuclear magnetic resonance
ABP	Athlete's Biological Passport
ACN	Acetonitrile
ADIPOR1	Adiponectin receptor 1
ADMA- DDAH- NOS	Asymmetrical dimethylarginine-dimethylarginine dimethylaminohydrolase- nitric oxide synthase
ADMA	Asymmetrical dimethylarginine
ADP	Adenosine 5'- diphosphate
ALAS2	Aminolevulinate, delta-, synthase 2
AMP	Adenosine monophosphate
ATP	Adenosine 5'- triphosphate
AUC	Area under the curve
BCAAs	Branched chain amino acids
BMI	Body mass index
CA1	Carbonic anhydrase I
CE	Capillary electrophoresis
CHO	Carbohydrate drink
CI	95% confidence intervals
CID	Collision Induced Dissociation
CoA	CoenzymeA
CON	Constant steady state exercise
CVD	Cardiovascular disease
DDAH	Dimethylarginine dimethylaminohydrolases

DIMS	Direct-infusion mass spectrometry
DNA	Deoxyribonucleic acid
DO	Day order
DPYSL5	Dihydropyrimidinase-like 5 or collapsin response mediator protein 5 (CRMP5)
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial NO synthase
EPO	Erythropoietin
ESA	Erythropoietin stimulating agent
ESI	Electrospray ionisation
eV	Electron volt
FADH	Flavin adenine dinucleotide
FTIR	Fourier transform infrared spectroscopy
g	Specific gravity
GC- MS	Gas chromatography-mass spectrometry
GC/TOF-MS	Gas chromatography time-of-flight mass spectrometry
GDP	Guanosine 5'-diphosphate
GMP	Guanosine monophosphate
GSH	Glutathione
GTP	Guanosine 5'-triphosphate
Hb	Haemoglobin
HBD	Haemoglobin, delta
HBE1	Haemoglobin, epsilon 1
HBM	Haemoglobin, mu or HBAP2
Hct	Haematocrit
HGPRT	Hypoxanthine-guanine phosphoribosyl-transferase
HIE	High intermittent exercise
HIF	Hypoxia-inducible factor

HIIT	High-intensity interval exercise
HMDB	Human metabolome database
HODE	9- and 13-hydroxyoctadecadienoic acids
HOME	12,13 hydroxyoctadec-9-(Z)-enoate
HPLC-ESI-MS-MS	High performance liquid chromatography- electrospray ionisation- tandem mass spectrometry
HPLC-UV	High performance liquid chromatography- ultraviolet detection
HR	Heart rate
IMP	Inosine monophosphate
IU	International Units
IV	Intravenous
kDa	kilo Dalton
LC-APCI-MS	Liquid chromatography- atmospheric pressure chemical ionisation- mass spectrometry
LCHO-P	Low- carbohydrate- protein beverage
LC-MS	Liquid chromatography-mass spectrometry
LMNA	Lamin A/C
LOC100131726	HCC-related HCC-C11_v3
LOC100288842	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5 pseudogene
LSVM	Linear support vector machine
m/z	Mass-to-charge ratio
m	Mass
MCT-1	H^+-monocarboxylate co transporter
miRNA	microRNA
MOD	Continuous moderate-intensity exercise
mRNA	messengerRNA
MSI	Metabolomics Standard Initiative
MSTUS	MS ‘Total Useful Signal’

NADH	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NO_3^-	Nitrate
OPLS-DA	Orthogonal partial least squares- discriminant analysis
PCA	Principal component analysis
PK	Pantothenate kinase
PLS-DA	Partial least squares- discriminant analysis
PTR MS	Proton transfer reaction mass spectrometry
QC	Quality control
RBC	Red blood cell
RhoA	GTP binding protein
r-HuEPO	Recombinant human erythropoietin
RNA	Ribonucleic acid
RNF213	Ring finger protein 213
ROC	Repeater operator characteristic
ROS	Reactive oxygen species
RP	Reversed phase
rpm	Revolutions per minute
RSD	Relative standard deviation
RT	Retention time
SC	Subcutaneous
SDMA	Symmetrical dimethylarginine
SELENBP1	Selenium binding protein 1
SERPINA13	Erpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 13 (pseudogene)
SLC4A1	Solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)

SNCA	Synuclein, alpha (non A4 component of amyloid precursor)
SoV	Sources of variance
STRADB	STE20-related kinase adaptor beta
TAN	Total adenine nucleotides
TESC	Tescalcin
THR	Threshold training distribution
TPRA1	Transmembrane protein, adipocyte associated 1 or GPR175
TT	Time trial
UCP2	Uncoupling protein 2
UHPLC/MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
UPLC-qTOF-MS	Ultra-performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry
v.e	Volitional exhaustion
v	Volume
VIP	Variable importance for projection
VT	Ventilator threshold
W	Watts
WADA	World Anti-Doping Agency
WHO	World Health Organisation
WR_{peak}	Peak work rate
ZIC-HILIC	Zwitterionic hydrophilic interaction liquid chromatography

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Chapter 1. General Introduction: Erythropoietin and its Use/ Detection in Sport, Practical Approaches of Metabolomics, as well as the Phenotypic Response of Physical Activity

Originally this metabolomics initiative, which will be outlined in the current thesis, stemmed from previous OMICs- based research projects funded by the World Anti-Doping Agency (WADA) which were as follows: in 2008 "A Gene Microarray Based approach to the Detection of Recombinant Human Erythropoietin Doping in Endurance Athletes" ($ 300,000) and a related project funded in 2010 "Application of a minimally-invasive method for RNA sampling and the addition of miRNA to the detection of recombinant human erythropoietin (r-HuEPO) use by athletes" ($ 200,000) as well as the Chancellors Fund for: "The application of whole- genome expression combined with metabolomic responses to drug testing in sport." (£ 9,000). These exciting projects were designed to provide the basis for the development of new drug (in sport) detection methods with improved discriminatory power, relative to current detection protocols using a "systems biology" approach (i.e., genomics, transcriptomics, metabolomics and proteomics) assessed by the very latest OMICs technology applied to whole blood, urine and saliva. The purpose, essentially, of the recently added metabolomics component was devised in order to identify biomarkers that could differentiate between recombinant human erythropoietin (r-HuEPO) administration (exogenous) and chronic altitude exposure (endogenous) using samples already collected from the aforementioned WADA funded study. The potential for insight into the full metabolome of a relatively homogenous group of endurance trained individuals allowed for several pertinent questions to be asked: whether there is a downstream metabolic effect of r-HuEPO? What are the natural and experimentally introduced sources of variance which contribute or mask the desired phenotypic outcome? Whether the "one test fits all" theory can be applied to the testing methods utilised to detect for r-HuEPO? Or is there an additional effect due to metabotyping- grouping metabolically linked subjects? As such an almost five year journey into understanding whether or not there is a metabolic effect of r-HuEPO in the Caucasian sub-group begins here.

As a result of this thesis, chapter 1 section 1.6. (*An Introduction to Physical Activity as a Source of Variance in Human Metabolomics Studies*) as well as chapter 5 (*A Study of the Effects of Exercise on the Urinary Metabolome Using Normalization to Individual Metabolic Output*) have been published, with detailed reference available

in appendix i. Additionally, all related oral/ poster presentation details can be found in appendix ii.

1.1. A Brief Introduction to EPO and its Recombinant Partner

Erythropoietin is of mainly renal origin, although 10 % has been attributed to the liver (Gaudard et al., 2003), and as a hormone its production is predominantly stimulated by low haemoglobin content (i.e. low oxygen levels in the blood) which in turn aims to regulate red blood cell mass (Jelkmann, 2007). Once released into the blood stream, from the renal cortex, the main EPO receptor target is located on the cell surface of the erythroid progenitor cells of the bone marrow (Debeljak et al., 2014). This in turn stimulates their proliferation and differentiation (Debeljak et al., 2014). A number of studies have demonstrated that self-reported mood, cognitive function and perceived physical condition were improved following r-HuEPO administration (Ninot et al., 2006; Miskowiak et al., 2008). Given the presence of EPO receptors across the human body it stands to reason that non-hematopoietic effects exist (Jelkmann, 2010; Debeljak et al., 2014), essentially highlighting the pleiotropic survival and the role of EPO as a growth factor (Jelkmann, 2007, 2010; Katz et al., 2010) in both normal and malignant cells (Debeljak et al., 2014). Recent clinical evidence highlights the fact that r-HuEPO administration exerts a protective effect against chemotherapy as well as negatively impacts the management of tumour progression (Debeljak et al., 2014).

The recombinant form of this 30.4 kDa glycoprotein has been used in the treatment of both renal (chronic kidney disease) and non-renal (malignant or autoimmune disease) anaemias (Jelkmann, 2007), and has proven to be efficient in reducing demand on the medical infrastructure in comparison to other erythropoiesis stimulating methods and homologous and autologous blood transfusion (Reichel and Gmeiner, 2010). In fact to begin the journey of isolating EPO for its therapeutic usages Miyake et al. (1977) explained a seven step procedure requiring 1,500 litres of urine to purify milligram amounts of the hormone from patients with aplastic anaemia (Miyake et al., 1977). In terms of its biochemical structure, serum EPO consists of a 165 amino acid chain, (Reichel and Gmeiner, 2010), with two disulphide bridges. There is a 40 %

carbohydrate component with both N-linked and O-linked glycans and this accounts for its glycosylation micro-heterogeneity (Azzazy and Mansour, 2007; Haase, 2013). The *in vivo* half-life of EPO is determined by its N-glycans composition, more specifically the sialic acid residues that aid in the removal of the glycoprotein by the galactose receptors of hepatocytes (Jelkmann, 2007). Thus the addition of more of them in the recombinant form (epoietin α, β, γ, δ, ε, ζ, θ, ι, ω as well as darbepoietin α (Gaudard et al., 2003; Reichel and Gmeiner, 2010)) prolongs biological activity. Endogenous EPO has a half- life of 6 hours (Reichel and Gmeiner, 2010), whereas the serum half- life of the recombinant forms are as follows: epoietin α between 4- 11 hours intravenously (IV) and 19- 25.3 hours subcutaneously (SC), epoietin β between 8.8- 10.4 hours (IV) and 24 hours SC, finally darbepoietin α 18- 25.3 hours IV and 48.8 hours SC (Reichel and Gmeiner, 2010). The biotechnological production of EPO followed from the advancement in cDNA technology that allowed the EPO gene, which was isolated from the liver, to be amplified, incorporated into a vector and used to transform various mammalian cell-lines. The α- and β- recombinant forms are produced by genetically transformed Chinese hamster ovary cells whereas the ω- form derives from genetically transformed hamster kidney cells, with the latter differing greatly in hydrophilicity (Gaudard et al., 2003). Epoietin δ originates from a genetically transformed human fibrosarcoma cell line and darbepoietin α, which is considered a novel erythropoietin stimulating agent, is produced by genetically transformed Chinese hamster ovary cells.

r-HuEPO administration has been widely investigated in terms of its ergogenic effects, which can be primarily attributed to increases in blood oxygen carrying capacity that lead to enhanced endurance performance in normobaric environments. Essentially it is the ‘quick’ version of the well-cited “living high-training low” (Stray-Gundersen et al., 2001) regime which sees athletes living at an altitude of ~ 2,000- 2,500 m for approximately 27- 30 days followed by high-intensity training at 1,500 m before competition at sea level (Stray-Gundersen et al., 2001). The improvements to maximal oxygen uptake ($\dot{V}O_{2max}$) are ~ 3 % which are modest in comparison to the purported r-HuEPO administration effects. It has been shown that 4 to 6 weeks of r-HuEPO administration increased $\dot{V}O_{2max}$ by ~ 8 % (Berglund and Ekblom, 1991; Audran et al., 1999; Birkeland et al., 2000; Parisotto et al., 2000; Russell et al., 2002; Connes et al.,

2003; Wilkerson et al., 2005; Durussel et al., 2013) as well as submaximal cycling capacity at 80 % $\dot{V}O_{2max}$ by ~ 54 % using time to exhaustion (Thomsen et al., 2007) and submaximal running performance by ~ 6 % using 3,000 m time trial (Durussel et al., 2013). Comparison of these studies in order to ascertain a global net effect is difficult due to the various exercise and administration regimes, however having said that the ergogenic effect is still prevalent across many research studies.

Due to the secondary benefits of r-HuEPO to exercise capacity, EPO and its related forms were banned in 1990 by the International Olympic Committee and were placed on WADA's prohibited list from the agency's early inception (Reichel and Gmeiner, 2010).

1.2. The Potential for Metabolomics in r-HuEPO Detection

"Sophisticated analysis methodologies"(Adamski, 2012) such as the OMICs that are directly related to the study of the central dogma (genomics, transcriptomics, proteomics and metabolomics) of complex hierarchal systems have greatly influenced the scope of research within the past decade (Ellis et al., 2007; Chorell et al., 2009; Burke et al., 2010; Michlmayr and Oehler, 2010; Reichel, 2011). We have certainly moved past the stage of examining the smell and colour of urine as a tool for disease diagnosis (published by Ullrick Pinder in 1506 *Epiphanie Medicorum* (Weiss and Kim, 2012)) however gene-environmental interactions entail the understanding of their unique and combined effect on population as well as individual level (Nicholson et al., 2008)- very similar to the multi-factorial level of information that could be retrieved in an OMICs-wide association study. The aim of any given doping pre-screening tool is to be as specific and sensitive as possible which is not always achievable with the ever- growing market of blood doping geared to mimicking, as far as possible, the natural internal bodily functions. However, metabolomics- based techniques have the potential for assessing a given organism's phenotypic response to immediate challenges or stimuli (Gieger et al., 2008; Nicholson et al., 2008; Michlmayr and Oehler, 2010; Adamski, 2012) and having the quickest turn-over rate in comparison to other OMICs technologies (Hunter, 2009; Perkel, 2011), allows for

some desirable analytical advantages. In theory, it is of importance to investigate the downstream effect of the series of intrinsic mechanistic processes that potentially lead to a targeted physiological response, which can be quantified in relation to its direct impact on the human phenotype (Nicholson et al., 2008). Thus, opening up the road to identifying novel biological targets (such as drug targets), individual profiling (personalised healthcare, nutrition management) as well as population profiling (metabolome-wide association studies) (Gieger et al., 2008; Nicholson and Lindon, 2008; Nicholson et al., 2008). These impact areas can be used to improve doping control by taking into account both sensitivity and specificity of the metabolome-based test in question. Of particular interest are the potential benefits of these impact areas in order to improve the Athlete's Biological Passport (ABP) - an electronic, longitudinal, haematological parameter monitoring system investigating the indirect use of a blood manipulation substance (recombinant human erythropoietin; r-HuEPO) or method (homologous blood transfusion). Essentially identifying whether a sample is 'suspicious' based on the intra-individual variability across a selected number of blood parameters (Sottas et al., 2010). These parameters include, haematocrit, haemoglobin, red blood cell count, percentage of reticulocytes, reticulocyte count, mean corpuscular volume/ haemoglobin/ haemoglobin concentration (Saugy et al., 2014). There is also the multi-parametric OFF-hr score which combines haemoglobin and reticulocyte score and finally the Abnormal Blood Profile Score (Patrick, 2012). The Abnormal Blood Profile Score includes a combination of the following: haematocrit, haemoglobin, red blood cells, percentage of reticulocytes, mean corpuscular volume/ haemoglobin concentration, serum EPO, serum soluble transferrin receptor, optical red blood cell, red cell distribution width, absolute reticulocyte count as well as immature reticulocyte fraction (Saugy et al., 2014). The ABP, although bearing a high annual cost as well as technical demands, has in fact worked as a successful deterrent of doping to most competitive athletes. Especially those among cycling teams (Patrick, 2012) as it was first introduced in the Union Cycliste Internationale (International Cycling Union) (Zorzoli et al., 2014)- in part as a response to the prevalent doping nature of the 1990s.

1.2.1. The Current Problems in r-HuEPO Detection

In spite of the recent advances made in r-HuEPO detection (Dehnes et al., 2014; Okano et al., 2014; Vogel et al., 2014), the recent doping allegation against Lance Armstrong sheds light on the problematic nature of doping detection. Training and altitude, especially when combined, have been identified as the factors most capable of influencing the sensitivity and specificity of the ABP (Saugy et al., 2014). Additionally, the individualized approach of the ABP focuses on indirect markers for altered erythropoiesis; rather than utilizing techniques which could directly detect the presence of a prohibited substance. This raises several legal and scientific concerns given that an athlete could be potentially banned from their sport with only circumstantial evidence (Hailey, 2010). The biological passport for cycling was implemented in 2008 in response to the doping scandals within cycling that questioned not only the credibility of the sport itself but that of WADA as well. This passport unfortunately does not follow the substance abuse *per se* rather the individualized athlete response over a long period of time based on a statistical model (Bayesian network) that makes comparisons to the athlete's 'true clean' baseline. This again raises issues with regard to whether or not a true baseline existed in the first instance yet makes it very difficult to develop, or indeed focus, on implementing and developing direct analytical tests for the prohibited substances themselves (Hailey, 2010).

This issue is carried over to the misuse of r-HuEPO in cycling whereby blood manipulation is popular mainly due to the difficulty in its detection by anti-doping authorities. Indirect detection has traditionally been based on haematological markers of altered erythropoiesis, which have not been entirely specific or sensitive (Malcovati et al., 2003; Ashenden, 2004; Sottas et al., 2006). On the other hand, direct isoelectric focusing, which profiles EPO glycoforms, is the only validated test for the detection of r-HuEPO, but the test has a rather limited detection window (approximately 36- 48 hours) and is also of limited sensitivity. To make matters more difficult there are several substances undergoing clinical testing in humans that have been shown to mimic the action of r-HuEPO (Lundby et al., 2012) along with potentially 100 other

EPO mimicking drugs on the market that go undetected by current aforementioned methods (Hailey, 2010; Mehaffey, 2012).

Furthermore, Ashenden et al. (2011) investigated the sensitivity of the ABP to detect micro- dose injections of r-HuEPO in healthy volunteers over a period of 12 weeks (enough to raise total haemoglobin mass values by 10%) (Ashenden et al., 2011). The authors found that the ABP software did not flag any of the subjects despite the fact that performance gains are still pertinent with this dose regime (Lundby et al., 2008a, 2012) giving rise to false negatives. Ojiambo et al. (2008) investigated haematological model scores that were applied to an elite East- African cohort, the authors found indications of systematic doping (Ojiambo et al., 2008) or what may be cases of "false-positives". This highlights the unwanted result of combining altitude dwelling and training. Finally, Reichel (2001) reviewed the latest in OMICs related projects funded by WADA as well as their findings. The author concluded that there is a clear need to improve analytical techniques to aid in the detection of r-HuEPO abuse by athletes and despite this new dimensionality of research that can be conducted within the "systems biology" approach none of the anti-doping related OMICs studies have directly led to their applications in routine doping control (Reichel, 2011).

1.3. Metabolomics-based Research into r-HuEPO

To date there have only been two studies, one of which is a pilot, utilizing metabolomics- based techniques to investigate the effects of r-HuEPO on the human metabolome. The first most recent study, and the only one to have combined r-HuEPO with training, investigated thirty-eight healthy but untrained males (details of study in table 1-1). The subjects were randomly assigned to either 10 weeks of placebo treatment (SP; N:9), darbepoietin α (SE, N:9), placebo with endurance training (TP; N:10) and endurance training with darbepoietin α (TE, N:10). The training involved supervised indoor cycling and each week consisted of three sessions that started with a 5 minute warm-up followed by a main session before ending with a 5 minute cool down. Each of the three main sessions per week could either be a) 40 minutes, b) 2 x 20 minutes (5 minute easy cycling break in between) or c) 8 x 5 minutes (1 minute

easy cycling break in between). Each main set was performed once a week. Blood samples were collected for metabolic analysis as well as skeletal muscle biopsies (*Vastus Lateralis*) mainly for gene-expression analysis. Liver fat fractions, intramyocellular lipid content as well as visceral and subcutaneous fat were measured with magnetic resonance techniques (^{1}H-MR and MR spectroscopy). In brief, resting energy expenditure increased with training and r-HuEPO treatment separately and in combination. In fact in a similar study by Christensen et al. (2012) a single dose of 400 IU.kg^{-1} of epoietin α significantly increased resting energy expenditure in healthy males (Christensen et al., 2012). In the most recent study, r-HuEPO treatment was also followed by increases in free fatty acid levels (opposite in training alone along with palmitate flux), intrahepatic lipid content, and UCP2 mRNA (uncoupling protein 2) after prolonged treatment in both groups treated with r-HuEPO. The authors highlighted an opposite lipolytic effect of r-HuEPO treatment and training that neutralised when the two were combined. Interestingly, UCP2 is involved in the transfer of anions from the inner to outer mitochondrial membrane (proton leak) and essentially separates oxidative phosphorylation from adenosine triphosphate (ATP) synthesis with energy released as heat (www.Genecards.org; GCID: GC11M073685). This heat production after r-HuEPO treatment may be linked to the rise in resting energy expenditure, however more research is warranted to see if this might contribute to detection of EPO abuse (Christensen et al., 2013).

Appolonova et al. (2008) conducted a thirteen day trial whereby two male subjects were injected with one 2000 U r-HuEPO dose (start of day four; details can be seen in table 1-1). Twenty- four hour urine samples were collected each day at specific time points and analysed via high-performance liquid chromatography tandem mass spectrometry for arginine, citrulline, asymmetrical dimethylarginine as well as symmetrical dimethylarginine (SDMA) considered to be part of the DDAH- ADMA NOS system (dimethylarginine dimethylaminohydrolase- asymmetrical dimethylarginine nitric oxide synthase) (Appolonova et al., 2008). There was a reference population added to this protocol of 15 healthy volunteers aged between 21-50 years (both female and male). Precision (coefficient of variance) was less than 10 %, with retention times for arginine being 6.0 ± 0.1 min; ADMA at 6.08 ± 0.1 min; SDMA at 6.08 ± 0.1 min; and citrulline at 6.5 ± 0.1 min. The mass spectra were

generated by Collision Induced Dissociation (CID). Both subjects showed a marked increase in both four targeted metabolites post administration however a specific detection window for its direct application into doping control analysis was not mentioned. It is worth mentioning that one of the subjects did not participate in sport (presumed non- active) and the second was a long distance runner (Appolonova et al., 2008). The percent concentration increase of the four metabolites post administration was approximately 5- 6 % higher in the long distance runner than the non-active male; however both showed a minimum of a two fold increase from baseline concentrations.

Table 1-1. Details of human metabolite profiling studies and supporting cell culture studies investigating targeted metabolites in relation to r-HuEPO administration.

Study	Intervention/ Administration	Dose	Human (H); Cell line (C)	Sample Type	Analytical Method	Metabolite(s)
Christensen et al. (2013)	Darbepoetin α/ SC	Once a wk at 40 µg for 3 wks then 20 µg for remaining 7 wks	H	Blood (Plasma, Serum) Skeletal Muscle	HPLC ^{1}H-MR Spectroscopy	SE: > Liver fat fraction, SE: > hepatic fat fraction % compared to TP TP/TE: > Glut4 not SE TE: tended > M-value/Insulin SE: >REE compared to SP TP/TE + clamp: >REE TE/SE: > UCP2 over time ESA blunted effects of training > cyt C ESA: > serum FFA then < after training ESA: > serum palmitate conc TE/TP: < palmitate flux compared to pre - No effect of ESA alone TE: < LDL after 10wk
Appolonova et al. (2008)[1]	r-HuEPO (Epocrine)/ IV	2000U.day^{-1}	H	Urine	HPLC-ESI/MS	> Arginine, > Citrulline, > ADMA, > SDMA
Scalera et al. (2005)[2]	Epoetin β & Darbepoetin α/ *NA*	(0, 0.1, 1, 10, 50, 100, and 200 U/ml)	C (cell supernatant)	Human Umbilical Vein Endothelial Cells	HPLC-MS	> ADMA (dose dependent), < DDAH, < NO synthesis (> oxidative stress) > Allantoin (>ROS)
Wang & Vaziri, 1999[2]	EPO/ *NA*	(0, 5, and 20 U/mL)	C	Human coronary artery endothelial cells	*NA*	< basal and acetylcholine-stimulated production of NO < eNOS protein abundance

SC: subcutaneous; IV: intravenous; U: International Units; SP: Sedentary Placebo, SE: Sedentary ESA, TP: Endurance Training Placebo, TE: Endurance Training ESA, HPLC-ESI/MS: High Performance Liquid Chromatography Tandem Mass Spectrometry (Electro Spray Ionization); ADMA: asymmetrical dimethylarginine; SDMA: symmetrical dimethylarginine; DDAH: dimethylarginine dimethylaminohydrolase; NO: Nitric Oxide; eNOS: endothelial NO synthase. [1] Metabolomics- based experiment; [2] Supporting evidence for targeted metabolite identification presented in 1

The DDAH- ADMA pathway (seen in Figure 1-1 below) is a major regulator of NO, which is generated from L-arginine. Studies have suggested that this system plays vital roles in endothelial cell motility and angiogenesis by its management of NO production (Fiedler, 2008). Essentially, ADMA (analogue of arginine) acts as a competitive inhibitor of NOS; responsible for the co-production of NO. In turn, ADMA is regulated by DDAH I and II. Inhibition of DDAH I and II has been shown in animal studies to suppress endothelial derived NO however re-activation of DDAH allows for restoration (*in vivo/ vitro*) of the suppression in RhoA- (GTP binding protein) dependent stress fibre formation; owing to its regulation of actin cytoskeleton (Cachero et al., 1998; Fiedler, 2008). In the study by Scalera et al. (2005) endothelial cells (*in vitro*) were incubated with various concentrations of Epoetin β and darbepoetin α for 24 hours (see table 1-1 for details). The cell supernatant was investigated for allantoin (marker of oxidative stress), ADMA as well as DDAH by high-performance liquid chromatography mass spectrometry (HPLC-MS). The results highlighted that r-HuEPO increased levels of ADMA in a dose-dependent manner which was inversely related to NO formation as well as DDAH activity over the same time frame (Scalera et al., 2005). In the highest concentration a marked dose-dependent increase of allantoin was indicative of oxygen free radical formation, which was supported by the increase in intracellular reactive oxygen species (ROS). Scalera et al. (2005) noted that the administration of EPO *in vivo* and *in vitro* aims to resist the vasodilatory effects of NO; this could contribute to the related hypertensive effects of r-HuEPO.

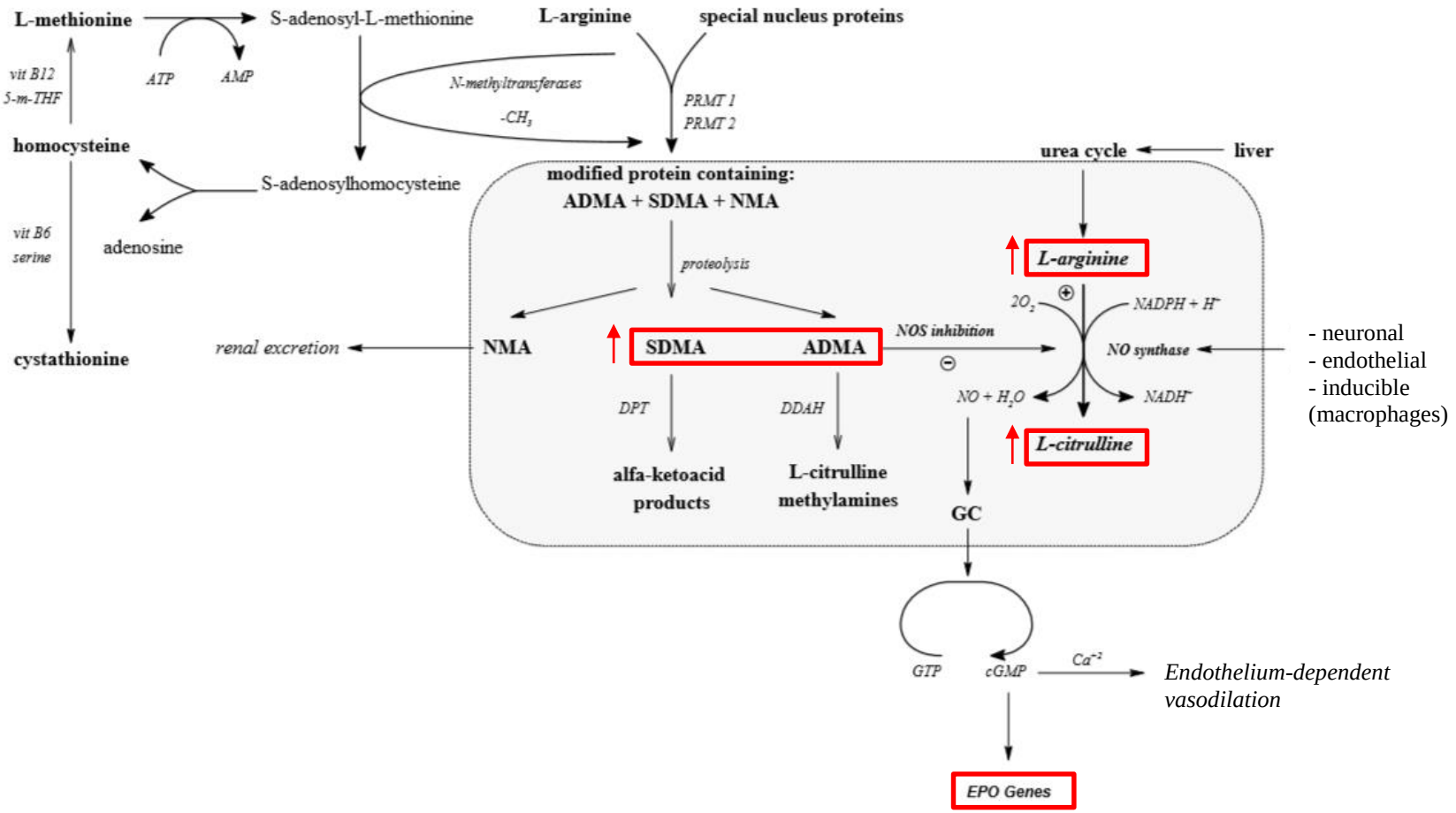


Figure 1-1. Simplified schematic taken from Appolonova et al. (2008) illustrating the pathway of the ADMA-DDAH-NOS system. Highlighted in red are the up-regulated metabolites of interest indicative of possible r-HuEPO misuse as outlined by Appolonova et al. (2008)

In patients and animals with chronic renal failure administration of r-HuEPO has been shown to induce hypertension (Wang and Vaziri, 1999; Lundby and Olsen, 2011) which can, in part, be explained by the EPO induced inhibition of eNOS production (Lundby and Olsen, 2011). A related study on the incubation of human coronary artery endothelial cells with subsequent administration of EPO highlighted a marked dose-dependent decrease in basal NO formation as well as eNOS protein expression (Wang and Vaziri, 1999). However, the authors also pointed to EPO's growth stimulatory effects resulting in a marked increase in DNA synthesis and proliferation.

1.3.1. Other Potential Metabolites of Interest

EPO function in normal humans has been shown to be of pleiotropic nature, however its distinctive role in metabolic action has not been widely investigated (Lundby and Olsen, 2011). Hypoxia induced elevation of endogenous plasma EPO has been correlated to enhanced lactate production in sedentary individuals (acutely introduced to 3,500 m compared to sea level) and has also been shown in simulated altitude (Sakata et al., 2000). The authors highlighted a > 3 fold increase in lactate, however aerobic fitness is intimately related to lactate production (increased lactate production noted at approximately 50 % - 60 % of $\dot{V}O_{2max}$ in sedentary compared to 70 % - 80 % of $\dot{V}O_{2max}$ in trained individuals) as well as lactate transport into erythrocytes (Skelton et al., 1995, 1998; Sakata et al., 2000). On the other hand, lower circulating plasma lactate levels have been speculated to occur with administration of r-HuEPO during training (Connes et al., 2004; Juel et al., 2007). Apart from the erythrocyte stimulating effects of r-HuEPO, its effects translate to the functioning and synthesis of erythrocyte membrane proteins MCT-1 (H^+-monocarboxylate co transporter) and band- 3 which influence acid- base balance alongside lactate (H^+) influx into the erythrocyte; ultimately away from the muscle. This gradient, or dilution effect of the plasma, will aid in lactate removal during intense exercise as well as increase rate of release from muscle (Connes et al., 2004). Both transporters have been shown to be significantly elevated in chronic hypoxia as well as in normoxia with systematic subcutaneous injections with r-HuEPO (Connes et al., 2004). EPO receptors have been shown to be

present in human skeletal muscle and thus the ion (acid-base) homeostasis of the erythrocytes was hypothesized to be in part controlled by the increased EPO level (Juel et al., 2007). A study conducted to evaluate the effects of prolonged r-HuEPO administration in trained individuals on muscle membrane transport systems showed that MCT-1 and other proteins involved in pH regulation, remained unchanged throughout the intervention (Juel et al., 2007). The studies above highlight the possibility for an underlying lactate influx system present in both hypoxia and r-HuEPO administration which is most pertinent in trained individuals; however more research is required.

Hepcidin, a hormone responsible for iron metabolism, has accumulated interest as a potential new anti-doping screening tool for r-HuEPO misuse. In brief, stimulation of erythropoiesis, either by subcutaneous injections or hypoxia, increases the demand for iron in healthy subjects hence increases in iron mobilization from skeletal muscle (higher bone marrow iron utilization) as well as observation of lower levels of myoglobin (Robach et al., 2009). Robach et al. (2009) investigated the effects of iron metabolism in 8 healthy volunteers. With artificially induced stimulation of erythropoiesis (systematic low dose r-HuEPO injections for 4 weeks) the main findings were increases in muscle iron and L-ferritin levels implying a change in muscle protein expression and regulation which was indeed observed by the suppression of hepcidin. This suppression is also translated in a single 5000 IU injection of r-HuEPO (Lundby et al., 2012). However there are intricate mechanisms which need to be taken into consideration with such methods, given that both inflammation and hypoxia induce hepcidin suppression. Therefore conclusions based on such observations need to be made with caution given that both aforementioned circumstances are common in training athletes. It would be of interest therefore to measure the extent of hepcidin suppression (or targeted metabolites related to iron metabolism) in inflammation compared to r-HuEO as well as altitude in trained individuals.

1.3.2. Potential Metabolite Targets from WADA Gene-Expression Study (unpublished)

Based on unpublished work from the gene-expression report filed with WADA, a list of 18 candidate genes were selected in the Caucasian sub-group as potential targets. As such table 1-2 below indicates, where possible, whether these gene targets could relate to a potential metabolic pathway.

Table 1-2. Potential metabolite targets for the 18 candidate genes selected by the WADA gene- expression study (full name and functionality of each gene available in appendix v).

From 18 Gene Candidates				
No.	ID (www.GeneCards.org)	Possible Metabolite Response	Other	UniProt.org ID
1	ADIPOR1	**Lipid metabolism** (*fatty acid oxidation*) **Glucose uptake** (*by adiponectin)*	-	Q96A54
2	ALAS2	**Heme Biosynthesis** **Porphyrin- containing compound metabolism** (porphobilinogen) **Protoporphyrin-IX biosynthesis** **Glycine, threonine and serine metabolism** *(5-aminolevulinate from glycine)*	Erythroid specific mitochondrially located enzyme. Catalytic activity (Pyridoxal phosphate: cofactor): Succinyl-CoA + glycine = 5-aminolevulinate + CoA + CO_2	P22557
3	CA1	Reversible hydration of carbon dioxide. Hydrates cyanamide to urea. Enzyme regulation: ***Activated by*** histamine, imidazole, L-adrenaline, L- and D-histidine, and L- and D-phenylalanine. ***Inhibited by*** coumarins, sulfonamide derivatives such as acetazolamide, benzenesulfonamide and derivatives (4-carboxyethylbenzene-sulfonamide, 4-carboxyethylbenzene-sulfonamide ethyl ester, 4-(acetyl-2-aminoethyl)benzene-sulfonamide, 4-aminoethylbenzene-sulfonamide), and 'prong inhibitors' BR15, BR17, BR22 and BR30.	-	P00915
4	DPYSL5	Beta-Alanine metabolism. Thymine and uracil degradation.	Neural development. Enzyme activity unsure.	Q9BPU6

5	HBD	-	-	P02042
6	HBE1	-	Globin family.	P02100
7	HBM	-	Pseudogene. Globin family. Erythroid tissues.	Q6B0K9
8	LMNA	Cellular protein metabolic process.	-	P02545
9	LOC100131726	-		
10	LOC100288842	-		
11	RNF213	-	Probable E3 ubiquitin- protein ligase that may play a role in angiogenesis. Association with ATPase activity.	Q63HN8
12	SELENBP1	-	Selenium- binding protein which may be involved in the sensing of reactive xenobiotics in the cytoplasm. May be involved in intra- Golgi protein transport.	Q13228
13	SERPINA13	-	Serpin family. Pseudoform. Protease inhibitor. Serine protease inhibitor. Glycoprotein.	Q6UXR4
14	SLC4A1	-	Band 3 is the major integral glycoprotein of the erythrocyte membrane.	P02730

15	SNCA	-	May be involved in the regulation of dopamine release and transport. Induces fibrillization of microtubule-associated protein tau. Reduces neuronal responsiveness to various apoptotic stimuli, leading to a decreased caspase-3 activation.	P37840
16	STRADB	-	Pseudokinase.	Q9C0K7
17	TESC	-	Co- factor in cell pH. Inhibits the phosphatase activity of calcineurin.	Q96BS2
18	TPRA1	-	UPF0359 family. Multi- pass membrane protein.	Q86W33

1.4. An Introduction to Metabolomics

1.4.1 Mass Spectrometry Methods

Mass spectrometry based metabolomics is a rapidly developing OMICs technology, which allows hundreds of metabolites (generally with a mass < 1500 Da, at ≤ 5 ppm mass deviation) to be investigated within each metabolome at any given time frame; essentially creating a 'snapshot' of the biological state of an organism (Michlmayr and Oehler, 2010). This unique fingerprint of cellular activity encompasses both inter-organ processes as well as environmental challenges. Metabolites are labile molecules that can reflect the functionality of a cell itself , and thus changes to the metabolome are expected to be amplified in relation to the proteome or transcriptome (Ellis et al., 2007). Metabolomics, present at the end stage of the OMICs cascade (Figure 1-2), was formally coined just over a decade ago, yet the fundamental analytical chromatographic techniques trace back to the 1960s (Ellis et al., 2007).

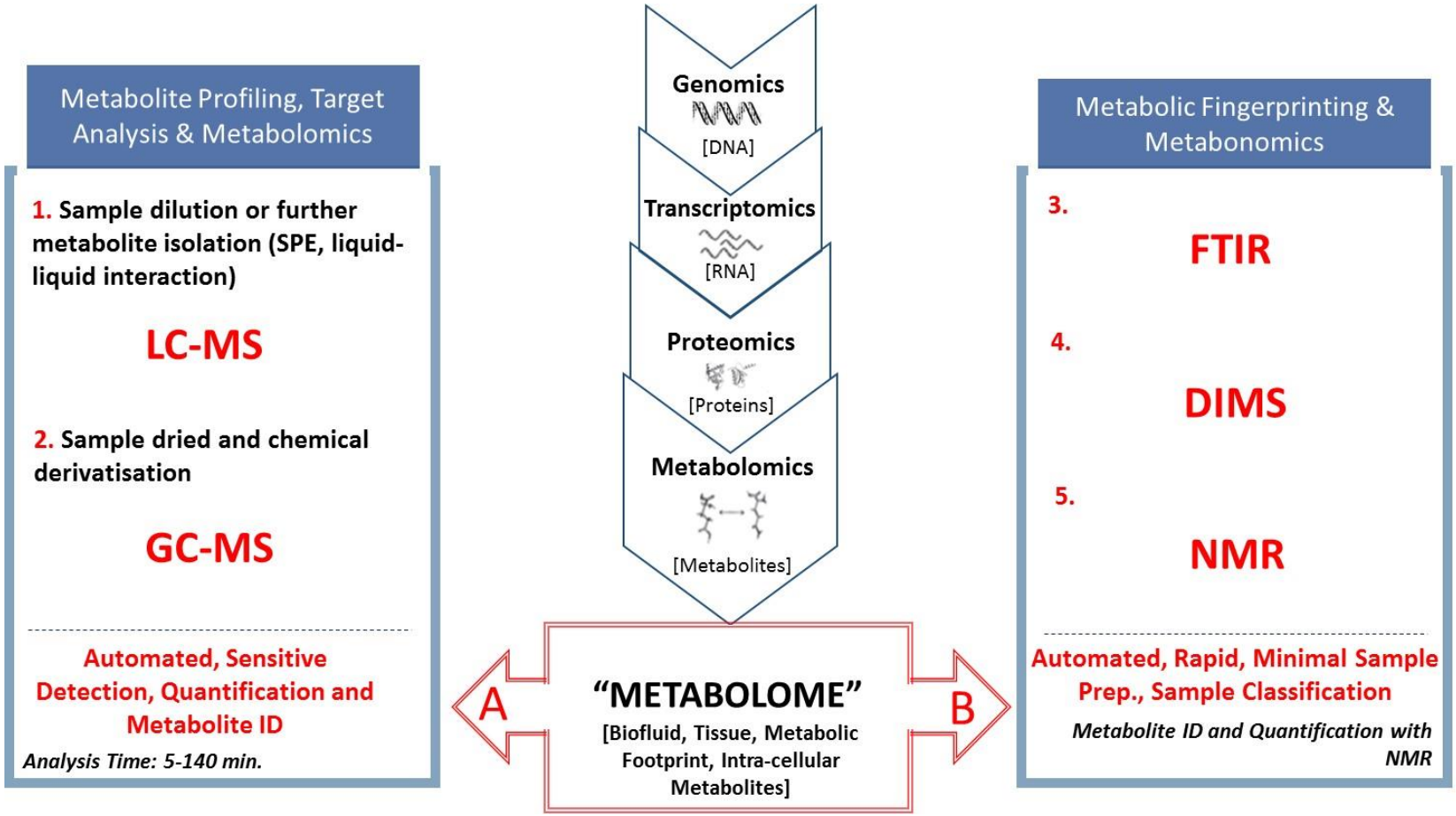


Figure 1-2. Diagrammatic representation of the OMICs cascade as well as some of the analytical techniques available within the field of metabolomics. Adapted from Michlmayr and Oehler, (2010) as well as Dunn and Ellis, (2005).

As illustrated in Figure 1-2 metabolomes of various origins can be analysed with a range of advanced analytical techniques depending, on the desired outcome. Option A allows the metabolome of a given sample to be analysed for metabolite profiling (identification/ quantification of pre-selected metabolites) or targeted analysis (qualitative/quantitative analysis of one or a few metabolites). The techniques used include LC-MS (1) which provides metabolite separation by liquid chromatography that is followed by electrospray ionization (or atmospheric pressure chemical ionization) in either positive or negative ion mode where positive ionization mode is often more sensitive than negative ion mode (Dunn and Ellis, 2005). Compared to gas chromatography mass spectrometry (GC- MS) (2) (originally thought of as the gold standard), LC-MS allows for lower temperatures for analysis as well as simpler sample preparation due to sample volatility not being required. Quantification can be achieved by external calibration or application of a response ratio (common in pharmaceutical analysis). It is possible to generate structural information by fragmenting the molecular ions generated under electrospray ionisation condition using CID. However identification with CID spectra is not always possible since mass spectral libraries for ESI tandem MS or MS^n are not commonly available (Dunn and Ellis, 2005). There have been recent developments in the past decade, however, in relation to available databases; a good example of this being the human metabolome database (http://www.hmdb.ca/). This online library comprises of more than 40, 000 metabolite entries with the option to view available metabolite structure and related pathway maps. Of particular interest is the ability to submit MS spectral files that can then be automatically searched against its database of MS/MS spectra- this assists in the identification process.

Electron impact ionization is generally used with GC-MS which provides fragments within the mass spectra generated at an standard energy of 70eV; this information allows for a distinct fingerprint to be developed that can be compared to the spectral libraries with mass/intensity association (Dunn and Ellis, 2005; Watson, 2010). The major advantage of GC-MS is the very high resolution chromatography available from capillary GC but its disadvantage is that many important metabolites are not volatile.

Option B allows for metabolite fingerprinting (sample classification) as well as metabonomics. Fourier transform infrared spectroscopy (FTIR) (3), based on vibrational (e.g. stretch and bend) spectroscopy, is mainly utilized as a diagnostic tool. Each unique vibrational response (of functional group) to an infrared beam results in an infrared absorbance spectrum that can ultimately be identified as a 'fingerprint' depended on the chemical/ biochemical substance under investigation. However the specificity and sensitivity of this technique is limited when compared to techniques mentioned in option A despite the rapidity of spectral collection (Dunn and Ellis, 2005; Ellis et al., 2007). Direct-infusion mass spectrometry (DIMS) (4) allows for one mass spectrum per sample to be collected which is representative of the sample in question however coverage of a full set of metabolites is directly related to the ability of each metabolite to be ionized. This technique has been applied to disease diagnostics (as a screening tool) as well as for classification (coupled with tandem MS) due to its rapid and automated approach.

Finally, nuclear magnetic resonance (NMR) (5) spectroscopy is based on the principal that almost every element will have an isotope with a magnetic spin greater than zero in order to undergo the 'NMR effect'- detection of radiation whilst nuclei are in the relaxation process following excitation with radiofrequency radiation. In general, it is a rapid as well as non- destructive method requiring minimal sample preparation. Chemical shifts are assigned to specific metabolites yet the pattern of the spectrum is generally utilized for classification of the sample which is very similar to FTIR and DIMS (Dunn and Ellis, 2005). The full set of the human metabolome, consisting of endogenous/ exogenous metabolites from drug/ chemicals as well as gut microflora, has not yet been experimentally categorized hence the difficulty in developing full mass spectral libraries of all metabolites- this can make identification procedures more complex and time consuming (Dunn et al., 2011).

1.4.2 Chromatographic Methods Used In Metabolomics

By achieving higher mass resolution and sensitivity, via high resolution MS platforms, one is inevitability extending the possibilities of untargeted metabolite coverage (Zhang et al., 2014). The separation techniques associated with these platforms provide

retentions time as an additive feature to identify each metabolite which aids to reduce ion suppression (Gika et al., 2014; Zhang et al., 2014). Whilst comparing GC, CE (capillary electrophoresis) and LC, the sample preparation, coverage and reproducibility favour LC methods (Kuehnbaum and Britz-McKibbin, 2013; Zhang et al., 2014). In general, LC-MS experiments will start with a sample preparation/ clean-up phase prior to injection. The LC column will allow compounds to separate based on either (a) size, (b) affinity to stationary phase, (c) polarity, or (d) hydrophobicity (Katajamaa and Oresic, 2005). One method will not be able to encompass all molecules (neutral, ionic, non-polar and so on), as some will ionise better in one mode of a particular method over another (Theodoridis et al., 2012). Additionally, molecules will co-elute at similar retention times, hence why MS can further differentiate analytes based on their mass-to-charge ratio (m/z) (Katajamaa and Oresic, 2005).

Reversed Phase (RP) LC, very commonly used in high resolution MS platforms, has the limitation in the retention as well as separation of polar metabolites- which form a large component in biological matrices (Zhang et al., 2014). For the superior retention of hydrophilic (polar) compounds with improved ionisation efficiency with ESI, Hydrophilic Interaction Liquid Chromatography (HILIC) is being increasingly applied (Cubbon et al., 2010; Zhang and Watson, 2013; Zhang et al., 2014). The currently available HILIC columns can be categorised based on their stationary phase composition: silica, charged and zwitterionic phases (Zhang et al., 2014). The zwitterionic stationary phase, allows for water retaining properties combined with relatively weak ion exchange interactions, owing to the low surface charge of the predominantly utilised sulfoalkylbetaine functional groups (Cubbon et al., 2010). The negatively charged sulfonic group at the end of the zwitterionic moiety creates an electrostatic attraction to positively charged metabolites (low pH levels). The combination of a polymer-based substrate has been shown to improve the chromatographic separation attracting negatively charged metabolites at a higher pH (Zhang et al., 2014). Pre-processing for generation of LC-MS file can be seen in Figure 1-3.

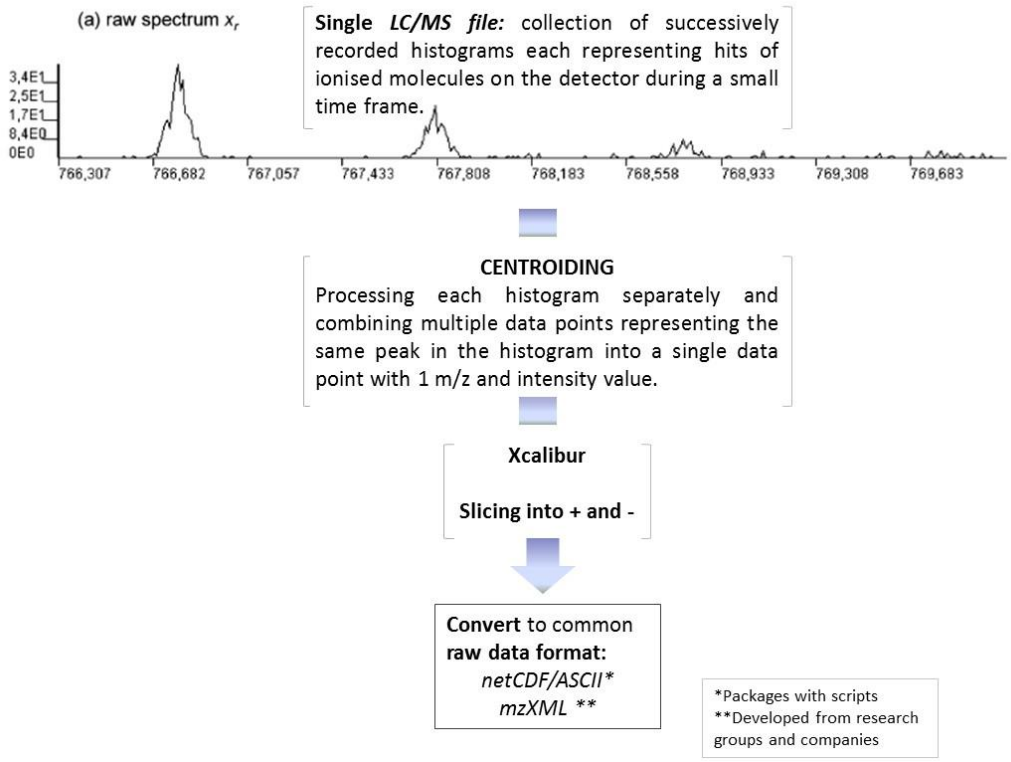


Figure 1-3. Simplified pre-processing pipeline of LC-MS file generation, illustration modified from Katajamaa and Oresic, (2007).

1.4.3. Metabolomics at Strathclyde

The metabolomics unit at the Strathclyde Institute of Pharmacy and Biomedical Sciences (http://www.metabolomics.strath.ac.uk/index.php) has a variety of techniques available for both targeted as well as untargeted analysis. Currently in use are an LTQ Orbitrap mass analyser for LC-MS as well as an Exactive Orbitrap (accurate mass capability but no fragmentation capability). Well-established hydrophilic interaction chromatography methods are used to carry out each investigation (Creek et al., 2011; Zhang et al., 2013, 2014); which use eluents containing acetonitrile in with aqueous modifiers either containing formic acid or ammonium carbonate. The chromatographic techniques provide relatively stable retention times, good peak shape and resolution (Zhang and Watson, 2013).

Data processing techniques vary from commercially available Sieve (Thermo Fisher Scientific) to open source programs which include: MzMine

(**mzmine**.sourceforge.net/), developed by Katajamaa and Oresic (Katajamaa and Oresic, 2005; Katajamaa et al., 2006; Pluskal et al., 2010) and MzMatch/PeakML (**mzmatch**.sourceforge.net/), developed by Richard Scheltema and Andris Jankevics (Scheltema et al., 2011; Creek et al., 2012). MzMatch has an accompanying graphical user interface for zwitterionic silica-based ZIC-HILIC and polymer-based ZIC-pHILIC experiments which is the IDEOM pipeline developed by Darren Creek (Creek et al., 2012). The data processing techniques for MS data follow the following basic structure post raw data file conversion: (a) peak/ feature identification (e.g. chromatogram deconvolution and deisotoping); (b) alignment (this stage can be followed by gap filling and in the case of MzMatch relative standard deviation of peak intensity is calculated prior to combining of data sets); (c) normalization (or scaling can be applied after putative identification prior to multivariate analysis); (d) identification (including adduct and complex search, fragment, as well as formula prediction) (Sugimoto et al., 2012).

The identification of putative metabolites in IDEOM is aided by the addition of a retention time calculator and the ability to simultaneously check whether a putative metabolite identified in the sample of question matches the retention time of an authentic standard in the standard mixtures run in the same batch as the samples. Where possible, especially in the absence of tandem mass spectrometry methods, this will enable a higher score level via the Metabolomics Standard Initiative (MSI; level 1 through to 4). These levels, aimed at clarifying the reporting of metabolite identifications (Fiehn et al., 2007; Salek et al., 2013), can be classified as follows (http://cosmos-fp7.eu/msi):

- Level 1 - Identified metabolites (matching to authentic chemical standard from researcher's laboratory via same analytical methodology)
- Level 2 - Putatively annotated compounds
- Level 3 - Putatively characterised compound classes
- Level 4 - Unknown compounds

1.5. Normalization and Sources of Variance

1.5.1. Brief Introduction to Normalization Techniques

There is some disagreement with regard to selection of the ideal normalisation technique especially as it relates to urine (Ryan et al., 2011a). In spite of the much lower protein content of urine in comparison to plasma, the variations of volume and strength leave the interpretation of the resulting data output open to question.

The gold standard of urine normalisation has been creatinine (Ryan et al., 2011b), however there are several other factors which influence this specific technique that require some attention mainly gender, age, stress, disease, training modality and diet (including supplement use with for example creatine (Yoshizumi and Tsourounis, 2004)) as well as ethnicity (Hsu et al., 2008; Warrack et al., 2009; Dunn et al., 2011). Creatinine is a breakdown product of creatine phosphate in muscle and is eliminated by the kidneys at a relatively constant rate per day. Osmolality, an important measure of metabolic output as well as hydration status, can be measured by employing a commonly utilized freezing-point method (Warrack et al., 2009). In addition to sample normalisation, the variability of the instrument must also be considered. The use of internal standards (spiking samples with externally prepared authentic stock solutions) is a common practice and these should be stable compounds which are not present in biological matrices. Alternatively, a pooled QC (quality control) sample which is representative of the sample to be analysed should be added to the run sequence in order to monitor progress within and between batches as well as instrument drift (Koek et al., 2011). The MSTUS (MS 'Total Useful Signal') is a method highlighted by Warrack et al. (2009), and carries out subtraction of all the background signals in the mass spectrometer present in blank runs and retains the useful signals associated with the samples (Warrack et al., 2009). However, there does seem to be a problem with this in that abundant metabolites such as creatinine tend to fluctuate throughout the day. Even the use of 24 hour samples is not fool proof due to the levels of creatinine, as well as the sensitivity of the metabolites associated with the purine pathway (Zieliński et al., 2009). Finally, duplicates or repeat runs can also be introduced for quality control within samples. However, method performance is also dependent on

biological fluid under investigation and physicochemical properties of these fluids (Koek et al., 2011).

1.5.2. Source of Variance in Human Metabolomics Studies

The inevitable innate variance of human biology coupled to ever-changing experimental procedures- this notion endlessly turns the wheels of the scientific mind whilst attempting to uncover new insights into basic and complex human processes. In the literature we find new and old concepts regarding sources of variance (SoV) (Ryan et al., 2011b; Sloutsky et al., 2013) that obscure or increase the elusive 'noise level' of metabolite data sets, especially as it relates to human investigations in a variety of biological fluids. The human metabolome, the end-stage of the OMICs long factory line, encompasses the intrinsic and extrinsic challenges that it faces on a day- to- day basis. As with any well- oiled factory lines, pitfalls are identified and managed accordingly. Yet in the complex hierarchal system of human biology this takes on new facets- and these are namely identified as SoV, or co-factors that contribute uniquely to the effect of the resulting data output. Each one could inevitably be part of the normalizing strategies for the resulting data set. However, each study requires careful thought and consideration into the selection of these co-factors, bearing in mind the type of biological fluid under investigation. Plasma is often considered as a spot test providing information on intrinsic inter-organ communications whereas urine, with much more abundant metabolite numbers is considered to provide an averaged response of the metabolic output, and gives an indication of the extrinsic (i.e. environmental) challenges as well. This means that if the population under investigation are regularly exercising males and the resulting profiles (or patterns) that emerge through the urinary metabolite *data x* are similar, than it may not be appropriate to utilize creatinine as a co-factor given the close relationship of muscle activity and creatinine release of that particular cohort (Ryan et al., 2011b). In fact, creatinine may as well as be a metabolite response of interest that could be used to further classify the subjects tested. On the other hand, if this was an inactive patient group then creatinine might be a possible option to combat the variations in urinary volume. It is often the case in metabolomics data that creatinine becomes the most

abundant feature across the metabolite matrix, especially in urine, with seemingly its own unique flux system. Taken as a whole, the pitfalls in this complex factory line must be considered as a culmination of factors working together based, in part, on the circumstances introduced by the experimenter. Figure 1-4 outlines a generalised equation for the common SoV that accommodates any given metabolite response.

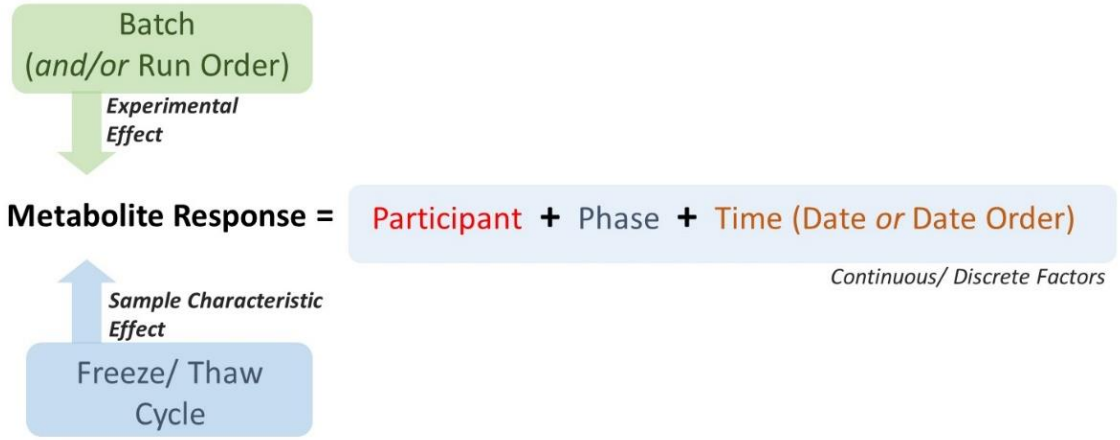


Figure 1-4. Generalised equation indicating the various co- factors (continuous/ discrete) which arise in large systematic metabolomics data sets that can be utilized for modelling in order to retrieve desired metabolic response. The participant accounts for the inter-individual variability, the phase accounts for experimental conditions such as pre- and post- drug treatment, finally the element of time can be described for example, as date or date order in relation to the start of an intervention. Other aspects which are present are the freeze/ thaw cycle(s) along with the instrument effect which could include batch and/ or run order of samples. Other co-factors not shown here but discussed in previous literature include, but are not limited to, sample preparation, instrument calibration and chromatographic column.

We observe that the common denominator in the SoV, if experimental conditions are appropriately controlled, is the inter-subject variability, or the participant effect (Figure 1-4). Given that this variability more often than not is exhibited in the outcome (both in pattern and degree of response), it might be worthwhile to consider the idea of ‘metabotyping’ by, essentially, creating global metabolome specific responses that could lead to new or even modified existing metabolomics data repositories. Originally addressed as metabolic phenotyping (Gavaghan et al., 2002) this concept, only a few

years old with regards to human metabolomics experiments (Krug et al., 2012; Pasikanti et al., 2013; Dumas et al., 2014; Ladep et al., 2014; O'Donovan et al., 2014), allows for clustering of metabolically linked observations. These observations could be response patterns or more precise quantitative measures (e.g. fold change) that will align subjects based on their unique metabolome instead of the use of, let us say, quantile normalization (Brodsky et al., 2010; Veselkov et al., 2011; Lee et al., 2012) in the hope that each participant will fit into the same bracket of 'normal' with reduced systematic error. This could provide an alternative to a biological passport since if an exercise stress test were used to 'type' someone, then discontinuities in the development of someone's profile might highlight interventions other than simply training.

1.6. An Introduction to Physical Activity as a Source of Variance in Human Metabolomics Studies

Physical activity and exercise are potent lifestyle interventions (Sarris et al., 2014) for the reduction of several psycho-somatic and physical risks, including cardiovascular disease (CVD) (WHO, 2009), diabetes (WHO, 2009) and mild-to-moderate depression (Sarris et al., 2014). Regular exercise, a relatively inexpensive treatment (Sarris et al., 2014), can modify but more importantly improve overall quality of life and expectancy. However, questions remain with regard to adequate duration, frequency and intensity, as well as what type of exercise is optimal for individuals of different cohorts (i.e. sedentary *vs.* already active), and what the molecular mechanisms underlying health improvement through exercise are. The answer may depend on various factors such as gender, age, life- style and body mass index (BMI). However, directly investigating the effect of exercise on the human metabolome, via metabolomics-based techniques, can provide new insight into novel phenotypic responses- giving rise to personalised training regimes that are reflective of the initial metabolic status of each individual.

Surprisingly, in spite of the importance of exercise in maintaining health, there are only around 64 papers in the literature that have investigated the effects of exercise on the metabolome during the past 15 years (1999- 2014). Increases in the power of MS and NMR techniques coupled to advances in data extraction and manipulation techniques have led to a rapid growth in publications on metabolomics. The most recurring theme with regard to the metabolic effects of exercise in the literature is related to purine metabolism and more specifically to hypoxanthine a purine derivative as well as a reaction intermediate in the adenosine metabolism and nucleic acid formation (salvage pathway). Adenosine metabolism is important in exercise because of the crucial role of ATP in muscle function. The earliest observed increase in "nonuric acid purine" in urine samples post-exercise was made in the early 1900s by Burian (as referenced in Nasrallah and Al- Khalidi (Nasrallah and Al-Khalidi, 1964)). Most investigations into these adenine nucleotides, as potent vasodilators and contributors in energy regulation, trace back as far as the techniques developed in the 1960s that have formed the basis of metabolomics. Starting with human studies, Forrester and Lind (1969) investigated the ATP content from venous effluent of the forearm muscle in response to exercise (i.e. 10 % and 20 % maximal voluntary contractions), illustrating an increase in ATP post-exercise compared to resting values (Forrester and Lind, 1969). Frog and other animal heart (intact and perfused) studies were used, in the 1930s, in order to demonstrate an improvement in heart beat after injections of nucleic acid derivatives (Forrester, 1967; Forrester and Lind, 1969). From around 1999 onwards the principal metabolite of interest in a variety of biological fluids for monitoring the effect of exercise is hypoxanthine.

A comprehensive review was conducted to identify research studies which have utilised a metabolomics- based technology to monitor the effects of exercise in predominantly healthy subjects in the period 1999- 2014. Based on these criteria 64 papers were selected through the following search engines, Google Scholar (http://scholar.google.co.uk/) and Pubmed (http://www.ncbi.nlm.nih.gov/pubmed). Figure 1-5 illustrates the main features of the included studies ranging from metabolomics technology, subject group demographics, gender groups as well as sample type tested.

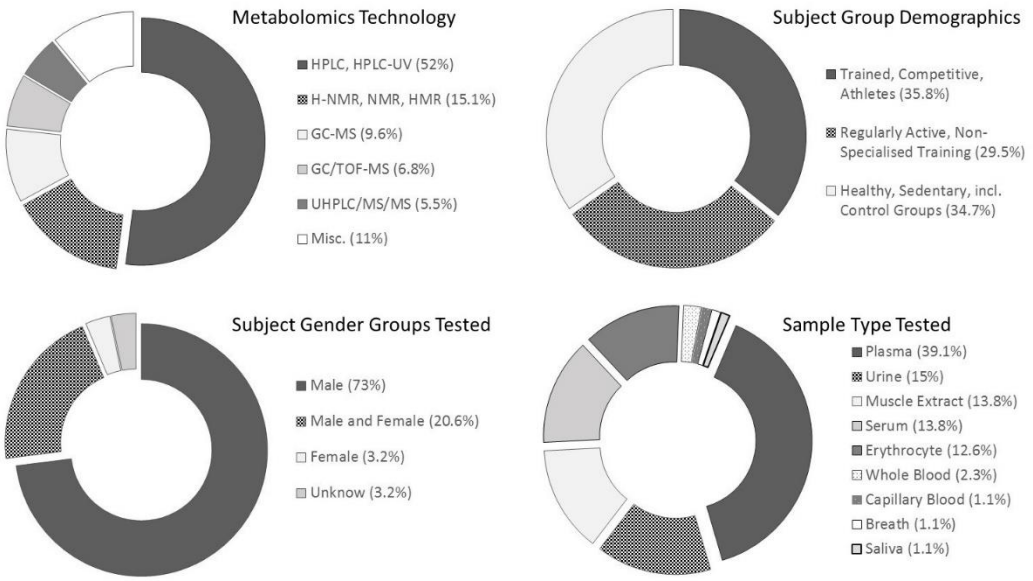


Figure 1-5. Main features of the research studies selected for review. The most prevalent metabolomics technology utilised is high performance liquid chromatography, via plasma of healthy trained/athlete and active male groups.
Abbreviations: HPLC-UV: high performance liquid chromatography- ultraviolet detection; ^{1}H-NMR: proton nuclear magnetic resonance; GC-MS: gas chromatography mass spectrometry; GC/TOF-MS: gas chromatography time-of-flight mass spectrometry; UHPLC/MS/MS: ultra-high performance liquid chromatography tandem mass spectrometry. Miscellaneous includes: LC-APCI-MS: liquid chromatography- atmospheric pressure chemical ionisation- mass spectrometry; (FIA)-MS/MS: flow injection analysis tandem mass spectrometry; PTR MS: proton transfer reaction mass spectrometry; (FIESI-ICR-FT)/MS: Flow injection electrospray ionization ion cyclotron resonance Fourier transform mass spectrometry; CE: capillary electrophoresis; HPLC-ESI-MS-MS: high performance liquid chromatography-electrospray ionisation- tandem mass spectrometry; UPLC-qTOF-MS: ultra-performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry

A summary table (table 1-3) has been created for each study identified highlighting the main metabolomics technology used, subject demographics, exercise regimen and sampling time-points. Table 1-4 contains a summary of the sample type tested along with main metabolites that were analysed via the metabolomics technology accompanied by relative response to exercise where appropriate. The majority of the studies are focused on particular pathways and most of the untargeted metabolomics studies on exercise, that have been published within the last four year, are in line with

the wider availability of high resolution mass spectrometry and more advanced NMR techniques. There is, however, a large variation in the depth of the studies as judged by their success in generating intriguing new hypotheses where there is a sense of the metabolome operating in a complex and dynamic fashion in response to a variety of exercise regimes. The discussion in this review will be focused more on the in depth studies, and the ones not included have been placed as supplementary tables S2 and S3 in appendix vi and vii, respectively.

Table 1-3. Detailed Study Features of Metabolomics-based Articles* since 1999 with Main Study Aim to Investigate Effect of Exercise on Different Human Bio-fluid Metabolomes.

Instrument	Exercise Test	Subject Characteristics (N, Gender, Age, Test Groups)	Sampling Time-Points	Reference
HPLC	**Session 1-** Incremental $\dot{V}O_{2max}$ test, cycle ergometer **Session 2-** 1. 5min warm-up @ 50% $\dot{V}O_{2max}$ 2. 75% $\dot{V}O_{2max}$ (60rpm) until v.e.	**10, Male, 23 – 32yrs** One Group- active	Plasma- rest (pre), during-exercise (every 20min and at exhaustion), 5min post-exercise Muscle (*lateral aspect* of the quadriceps) extracts- pre-exercise and post-exercise	(Sahlin et al., 1999)
HPLC	**Session 1-** $\dot{V}O_{2max}$ test, cycle ergometer 1. 10min warm-up @ 100W, 80rpm 2. 3min rest 3. 6min @ 150 – 170W 4. 5min rest 5. 6min @ 225 – 275W 6. 5min rest 7. cycle to exhaustion @ 275W w/ 25W increase ever 45s **Session 2-** Cycle 90% $\dot{V}O_{2max}$ until exhaustion **Session 3-** Work load adjusted to elicit exhaustion after 15-20min **Session 4-** 2x cycle to exhaustion @ 263±13W, bouts separated by 1hr	**8, Male, 21 – 25yrs** One Group- active	Plasma- rest (pre), 1, 5, 10, 15, and 17.5min during-exercise, and immediately before exhaustion, as well as at 1, 2, 4, 9, 20, 30, 45, and 60min post-exercise Muscle (*vastus lateralis*) extracts- rest (pre) and at exhaustion [samples taken at session 4 for each bout]	(Hellsten et al., 2001)

HPLC [Merck Hibar Li-chrosphere 100 CH-18/2 250mm x 4mm column]	**Session 1-** $\dot{V}O_{2peak}$ test, cycle ergometer **Session 2-** 1wk sprint training [twice each day 1 set of 15x 10s 'all-out' sprints w/ 50s break between sprint, two sets separated by minimum 6hr rest] Performance Tests (PT): Pre-sessions 2 a 30s 'all-out' sprint was performed followed by another post 1wk training	**7, Male, 23.1 ± 1.8yrs** One Group- active, non-specialised training	Plasma- rest (pre), 0, 10, 15, 20, 30, 60 and 120min post-PT Urine- 12hrs pre and 2, 6, and 16hrs post-PT Muscle extract- rest, immediately post-exercise and 10min into recovery	(Stathis et al., 2006)
HPLC [Hypersil ODS 100mm x 4.6mm x 5µm column and a Hypersil ODS 20mm x 4mm x 5µm pre-column]	**4 testing sessions across 1yr training cycle** [preparatory phase: 1; competition phase: 1; transition phase: 1; end of 1yr training cycle and during next yr preparatory phase: 1] **Session 1, 2, 3 and 4-** Incremental $\dot{V}O_{2max}$ test, treadmill [10km/hr^{-1} start, then 2 km/hr^{-1} increase every 3min until v.e.]	**9, Male, 22.9 ± 0.6yrs** One Group- long distance runners	Rest, immediately pre-exercise and 5min post-exercise	(Zieliński et al., 2009)
HPLC [Hypersil ODS 100mm x 4.6mm x 5µm column and a Hypersil ODS 20mm x 4mm x 5µm pre-column]	**4 testing sessions across 1yr training cycle** [preparatory phase: 1 pre and post 1st specific subphase; competition phase: 1 pre; and transition phase: 1 post] **Session 1-** Incremental $\dot{V}O_{2max}$ test, treadmill	**Group 1- 11, Male, 22.3 ± 0.7yrs** Middle-distance runners **Group 2- 11, Male, 24.1 ± 1.9yrs** No prior/current competitive sport experience, control group	Rest, immediately pre-exercise and 5min post-exercise	(Zieliński et al., 2011)

	[10km/hr^{-1} start, then 2 km/hr^{-1} increase every 3min until v.e., measuring VT and HR] For **sessions 2,3 and 4** training loads adjusted based on 5 energetic zones			
HPLC [Hypersil ODS 100mm x 4.6mm x 5µm column and a Hypersil ODS 20mm x 4mm x 5µm pre-column]	**4 testing sessions across 1yr training cycle** [preparatory phase: 2; competition phase: 1; and transition phase: 1] **Session 1,2,3and 4-** Incremental $\dot{V}O_{2max}$ test, treadmill [10km/hr^{-1} start, then 2 km/hr^{-1} increase every 3min until v.e.]	**Group 1- 10, Male, 20 – 29yrs** Sprinters **Group 2- 10, Male, 21 – 28yrs** Triathletes Highly trained competitive athletes	Rest, immediately pre-exercise and 5min post-exercise	(Zielinski and Kusy, 2012)
HPLC [Hypersil ODS 100mm x 4.6mm x 5µm column and a Hypersil ODS 20mm x 4mm x 5µm pre-column]	1yr training cycle incl. preparatory, competitive and transition phases **Session 1-** Incremental $\dot{V}O_{2max}$ test precompetitive phase, treadmill [start @ 10km/hr^{-1} w/ 2 km/hr^{-1} increase every 3min until v.e.]	**71, Male, Healthy and Highly Trained Athletes** Group 1- 28, 19 – 28yrs, triathletes Group 2- 12, 20 – 26yrs, long-distance runners Group 3- 13, 20 – 26yrs, middle-distance runners Group 4- 18, 19 – 29yrs sprinters	Pre-exercise and 5min post-exercise	(Zieliński et al., 2013b)
HPLC [Hypersil ODS 100mm x 4.6mm x 5µm column and a Hypersil ODS 20mm x 4mm x 5µm pre-column]	**3 testing sessions across 1yr training cycle** [preparatory phase: 1; competition phase: 1; and transition phase: 1] **Session 1-** Incremental $\dot{V}O_{2max}$ test, treadmill	**30, Male, Middle-aged Runners** Group 1- 11, 46 ± 3.8yrs, elite master runners Group 2- 9, 45.1 ± 4.7yrs, amateur runners Group 3- 10, 45.9 ± 6.1yrs, recreational runners	Pre-exercise and 5min post-exercise	(Zieliński et al., 2013a)

	[start @ 10km/hr^{-1} w/ 2 km/hr^{-1} increase every 3min until v.e.] **Training Loads:** Group 1- 7 times a week, total weekly training time of 8–10hrs, number of training session in 1-year cycle was 315 Group 2- 5 times a week, total weekly training time of 5hrs, number of training session in 1-year cycle was 221 Group 3- 3 times a week, total weekly training time of 3hrs, number of training session in 1-year cycle was 123			
HPLC	**Session 1-** Continuous effort test w/ progressively increasing intensity 1. 5min @ 25W, warm-up 2. Start→ 70W, cadence 70rpm 3. 20W increase every 3min until max effort reached	**22, Male, 21.9 ± 2.33yrs** One Group- healthy	Immediately pre- and post-exercise, and 30min post-exercise	(Dudzinska et al., 2010b)
HPLC	**Group 1-** All out 4min aerobic/ anaerobic exercise **Group 2-** Soccer match **Group 3-** Marathon	**52 athletes** Group 1- 8, cycle pursuers, 20-25yrs Group 2- 19, soccer players, 17-20yrs Group 3- 25, marathon runners, 22-34yrs	Pre-exercise and 5min of cessation	(Bianchi et al., 1999)
HPLC [Gemini C18, 5 µ (150 × 4 mm) column]	**Session 1-** $\dot{V}O_{2peak}$ 1. 3x3min submax. workloads @ 50, 100 and 150W 2. 1min workload increments of 25W until v.e. [rpm btw 80-90] **Session 2-**	**8, Male, 18- 35yrs** One Group- healthy untrained	**Plasma** at rest, during exercise at 1, 3, 5, 10, 15, and 30min and at 5, 10, 20, 30, and 60min post-exercise **Urine** collected overnight before trial and following 60min post-exercise.	(Gerber et al., 2014)

	A: *CON* 1. 30min @ 50% $\dot{V}O_{2peak}$ 2. 60min recovery in supine position or B: *HIIE* 1. 1min blocks of 20s @ 150% $\dot{V}O_{2peak}$ separated with 40s passive recovery, 30min total length 2. 60min recovery in supine position **Session 3-** A or B [sessions 2 and 3 separated by 1wk]			
HPLC	**Session 1-** $\dot{V}O_{2peak}$ test, cycle ergometer [3min at 3 submx work rates followed by > work rate every min until v.e.] **Session 2- 5 days of ingestion of placebo (calcium carbonate) OR allopurinol (300mg tablet) followed by** 8 x 10s 'all-out' sprints w/ 50s break between sprints. **Session 3- 5 days of ingestion of placebo OR allopurinol (reversed) followed by** 8 x 10s 'all-out' sprints w/ 50s break between sprints.	**7, Male, 24.9 ± 3yrs** **Active but non-specifically trained** [$\dot{V}O_{2peak}$ of 48.1 ± 6.9mL.kg^{-1}.min^{-1}]	Plasma- rest (pre), 0, 10, 15, 20, 30, 60 and 120min post- final sprint Urine- 12hrs pre and 2, 6, and 16hrs post-PT	(Stathis et al., 2005)
HPLC	**10 Day Summer Training Camp** Day 1-4: dry land training activities at sea level	**17, Male** U.S. National Alpine Ski Team	Day 1 first thing in the morning, Day 2 post-training, and Day 10 post-training session	(Subudhi et al., 2001)

	Day 5: travel and rest Day 6-10: on snow training btw 2000-2900m			
HPLC [Superpher 100RP-18, 5µm column 250 x 4mm, Merck]	**Monitored across a two wk period as part of a 6 month training program**, with a 5 days/wk training regime Exercise involved a 50 and 80km fast paced march across unpaved terrain with additional load of 35kg.	**31, Male, 19 ± 1yr** Healthy [$\dot{V}O_{2max}$ of 65.7 ± 0.6 mL.kg^{-1}.min^{-1}]	Pre-exercise and immediately post the 50 and 80km marches	(Chevion et al., 2003)
HPLC	**Session 1-** 1. Familiarisation with the 10 exercises of the resistance exercise circuit (i.e.bench press, leg press, latissimus dorsi pull, leg extension, shoulder press, triceps exercise, crunch, vertical row, biceps curl and pull up) 2. one repetition maximum for each exercise evaluated 3. for exercise crunch max number of repetitions evaluated **Session 2-** 1. warm- up on a cycle ergometer (15 min, 75W) 2. submax resistance exercise circuit at 75% of 1 repetition maximum. Recovery between stations set to 1min.	**Group 1- 7, Male, 31.3 ± 10.2yrs**, resistance trained **Group 2- 10, Male, 28.2 ± 3.9yrs**, non-resistance trained	30min pre- and post- exercise	(Ramel et al., 2004)
HPLC [Discovery C18, 150 x 4mm i.d., 5 µm (Supelco) and	**10min run** @ speed of ~9.6 ± 0.3km.hr^{-1} on flat ground (4 x 400m oval track; 1600 ± 50 m)	**30, Male and Female** Female (N:15) 22-36yrs Male (N:15) 21-40yrs	Pre-exercise, immediately post- and 60min post-exercise.	(Kanďár et al., 2014)

LiChroCart 125 x 4, Purospher Star RP-18e, 5µm; Merck columns]		Healthy		
HPLC-UV [Supelco Discovery DS 18 150mm x4.6mm, i.d. 3µm particles, 120 Å pore diameter] **CE**	**Competitive mountain bike race of ~102km**	**Group 1- 21, Male, 17.3 ± 0.5yrs**, well- trained cyclists **Group 2- 10, Male, 24.6 ± 3.6yrs**, sedentary controls	Group 1- Rest, Pre- race (10-15min), and 10-15min after completion Group 2- Samples taken at similar time during the day	(Gatti et al., 2005)
HPLC-ESI-MS-MS [Phenomenex LUNA C8 2 x 50 mm, 3µm particle size column] **HPLC-UV** [C18 reversed-phase column (Lichrocart 250-4, Lichrospher 100 RP-18, 5µm, VWR)	**Session 1 –** $\dot{V}O_{2max}$ [ergobike] and Maximal upper limbs strength [incremental max single lifting test on horizontal bench, with 3min rest btw reps] **Session 2-** randomly assigned to Group 1: Endurance then strength tests Group 2: Strength then endurance **Session 3-** Group 1: Strength then endurance Group 2: Endurance then strength tests [Session 2 and 3 separated by 1wk] **Tests-** Endurance: 1. 10min warm-up @ 40% $\dot{V}O_{2max}$ 2. 30-40min @ 80% $\dot{V}O_{2max}$	**15, Male, 28 ± 1yr** Healthy trained	6hr urine samples btw 10:00-16:00 on each experimental day	(Dovio et al., 2010)

	3. 10min cool down @ 50W plus stretching Strength: 1. 10min warm-up 2. (x2) warm-up on the bench (1 x 10 reps of the weight-free bar and 1 x 6 reps @ 40% of 1 repetition maximum) 3. 4 max series (3-6 reps until v.e.) @ 80% of 1 repetition maximum 4. 3min rest w/in the series plus stretching			
HPLC	**Session 1 –** $\dot{V}O_{2max}$ [ergometer, no specific protocol described] Then assigned to: Group 1 (N:15)- 45min @ 55% $\dot{V}O_{2max}$ Group 2 (N:15)- 1hr @ 70% $\dot{V}O_{2max}$	**30, Female, 20-24yrs** Healthy sedentary untrained with not involved in exercise program in the previous 24 months pre testing [Testing occurred during follicular phase of menstrual cycle]	Pre-exercise, immediately post-exercise and 24hr post	(Giraldo et al., 2009)
HPLC [C18 column]	**Session 1-** 1. 20min warm-up 2. 10min rest 3. 5min judo match	**16, Male, 18.4 ± 1.6yrs** National judoists [$\dot{V}O_{2max}$ of 55.7 ± 0.5 $mL.kg^{-1}.min^{-1}$]	Rest, 3min, 1 and 24hr post-match	(Degoutte et al., 2003)
HPLC [Waters Symmetry C18, 3µm, 2.1 x 150mm column]	**Session 1-** Exercise test on cycle ergometer 1. initial load set to 25W for 2min 2. > 25W every 2min until subject theoretical heart rate max reached	**9, Male and Female, 29.3 ± 3.5yrs** healthy	Pre-exercise, 3min post-exercise, 6 and 24hr into recovery	(Gangemi et al., 2003)

HPLC/MS [Hewlett Pckard octadecyl silica Hypersil colum (100 x 4.6mm i.d., 5µm) reversed-phase]	**Group 1 and 2-** Either on treadmill or cycle ergometer 1. 5min warm-up 2. > work rate to elicit heart rate of 70-80% of max 3. sustain for 45min **Group 3-** Seated for 50min	**24, Males, 23.7 ± 9.4yrs** Exercising for 6 months pre-trial (≥30min running/cycling, ≥4/wk) Assigned to following groups depending on pre-training regime: Group 1 (N:8)- Running Group 2 (N:8)- Cycling Group 3 (N:8)- Control	Pre- and post- exercise sessions	(Sparling et al., 2003)
HPLC	**Continuous effort and progressive cycle test** 1. 5min warm-up @ 25W 2. Increase to 70W w/ 70rpm 3. >20W every 3min until v.e. or until unable to maintain 70rpm.	**44, Male, 18.5 ± 2.92yrs** One Group- healthy [$\dot{V}O_{2max}$: 50.2 ± 6.26mL.kg^{-1}.min^{-1}]	Pre-exercise, immediately post- and 30min post-exercise.	(Dudzinska et al., 2013)
LC/MS/MS[a]	**Group 1-** Cardiopulmonary exercise testing [bicycle ergometer, 10-25W/min with > ramp cycle] **Group 2-** Exercise treadmill testing [Bruce protocol] **Group 3-** Boston Marathon	**Group 1-** 8, Male and Female, 48 ± 14yrs **Group 2-** (a) 40, Male and Female, 58 ± 13yrs (b) 25 (validation group), Male and Female, 59 ± 12yrs **Group 3-** 25, Male and Female, 42 ± 9yrs Amateur runners	Group 1- **Plasma** from the superior vena cava and pulmonary artery at baseline, peak exercise and 60min post-exercise Group 2- **Plasma** at baseline, peak exercise and 60min post-exercise Group 3- **Plasma** at pre-exercise and post-exercise	(Lewis et al., 2010)
GC/TOF-MS	**Session 1-** $\dot{V}O_{2peak}$ test, cycle ergometer **Session 2-** 90min cycle	**24, Male, 25.7 ± 2.7yrs** One Group- healthy, regularly trained	Pre-exercise and immediately post-exercise	(Pohjanen et al., 2007)

	[each 90min cycle consists of 9 x 10min equal sets] Sets of 10min each: 1. 2min @ 40% 2. 6min @ 60% 3. 2min @ 85% $\dot{V}O_{2peak}$ [**Session 2 completed in total 4 times**, 1wk apart permitted per session]	$\dot{V}O_{2peak}$: 59.1 **±** 7.3 mL.kg^{-1}.min^{-1}		
GC/TOF-MS	**2wk Training Program** [11 sessions/wk, 30hrs in total of technical and aerobic exercise]	**Male** Group 1- junior trained rowers, 20.2 ± 1.1yrs Group 2- senior trained rowers, 23.0 ± 2.7yrs Group 3- sex and age matched controls, healthy	Pre-training, at 1wk and post 2wk training	(Yan et al., 2009)
^{1}H-NMR [5mm inverse probe]	**Group A Session-** 3 sets of 2x 80m max runs, the two runs separated by 10s rest **Group B Session-** 3 sets of 2x 80m max runs, the two runs separated by 1m rest [20min rest between sets for both groups]	**12, Male, Moderately Trained** Group A- 21 ± 2yrs Group B- 20 ± 1yrs	Pre-exercise and 35min post-exercise	(Pechlivanis et al., 2010a)
^{1}H-NMR [5mm inverse detection probe]	**8wks training programme** with 3 exercise sessions per week. 1. First 4wks = each session incl. 2 sets of 80min max runs	**14, Male, Healthy and Moderately Trained** Group A- 21 ± 2yrs, each run per session separated by 10s rest	Pre-exercise and 30min post-exercise [Before and after 8wks of training]	(Pechlivanis et al., 2013)

	2. Second 4wks = each sessions incl. 3 sets of 80min max runs	Group B- 20 ± 2yrs, each run per session separated by 1min rest		
^{1}H-NMR [5mm broadband probe]	**Session 1-** $\dot{V}O_{2peak}$ **Session 2-** 1. 6min cycling @ ~25W with cadence 80rpm, 2. 45min cycling @ 60% WR_{peak}, 3. 90% WR_{peak} until v.e.	**17, Male, 50- 60yrs** Group 1- 9 master athletes Group 2- 8 healthy untrained	Pre-exercise, immediately post-exercise and 24hr post-exercise	(Mukherjee et al., 2014a)
^{1}H-NMR [5mm triple resonance probe]	**Session 1-** 1. Warm-up (running) 2. YO-YO Test, 2 x 20m shuttle runs with 10s recovery between bouts, starting speed 10km/hr^{-1}	**14, Male, 23 ± 3yrs** One Group- healthy elite soccer players	Pre-exercise and post-exercise	(Santone et al., 2014)
UHPLC/MS/MS [method for acidic and basic feature identification] **GC-MS**	**Session 1-** $\dot{V}O_{2max}$ test, treadmill [following Bruce protocol (1971)]	**77, Male and Female, 18 – 35yrs** One Group- healthy	Pre-exercise sample	(Lustgarten et al., 2013)
^{1}H-NMR	**Longitudinal physical activity** measured with the Metabolic Equivalent (MET) index.	**PART 1:** **Finnish Twin Cohort Study** 32, male and female same-sex twins, 50 – 74yrs Group 1- 16 healthy active Group 2- 16 healthy inactive **PART 2:** **Pieksämaki Cohort Study** 230, male and female, 41-62yrs Group 1- 115 healthy active Group 2- 115 healthy inactive	PART 1: Single sample post 32yrs of follow-up (2007) PART 2: Single sample post 5yrs of follow up (2003 and 2004) PART 3: Single sample post 5yrs of follow up (2007) PART 4: Single sample post 5yrs of follow up (1980 and 1997-1998)	(Kujala et al., 2013)

		PART 3: **Young Finns Study** 600, male and female, 30 – 45yrs Group 1- 300 healthy active Group 2- 300 healthy inactive **PART 4:** **Northern Finland Birth Cohort 1966** 1244, male and female, 31yrs Group 1- 622 healthy active Group 2- 622 healthy inactive		
Targeted MS/MS[b]	**Session 1-** Incremental bicycle ergometer test[c] [Increments increase by 25W per step until maximum capacity reached]	**30, Male and Female, 38.33 ± 7.16yrs** One Group- active and physically fit	Pre-exercise, at each step until (incl.) max performance	(Netzer et al., 2011a)
UPLC-qTOF-MS **HPLC**	**Session 1-** 1. Running velocity @ individual anaerobic threshold, determined with an incremental exercise test, treadmill [6km/h^{-1}, > 2km/h^{-1} every 3min] 2. $\dot{V}O_{2max}$, ramp test [8km/h^{-1}, > 1km/h^{-1} every 0.5 min until v.e.] **Session 2-** Group 1: 60min continuous run of moderate intensity 93% of the VIAT (~75% of $\dot{V}O_{2max}$) Group 2: 120min continuous run 70% of the VIAT (~55% $\dot{V}O_{2max}$)	**Group 1- 13, Male, 32.6 ± 6.1yrs** $\dot{V}O_{2max}$: 56.5 **±** 1.4mL.kg^{-1}.min^{-1} **Group 2- 8, Male, 30.9 ± 5.8yrs** $\dot{V}O_{2max}$: 63.0 **±** 2.0mL.kg^{-1}.min^{-1} [both groups consisted of healthy, lean subjects]	Pre-exercise and immediately post, 3hr post and 24hr post	(Lehmann et al., 2010a)

^{1}H-NMR [5mm broadband inverse probe]	**Session 1-** (a) Progressive incremental $\dot{V}O_{2max}$ test, cycle ergometer 1. 4min warm-up @ 75W 2. Power output increased by 25W every min until v.e. (b) Four 6s sprints against increasing braking force (1st set 2kg then > by 1kg per sprint), 5min recovery after each sprint **Session 2-** 1. 30s sprint on cycle ergometer @ max speed, individually determined resistance 2. 90min rest in recumbent position **Session 3-** 1. 4min warm-up, 75W 2. 70% $\dot{V}O_{2max}$ until v.e. 3. 5min active recovery 4. 90min rest in recumbent position	**22, Female** [on oral contraceptive pills for 6 months] **Group 1- 9, 22.1 ± 0.6yrs,** untrained recreational exercisers **Group 2- 12, 21.8 ± 1.0yrs,** trained (6: judoists; 6: cyclists/triathletes)	Pre-exercise and 30min post each exercise session	(Enea et al., 2010)
^{1}H-NMR	**29wk Training Program** 1. 2wks general testing [2x 40km time trial, peak power output test, cycle ergometer] 2. 4wks detraining 3. 1wk general testing 4. 6wks POL or THR, 3days/wk 5. 1wk general testing 6. 4wk detraining 7. 1wk general testing	**12, Male, 37 ± 6yrs** One Group Cross Over Design-Trained road cyclists	[during general testing wk]	(Neal et al., 2013)

	8. 6wks POL or THR, 3days/wk 9. 1wk general testing Polarised training model (POL) training intensity: 80% low intensity, 0% moderate intensity, 20% high-intensity Threshold training distribution (THR) training intensity: 57% low intensity, 40% moderate intensity, 0% high-intensity			
HPLC **GC-MS** [ZB1701(Zebron) capillary column (30m x 250 µm(ID) x 0.15 µm film thickness]	**Session 1, 2-** $\dot{V}O_{2max}$ Graded exercise test on cycle ergometer until v.e. 1. 5-10min @ self-selected intensity 2. Test start at 100W and power > by 15W every 30s until v.e. [cadence kept at ≥60 r.p.m] **Session 3-** High-intensity interval training (HIIT) familiarisation 10 x min intervals @ power output corresponding to ~80% $\dot{V}O_{2max}$ within a 2min recovery @ 50W btw intervals. [Familiarisation conducted 48hr post 2nd $\dot{V}O_{2max}$ and used to determine duration required to complete the same work rate on a cycle with continuous power output ~65%$\dot{V}O_{2max}$ which is for the moderate-intensity continuous exercise (MOD)	**10, Male, 33.2 ± 6.7yrs** Well trained cyclists and triathletes [$\dot{V}O_{2max}$: 4.8 ± 0.3L/min]	Pre-exercise, immediately post-exercise, 1 and 2hrs post-exercise	(Peake et al., 2014)

	Session 4- randomly assigned to HIIT or MOD then reversed [at least 1wk post familiarisation and 7 day break between MOD or HIIT]			
UHPLC/MS/MS [method for acidic and basic feature identification] **GC-MS**	**Session 1-** Graded $\dot{V}O_{2max}$ test, treadmill **Session 2-** Day 1: 70% $\dot{V}O_{2max}$ for 2.5hr Day 2: *as above* Day 3: *as above*	**15, Male and Female, 19 – 45yrs** One Group- healthy trained competitive long distance runners	Pre-exercise, post-exercise on day 3 and 14hr post-exercise after recovery rest	(Nieman et al., 2013b)
UHPLC/MS/MS [method for acidic and basic feature identification] **GC-MS**	**Session 1-** Graded exercise test (25W increment every 2min, starting at 150W) **Session 2-** 75km cycling on pre-programmed mountainous course with moderate difficulty	**19, Male, 27- 49yrs** One Group- healthy competitive road racers	Pre-exercise, immediately post-exercise, 1.5hr post-exercise and 21hr post-exercise	(Nieman et al., 2014)
GC/TOF-MS [10m x 0.18mm i.d. fused silica capillary column]	**Session 1-** $\dot{V}O_{2max}$ test **Session 2-** Nutritional load (all subject) Blood samples collected in the fasting state (pre) and every fifteen minutes for 90 minutes following a nutritional load. The nutritional load consisted of 1g CHO, 0.2g proteins and 0.1g fat per kg body weight. **Session 3-** Exercise with or without nutritional load 65min strenuous ergometer cycling session with a workload related to their own $\dot{V}O_{2max}$ 1. 20min @ 55% $\dot{V}O_{2max}$	**27, Male** **Group 1-** 7, 28.16 ± 2.7yrs, High fitness (nutrition) with $\dot{V}O_{2max}$ 63.20 ± 2.93mL.kg^{-1}.min^{-1} **Group 2-** 6, 25.58 ± 1.77yrs, High fitness (water) with $\dot{V}O_{2max}$ 63.67 ± 2.8mL.kg^{-1}.min^{-1} **Group 3-** 7, 26.30 ± 5.30yrs, Low normal (nutrition) with $\dot{V}O_{2max}$ 44.57 ± 5.62mL.kg^{-1}.min^{-1} **Group 3-** 7, 24.04 ± 1.83yrs, Low normal (water) with $\dot{V}O_{2max}$ 42.71 ± 2.87mL.kg^{-1}.min^{-1}	For first exercise session: Pre-exercise, during resting (pre), immediately after nutritional load/water intake as well as every 15min min for the following 60min. For second exercise session: Pre- (during resting), immediately post- exercise and every 15min for the following 45min.	(Chorell et al., 2012)

	2. 25min @ 70% $\dot{V}O_{2max}$ 3. 10min @ 55% $\dot{V}O_{2max}$ 4. 2min all-out 5. 8 min @ 30% $\dot{V}O_{2max}$ 6. Given either nutritional load OR water 7. 6h recovery 8. Exercise session (as above) followed by nutritional load (only for subjects that were given nutritional load earlier)			
GC/TOF-MS	**Session 1-** $\dot{V}O_{2peak}$ test, cycle ergometer **Session 2-** 90min cycle [each 90min cycle consists of 9 x 10min equal sets] Sets of 10min each: 1. 2min @ 40% 2. 6min @ 60% 3. 2min @ 85% $\dot{V}O_{2peak}$ [**Session 2 completed in total 4 times**, 1wk apart permitted per session]	**24, Male, 25 ± 2.7yrs** One Group- healthy, regularly training	Pre-exercise and immediately post-exercise	(Thysell et al., 2012)
GC-MS	**Session 1-** $\dot{V}O_{2max}$ test, cycle ergometer 1. 3min warm-up, 2. 4min steady state workloads @ 15%, 35%, 55% and 75% (of estimated max power)	**65, Male and Female, 18 – 61yrs** Group 1- healthy high fitness Group2- healthy low fitness [groups created based on lower and upper quartiles of $\dot{V}O_{2max}$ results]	Pre-exercise sample	(Morris et al., 2013)

	[workloads estimated from theoretical $\dot{V}O_{2max}$]			
^{1}H-NMR [5mm triple resonance cryoprobe]	**18 months training program** incl. 3 x weekly exercise sessions on non-consecutive days. Each session should be a combination of multi-component progressive resistance training and weight bearing activities	**80, Male, Middle-aged, Healthy** **Group 1-** 20, exercise and calcium + vitamin- D_3 fortified milk **Group 2-** 20, exercise alone **Group 3-** 20, calcium + vitamin- D_3 fortified milk alone **Group 4-** Control [Emphasis made by authors between groups 1,2 *vs.* control and baseline samples from groups 1,2]	Pre-exercise (baseline) and 18 months post training program	(Sheedy et al., 2014)
UHPLC/MS/MS^{2} [method for acidic and basic feature identification] **GC-MS** [C18 Sep Pak column]	**Session 1-** Graded $\dot{V}O_{2max}$ test, treadmill **Session 2-** 2wks of supplementation with either polyphenol soy protein complex or placebo accompanied by normal training. **Session 3-** 3 days running bout exercise (2.5h/day on treadmill @ ~70% $\dot{V}O_{2max}$) accompanied by supplements.	**31, Male and Female, Competitive half and full marathon road racers** **Group 1-** 16, Male and Female, 33.7 ± 6.8yrs, intervention group **Group 2-** 15, Male and Female, 35.2 ± 8.7yrs, placebo group	Pre-day 1 exercise, post- day 3 exercise and 14hrs post-exercise at day 4	(Nieman et al., 2013a)
GC-MS [C18 Sep Pak column]	**Session 1-** Graded exercise test (25W > every 2min, starting at 150W) **Session 2-** 75km time trial (1wk post session1)	**14, Male, 18–45yrs** Competitive road cyclists	Pre-exercise and supplementation, immediately post- 75km time trial, and 1hr post-exercise	(Nieman et al., 2012)

	Randomly assigned to bananas OR 6% CHO **Session 3-** 75km time trial (3wk post session2) Reversed supplement conditions. [Prior to session 2 and 3 subjects ingested 0.4 g/kg carbohydrate from bananas OR from a standard 6% CHO. During exercise subjects ingested 0.2 g/kg body weight every 15min of bananas or CHO]			
GC/TOF-MS [10m x 0.18mm i.d. fused silica capillary column]	**Session-** $\dot{V}O_{2peak}$ **Session 2-** 90min of ergometer cycling Each 10min set repeated 9 times without rest: 2min @ 40%, 6min @ 60% and 2min @ 85% $\dot{V}O_{2peak}$ 1 of 4 beverages immediately post completion of the 90min cycling, each subject completed all 4 tests in a randomized order, ingesting 1 beverage per test. 100 mL of water was ingested as well as at +15, +30, and +60 min post- exercise Beverage 1- 5 dL water Beverage 2- low low CHO (1g CHO per kg body weight in water (16% w/v)) Beverage 3- high CHO (1.5g CHO per kg body weight in water (24% w/v))	**24, Male, 25.7 ± 2.7yrs** [$\dot{V}O_{2peak}$ 59.1 ± 7.3mL.kg^{-1}.min^{-1}]	Pre-exercise, immediately post and at 15, 30, 60 and 90min post-exercise	(Chorell et al., 2009)

	Beverage 4- low CHO-protein (1g CHO per kg body weight in water (16% w/v) + 0.5g protein per kg body weight)			

Abbreviations: $\dot{V}O_{2max}$: maximal oxygen consumption; CON: constant steady state exercise; HIE: high intermittent exercise; W: Watts; WR_{peak}: peak work rate; v.e.: volitional exhaustion; rpm: revolutions per minute; VT: Ventilator threshold; HR: heart rate; TT: time trial.* Articles have been presented in order of appearance. [a] triple quadrupole mass spectrometer operated in electrospray ionization mode using a turbo ion spray LC-MS interface. [b] No details given by authors regarding specific metabolomics-based technology and method. [c] No details given by authors regarding specific exercise testing protocol.

Table 1-4. Metabolite Details as Presented from the Exercise Metabolomics Literature and Identified Through a Metabolomics-based Technology.

Sample Tested (Plasma:P; Erythrocytes:E; Urine:U; Serum: S; Muscle Extract:ME; Saliva:Sa, Breath:B; Whole Blood:WB; Capillary Blood:CB)	**Metabolite Pathways** (↓: reduced post- exercise, ↑: increased post-exercise)	**Reference**
P and ME	Hypoxanthine, **↑ (P)** Xanthine, **↑ (P)** Urate, **↑ (P)** IMP ATP AMP	(Sahlin et al., 1999)
P and ME (*Vastus Lateralis*)	IMP, **↑ (ME)** Hypoxanthine, **↑ (P)** Urate, **↑ (ME)** Allantoin, **↑ (ME)**	(Hellsten et al., 2001)
P, U and ME (*Vastus Lateralis*)	ATP, **↑ (ME)** IMP, **↓ (ME)** Inosine, **↓ (P) and ↑ (U) 2 hour post-exercise** Hypoxanthine, **↓ (P) and ↑ (U) 2 hour post-exercise** Uric acid, **↓ (P) and ↑ (U) 2 hour post-exercise**	(Stathis et al., 2006)
P and E	Hypoxanthine (P) Xanthine (P) Uric Acid (P) HGPRT (E) *(Significant changes observed for each metabolite across the 1 year training cycle)*	(Zieliński et al., 2009)
P and E	Hypoxanthine (P) Xanthine (P) Uric acid (P) HGPRT (E) *(Higher anaerobic exercise training decreases post-exercise hypoxanthine and uric acid with an increase in HGPT- this relationship is reversed when athlete reaches transition phase)*	(Zieliński et al., 2011)
P and E	Hypoxanthine, **↑ (P) levels in group 1 vs. group 2 during rest and post-exercise across 1yr training program** Xanthine (P) Uric acid (P) HGPRT, **↑ (E) during rest with ↑exercise training intensity**	(Zielinski and Kusy, 2012)

P and E	Hypoxanthine **(P)**, **good predictor of fitness** Xanthine (P) Uric acid (P) HGPRT (E)	(Zieliński et al., 2013b)
P and E	Hypoxanthine (P), **Group1 continuous ↓ across three tests, Group2 ↓ in 1st and 2nd tests, Group 3 no change** HGPRT (E), **Group1 continuous ↑ across three tests, Group2 ↑ in 1st and 2nd tests, Group 3 no change**	(Zieliński et al., 2013a)
WB and E	ADP, **↓ (E) post 30min recovery** AMP, **↓ (E) post 30min recovery** IMP, **↑ (E) immediately post and ↓ (E) post 30min recovery** Inosine, **↓ (WB) post 30min recovery** Hypoxanthine, **↑ (WB) immediately post, ↑ (WB) post 30min recovery**	(Dudzinska et al., 2010b)
P	Hypoxanthine, **↑**	(Bianchi et al., 1999)
P and U	Hypoxanthine, **↑(P)** Uric acid, **↑(P&U)**	(Gerber et al., 2014)
P and U	Inosine, **↑ (P)** Hypoxanthine, **↑ (P) greater in allopurinol *vs.* placebo same in (U)** Xanthine, **↑ (U) greater in allopurinol *vs.* placebo**	(Stathis et al., 2005)
P	α- and γ-tocopherol, **↓ over training camp**	(Subudhi et al., 2001)
P	Uric Acid, **↑** Ascorbic acid Dehydroascorbate	(Chevion et al., 2003)
P	α- and γ-tocopherol β-carotene Lycopene Ascorbic Acid Malondialdehyde, **↑ in Group1, 2** Linoleic acid	(Ramel et al., 2004)
P and E	Allantoin, **↑(P&E)** Uric acid, **↑(P&E)** Malondialdehyde, **↑(E)**	(Kanďár et al., 2014)
U	Cortisol, **↑** Cortisone, **↑** Creatinine	(Gatti et al., 2005)
U	Total Cortisol Cortisone Tetrahydroderivatives Unconjugated Cortisol and Cortisone [tetra- hydrocortisol (THF) + alloTHF]/tetrahydrocortisone ratio, **↑**	(Dovio et al., 2010)
P	Norepinephrine, **↑ post-exercise vs. pre in Group1, 2 and ↓ post 24 hours recovery in Group1, 2**	(Giraldo et al., 2009)

	Epinephrine, ↑ **post-exercise vs. pre in Group2 and ↓ post 24 hours recovery in Group2**	
P	Xanthine, ↑ Hypoxanthine, ↑	(Degoutte et al., 2003)
U	Lipoxin Tetraene	(Gangemi et al., 2003)
P	Anandamide, ↑ **in runners and cyclists**	(Sparling et al., 2003)
P and E	Uridine, ↑	(Dudzinska et al., 2013)
P	**Group 1 (pulmonary artery (PA) and superior vena cava (SVC))** *steep instantaneous PA-to- SVC gradients at peak exercise:* Purine degradation products Span 2 TCA cycle intermediates *Differentially expressed:* Amino Acids (SVC & PA) Acetoacetate (SVC & PA) Glucose-6-phosphate (SVC & PA) **Group 2 (P)** Lactate, ↑ Pyruvate, ↑ Glycerol, ↑ Alanine, ↑ Glucose-6-phosphate, ↑ Acetoacetate, ↓ Pantothenate, ↑ **in more fit individuals** Methionine, ↑ **in less fit individuals** Glutamine, ↑ **in less fit individuals** **Group 3 (P)** Alanine, ↓ Threonine, ↓ Serine, ↓ Proline, ↓ Valine, ↓ Histidine, ↓ Glutamine, ↓ Asparagine, ↓ Glucose-6-phosphate, ↑ Glycerol, ↑ β-hydroxybutyrate, ↑ Allantoin, ↑ Kynurenate, ↑ Quinolinate, ↑ Anthranilate, ↑ Lactate, ↑ Pyruvate, ↑	(Lewis et al., 2010)
S	34 changing metabolites *[not presented in paper by authors]* Glycerol and Asparagine confirmed only	(Pohjanen et al., 2007)

S	Alanine, ↓ **post 2wks training Group 2** Pyroglutamic acid, ↓ **post 2wks training Group 1** β-D-methylglucopyranoside, ↑ **post 2wks training Group 2** Valine, ↓ **post 2wks training Group 2** Uric acid, ↓ **post 2wks training Group 2** Glutamine, ↓ **post 2wks training Group 2** Glutamic acid, ↓ **post 2wks training Group 2** Phenylalanine, ↓ **post 2wks training Group 1** Tyrosine, ↓ **post 2wks training Group 1** Threonic acid, ↑ **post 2wks training Group 2** Ornithine, ↓ **post 2wks training Group 1**	(Yan et al., 2009)
U	2-Hydroxyisovalerate, ↑ 2-Hydroxybutyrate, ↑ 2-Oxoisocaproate, ↑ 3-Hydroxyisobutyrate, ↑ 3-Methyl-2-oxovalerate, ↑ 2-Oxoisovalerate, ↑ Lactate, ↑ Alanine, ↑ Pyruvate, ↑ Citrate, ↓ 2-Oxoglutarate, ↑ Trimethylamine N-oxide, ↓ Glycine, ↓ Fumarate, ↑ Tryptophan, ↓ Hypoxanthine, ↑ Formate, ↓ Histidine	(Pechlivanis et al., 2010a)
S	**Elevated with training Group A:** 1. Methylguanidine 2. Citrate 3. Glucose 4. Taurine 5. Trimethylamine N-oxide 6. Choline-containing compounds 7. Histidines 8. Acetoacetate/acetone **Elevated with training Group B:** 1. Acetoacetate/acetone 2. Methylguanidine 3. Valine 4. Choline-containing compounds *Lactate and lipid metabolites lower post training in Group A > Group B*	(Pechlivanis et al., 2013)
U	**Information based on network analysis showing differentially expressed**	(Mukherjee et al., 2014a)

	metabolites between experimental and control groups (by Metacore): Carbohydrate metabolism 1. TCA and tricarboxylic acid [t.] 2. Pyruvate metabolism [p.] 3. Propionate metabolism [p.] D-Glucuronic acid [p.] Amino acid metabolism 1. L-Alanine [p.t.] 2. Glycine [p.t.] 3. L-Ornithine [p.t.] 4. Alanine, Glycine, Cysteine metabolism [t.] 5. Ala, Ser, Cys, Met, His, Pro, Gly, Glu, Gln metabolism [t.] Glycosphingolipid metabolism	
Sa	Urea Glucose Lactate Citrate Acetate Glycerol Glutamate Leucine Alanine Lysine Ornithine Myo-inositol Glutamate Creatine Choline Tyrosine Glutamine Aspartate	(Santone et al., 2014)
S	**Metabolites positively associated with $V\dot{O}_{2max}$:** Pyridoxate 2-oleoylglycerophosphoethanolamine 2-hydroxyisobutyrate Erythrulose Tryptophan Leucylleucine Glycerate 4-ethylphenylsulfate Pantothenate 4-vinylphenol 57ulphate Erythronate N-acetylornithine 7α-hydroxy-3-oxo-4-cholestenoate α-hydroxyisovalerate Ergothioneine Tryptophan betaine Phenyllactate Phenol 57ulphate	(Lustgarten et al., 2013)

	Indolelactate **Metabolites negatively associated with $V\dot{O}_{2max}$:** γ-tocopherol 5α-pregnan-3β,20α-diol disulfate Stearoyl sphingomyelin Pentadecanoate	
S	Lipoprotein 1. Particle concentration 2. Particle size Apolipoprotein Triglycerides Cholesterol Fatty acids 1. Omega-3 FA, **↑ in active persons Part1** 2. Docosahexaenoic acid, **↑ in active persons Part1, 3** 3. Omega-6 FA, **↓ in active persons Part3** 4. Omega-7,9 and saturated FA, **↓ in active persons Part1, 3, 4** 5. Monounsaturated FA, **↓ in active persons Part1, 3, 4** Metabolic substrates 1. Glucose, **↓ in active persons Part1, 2, 3** 2. Acetoacetate, **↓ in active persons Part1 and ↑ Part 3** 3. 3-hydroxybutyrate, **↓ in active persons Part1 and ↑ Part 4** 4. Acetate, **↑ in active persons Part3** 5. Citrate, **↑ in active persons Part2, 3, 4** 6. Lactate, **↑ in active persons Part4** 7. Pyruvate, **↓ in active persons Part3, 4** Amino Acids 1. Isoleucine, **↓ in active persons Part2, 3, 4** 2. Valine, **↓ in active persons Part1** 3. Phenylalanine, **↓ in active persons Part4** 4. Tyrosine, **↓ in active persons Part2, 4** Misc. 1. Creatinine, **↑ in active persons Part3, 4** 2. Urea 3. Albumin	(Kujala et al., 2013)

	4. a1-acid-glycoprotein, **↓ in active persons Part1, 3, 4**	
CB (earlobe)	**Increased connectivity in relation to physical activity:** 1. Lactate 2. Alanine 3. Acyl-carnitines (C2 and C3) **Other relations:** 1. Glycine	(Netzer et al., 2011a)
P and ME	Hippurate, **↑ (P)** C6:0 carnitine, **↑ (P)** C8:0 carnitine, **↑ (P)** C10:1 carnitine, **↑ (P)** C10:0 carnitine, **↑ (P)** C14:2 carnitine, **↑ (P)** C12:0 carnitine, **↑ (P)** Norepinephrine, **↑ (P)**	(Lehmann et al., 2010a)
U	Lactate, **↑** Alanine, **↑** Acetate, **↑** Pyruvate, **↑** Succinate, **↑** Hypoxanthine, **↑**	(Enea et al., 2010)
U (first pass)	Hippuric acid, **↓ post THR** Creatinine, **↑ post THR** Dimethylamine, **↑ post THR** 3-methylxanthine, **↑ post THR** Hypoxanthine, **↓ post THR**	(Neal et al., 2013)
P	Succinic acid, **↑ HIIT** Aconitic acid, **↑ HIIT** 4-methyl-2-oxopentanoic acid, **↑ HIIT** 3-methyl-2-oxopentanoic, **↑ HIIT** Alanine, **↑ HIIT** Glutamate, **↑ HIIT** Tyrosine, **↑ HIIT** Myristoleic acid, **↑ HIIT** Oleic acid, **↑ HIIT** Epinephrine, **↑ HIIT** Linolenic acid, **↑ MOD** Dodecanoic acid, **↑ HIIT & MOD** Decanoic acid, **↑ HIIT & MOD** Malonic acid, **↑ HIIT & MOD** Palmi- toleic acid, **↑ HIIT & MOD** Heptadecenoic acid, **↑ HIIT & MOD**	(Peake et al., 2014)
S	**Positive fold change across 75 metabolites:** Lipid and carnitine metabolism Amino acid and peptide metabolism Haemoglobin and porphyrin metabolism Krebs cycle intermediates	(Nieman et al., 2013b)
P	**80 Total Metabolites Across:** Lipid metabolism Carnitine metabolism Ketones	(Nieman et al., 2014)

	Dicarboxylate and long chain fatty acids 1. Linoleate, ↑ 2. Linolenate, ↑ 3. Dihomo-linolenate, ↑ 4. Arachidonate, ↑ 5. Adrenate, ↑ 6. Docosapentaenoate, ↑ 7. 13- and 9-hydroxy-octadecadienoic acid, ↑ 8. (Z)-9,10-dihydroxyoctadec-12-enoic acid and (Z)-12,13-dihydroxyoctadec-9-enoic acid, ↑	
P	**Response to Exercise:** Hypoxanthine, ↑ Taurine, ↑ Ribose, ↑ Beta-D- methylglucopyranoside, ↑ Inositol, ↑ Citric acid, ↑ Beta-alanine, ↑ Malic acid, ↑ Tryptophan, ↓ Threonine, ↓ Threonate, ↓ Valine, ↓ Isoleucine, ↓	(Chorell et al., 2012)
S	**Elevated post-exercise *vs.* pre:** 1. Fatty acids 2. Alanine 3. Inosine **Decrease post-exercise *vs.* pre:** 1. Asparagine 2. Lysine 3. Serine 4. Phenylalanine 5. Methionine 6. Arginine 7. Ornithine 8. Proline 9. Histidine 10. Allothreonine 11. Tryptophan 12. Valine 13. Isoleucine	(Thysell et al., 2012)
U (second void) and P	**Significant group and gender interaction (U) with fitness:** Cystathionione L-alanine Glycine Ethanolamine L-methionine L-aspartic acid Homocysteine	(Morris et al., 2013)

	Creatinine **↓ in high fitness group (P):** Branched chain amino acids *(also in urine)* Leucine Isoleucine Valine	
U (second void)	**Comparing baseline to 18 month exercise program (↑ post training):** 1. Acetate 2. Choline 3. Dimethylamine 4. Guanidinooacetate 5. Hypoxanthine 6. Lactate 7. Malonate 8. Methylamine 9. Methylmalonate 10. N,N-dimethylglycine 11. Trigonelline 12. Trimethylamine 13. Tryptophan Amino acid excretion 1. Isoleucine 2. Leucine 3. Lysine 4. Methionine 5. Tyrosine Creatinine, **↓ in exercise group *vs.* control**	(Sheedy et al., 2014)
S	12,13-hydroxyoctadec-9(Z)- enoate, **↑ Group1,2** Cortisol, **↑ Group1,2** Hippurate, **↑ Group1** 4-methylcatechol sulphate, **↑ Group1** 4-hydroxyhippurate, **↑ Group1** Cinnamoylglycine, **↑ Group1** 2-hydro- xyhippurate, **↑ Group1** 3-hydroxyhippurate, **↑ Group1** Catechol sulphate, **↑ Group1** O-methylcatechol- 61ulphate, **↑ Group1** Arabinose, **↑ Group1** Caffeine, **↑ Group1**	(Nieman et al., 2013a)
S	Dopamine, **↑ post- exercise in banana supplementation group** **Immediate response to exercise:** Palmitoleic Acid 2,3,4-Trihydroxybutanoic Acid Malic Acid Succinic Acid Palmitic Acid Oleic Acid Heptadecanoic Acid D-Fructose	(Nieman et al., 2012)

	2-Hydroxybutyric Acid L-Isoleucine L-Glutamic Acid 2-Aminobutyric Acid L-Methionine Pyruvic Acid L-Proline	
S	3-methylhistidine, **↓ with low CHO + protein** Pseudouridine, **↑ with low CHO + protein** 4-deoxyerythronic acid, **↑ with low CHO + protein** Glycine, **↓ with low and high CHO**	(Chorell et al., 2009)

Abbreviations: [t.]: transport; [p.]: pathway(s); HGPRT: hypoxanthine-guanine phosphoribosyl-transferase; ADP: adenosine 5' diphosphate; AMP: adenosine monophosphate; IMP: inosine monophosphate; ATP: adenosine 5' triphosphate; THR: threshold training distribution

1.6.1. Studies Focused on Purine Metabolism

Observations on the effects of exercise on purine metabolism go back over 40 years. Normally during exercise the energy expenditure is closely related to ATP utilisation. However, during high-intensity exercise the demand for ATP outstrips supply and this causes degradation of total adenine nucleotides [TAN = ATP + ADP (adenosine diphosphate) + AMP] via deamination of AMP (adenosine 5'-monophosphate) to form IMP (inosine monophosphate) which produces ammonia as a side product (Sahlin et al., 1999). The IMP can then be lost from the muscle cells in the form of hypoxanthine which accumulates in plasma. Yet, hypoxanthine can be salvaged by hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Several papers have observed that the hypoxanthine salvage process tends to be more efficient in trained individuals and that, along with other purine metabolites, its concentrations in blood can provide an indication of the effectiveness of a training regimen- that can also be illustrated by an improvement in the level of ATP re-synthesis (Hellsten et al., 2001). Following exhaustive exercise muscle urate levels were also shown to increase (Stathis et al., 2006). Interestingly, the non-enzymatic oxidation of urate results in the formation of allantoin which makes it possible to assess to what extent it is acting as an antioxidant.

Changes in purine metabolism were monitored in long-distance runners during their yearly training cycle (preparatory, competitive and transition phases). As training

progressed the levels of post-exercise xanthine decreased and the levels of the salvage enzyme HGPRT increased (Zieliński et al., 2009). Observing the effect of training load and structure this time in middle-distance runners, it was proposed that plasma hypoxanthine concentration during and after exercise was affected by several factors including: the intensity of the exercise, the rate of reconversion of hypoxanthine to IMP by HGPRT, conversion of hypoxanthine to IMP by erythrocyte HGPRT, the rate of extraction by the liver and the rate of excretion. The main outcome of the study was that training resulted in less excretion of hypoxanthine (Zieliński et al., 2011). The same authors then compared high level sprinters and triathletes and found that hypoxanthine levels were lower in the trained sprinters in comparison with the triathletes while HGPRT levels were higher in sprinters as compared to the triathletes (Zielinski and Kusy, 2012). This suggested a more effective use of anaerobic energy resources resulting from sprint training than from triathlon training. Interestingly, in a study comparing triathletes, sprinters, middle- and long-distance runners, low hypoxanthine levels post-exercise correlated with performance to a greater extent than lactate levels and aerobic capacity (Zieliński et al., 2013b). Finally, these differences in purine metabolism were observed in not only elite but in amateur and recreational runners over similar 1 year training cycle. The lowest hypoxanthine levels and the highest HGPRT levels were observed in the elite runners, significant changes in purine metabolism with time were only observed after using high-intensity exercise in their training schedules, there was no significant change following constant low intensity exercise (Zieliński et al., 2013a).

Erythrocytes are unable to synthesise purine nucleotides *de novo*. Measurement of adenine, guaninine nucleotides, inosine and IMP in erythrocytes post-exercise showed that AMP, ADP and IMP were decreased at 30 minutes post-exercise supporting the idea that they are taken up by muscle and used to re-synthesise ATP. Hypoxanthine levels were high in RBC immediately post-exercise but inosine levels were elevated at 30 minutes post-exercise indicating differences between the two metabolites in terms of rate of entry into blood (Bianchi et al., 1999; Stathis et al., 2005; Atamaniuk et al., 2010; Dudzinska et al., 2010a; Gerber et al., 2014).

1.6.2. Studies focused on Metabolites Relating to Oxidative Stress

Free radical production during exercise may be caused by a variety of mechanisms including a switch from xanthine dehydrogenase, which uses nicotinamide adenine nucleotide (H^+) as a cofactor, to xanthine oxidase, which uses oxygen as a co-factor, and increased cyclooxygenase activity. Tocopherols and ascorbic acid protect against oxidative stress as well as compounds produced by the body such as uric acid and glutathione along with protective enzymes (Ramel et al., 2004).

Oxidative stress in alpine skiers was evaluated after 1 day of training and 10 days of training. The markers of oxidative stress tocopherols, glutathione (GSH), malondialdehyde (produced from lipid oxidation), lipid peroxides and uric acid were determined. Differences in this compounds pre- and post-training were small (Subudhi et al., 2001). The effects of extreme exercise (monitored over a two week period as part of a 6 month training programme) were assessed with regard to the antioxidant capacity of plasma. The levels of reduced ascorbic acid, total ascorbate, and dehydroascorbate were not changed by the exercise programme but uric acid levels were increased. There was a 10 fold increase in the level of creatine phosphokinase into the plasma and also a 4 fold increase in aspartate transaminase, a characteristic marker of liver injury. Plasma protein carbonyl content, a marker of protein oxidative damage, decreased significantly during exercise (Chevion et al., 2003). The levels of anti-oxidants and lipid peroxidation products were measured before and after submaximal exercise in resistance trained and non-resistance trained males. α-tocopherol, γ-tocopherol, β-carotene, and lycopene (fat soluble antioxidants) concentrations increased after exercise. The concentrations of the lipid peroxidation products malondialdehyde and conjugated dienes were also elevated after exercise in both groups (Ramel et al., 2004). Plasma uric acid and allantoin as well as RBC malondialdehyde were measured in individuals following a short 10 minute run. It was found that uric acid and allantoin were elevated by exercise but there was no effect on plasma malondialdehyde, however, RBC malondialdehyde was elevated indicating potential lipid peroxidation at the RBC membrane during a strenuous challenge such as exercise (Kanďár et al., 2014).

1.6.3. Studies Focused on Steroid Metabolism

Hydrocortisone is responsible for maintaining homeostasis within the body when it adapts to stress (de Kloet et al., 2008) and thus in the short term its levels may become elevated as a result and give an indication of the adaptive response to stress. Cortisone is an inactive form of cortisol present in tissues which may be activated by conversion into cortisol. The levels of cortisol and cortisone were assessed post-race in elite cyclists and were found to be significantly increased post-competition (Gatti et al., 2005). Exercise was found to increase the ratio of tetrahydrocortisol to tetrahydrocortisone, indicating increased generation of cortisol from cortisone at the tissue level which may be part of the adaptive response to exercise (Dovio et al., 2010).

In sedentary women cortisol levels decreased following both moderate and intense exercise and returned to normal 24 hours in recovery, thus the levels determined may depend on the duration of exercise as well as when the sample was taken (Giraldo et al., 2009). In addition, in this group, norepinephrine levels in serum were increased post-exercise whereas epinephrine levels were only increased by intense exercise. Estradiol levels were also shown to be elevated only by intense exercise (Giraldo et al., 2009). There has also been a long standing interest in levels of plasma androgens because of their influence on the anabolic response in muscle and bone post- exercise (Pritchard et al., 1999). Androgens were observed to increase in response to exercise whereas cortisol only increased after exercise. Endurance-trained subjects showed less pronounced changes in hormone concentrations in response to exercise than resistance-trained subjects. It would seem that the endogenous steroid hormone profile of men depends more on exercise mode or intensity than exercise volume as measured by caloric expenditure (Tremblay et al., 2004).

1.6.4. Miscellaneous Targeted Studies

The effects of a judo match on levels of selected plasma metabolites were determined at 3 minutes, 60 minutes and 24 hours post- match. The levels of glycerol, triglycerides and free fatty acids increased 3 minutes post-exercise although this might have been due to the subjects of the study having a low carbohydrate diet. Cholesterol levels were

also elevated post-exercise. Serum creatinine levels rose post-exercise and this was attributed to a reduction in glomerular filtration. There was a rise in ammonia post-exercise which might be attributed to the production of xanthine and hypoxanthine but could also be attributed to protein breakdown. Levels of uric acid were elevated but not until 60 minutes after the match. At 60 minutes post-exercise the levels of glycerol, triglycerides and free fatty acids had decreased but remained higher than the resting values. This suggested that lipolysis was participating in the re-synthesis of muscular glycogen stores. Apart from uric acid and urea, the levels of the remaining markers had returned to baseline by 24 hours (Degoutte et al., 2003). Furthermore, the effect of physical activity on urinary lipoxins (derived from eicosapentenoic acid) were measured and found to be elevated after exercise. These compounds are anti-inflammatory and lipoxin production may counterbalance inflammatory mediators produced by exercise (Gangemi et al., 2003). The endogenous cannabinoids anandamide and 2-arachindonylglycerol were measured in plasma following exercise and there were marked elevations in these compounds in both runners and cyclists post-exercise (Sparling et al., 2003). Finally, uridine levels in blood were measured after exercise and were found to correlate with increased levels of insulin and glucose in the blood. It was proposed that the increased levels of insulin post-exercise could be due to increased secretion of catecholamines. Insulin resistance of subjects was increased post-exercise and it was suggested, on the basis of a number of previous studies, that uridine has a role in insulin resistance (Dudzinska et al., 2013).

1.6.5. Untargeted or Semi-targeted Metabolomics Studies

The untargeted studies are reported in some detail below since it is difficult to observe clear themes due to the diverse nature of protocols and outcome of the studies themselves. So rather than miss what might be an important observation the findings for each paper have been summarised. In the discussion/ conclusion we have attempted to draw together some of these themes.

The study by Lewis et al. (2010) is by far the most comprehensive in the literature and involved around 500 subjects, although 302 of the plasma samples were pre-existing and from the Framingham Heart Study. Plasma samples were either spot samples or

several blood samples were taken over a short period of time using an in-dwelling catheter. The study utilised targeted mass spectrometry methods so that any metabolite outside the panel of 200 selected metabolites were not monitored. The metabolites which changed following exercise highlighted the molecular pathways that might modulate the beneficial effects of exercise (Lewis et al., 2010). This study was very detailed and the various observations are summarised below for the different exercise regimens which are detailed in table 1-3. In group two twenty three metabolites were significantly changed at the peak exercise time. There were the expected elevations in purine metabolites, lactate and pyruvate (glycolysis), glycerol (lipolysis) as well as alanine and glutamine (gluconeogenesis). The TCA cycle intermediates malate, succinate and fumarate were elevated and the glycolysis intermediates glucose-6-phosphate and 3-phosphoglycerate were also elevated. Nicotinamide (a tryptophan metabolite) which is known to enhance insulin release was elevated in this group post-exercise. Citrulline, which is a marker of nitric oxide formation, fell post- exercise and was present to a lesser extent in the plasma of fitter individuals. Pantothenic acid, which is required for CoA biosynthesis, was elevated post-exercise and was closely correlated with fitness. Allantoin which results from the oxidation of uric acid was lower post-exercise. In group 1, the metabolic changes were similar to those in group 2 with the change being greater in the pulmonary artery compared to superior vena cava plasma suggesting that the metabolic changes derive mainly from the heart muscle or from the lower limbs. Finally, group three measurements were taken after a 26.2 mile Boston marathon and marked elevations in glycerol and β-hydroxybutyrate were consistent with extensive lipolysis and ketone body production, respectively. In contrast to the observations after acute exercise endurance exercise was followed by a reduction in the gluconeogenic amino acids alanine, threonine, serine, proline, valine, histidine, glutamine and asparagine. An unexpected observation was an increase in the tryptophan metabolites kynurenate, quinolinate, anthranilate (Lewis et al., 2010).

The effect of strenuous exercise on metabolites in human serum were studied in healthy regularly trained males. Analysis was carried out by GC-TOF and followed by data extraction and multivariate statistics. Thirty four metabolites were affected by exercise although only glycerol and asparagine were reported as being fully characterised (Pohjanen et al., 2007). Changes in the metabolome of rowers

undergoing training were investigated by using GC-TOF. Samples were taken after 1 week and 2 weeks of training. The metabolic profile of serum from professional rowers was compared with a control group. Compared with control subjects, serum of professional rowers had higher levels of alanine, lactate, cysteine, glutamic acid, valine, glutamine, and lower levels of citric acid, palmitic acid, linoleic acid and oleic acid. Alanine and tyrosine were lowered by training after both the first and second week. Pyroglutamic acid, threonic acid, glutamic acid, phenylalanine and ornithine were stable in the first week but declined after the second week. Palmitic, oleic and linoleic acid were elevated after the first week of training but returned to normal at the end of the second week. Higher levels of fatty acids in rowers compared with controls were consistent with increased energy demand as was the elevation of fatty acids after the first week of training. Alanine was higher in rowers than in the control which might be consistent with its role in the alanine-glucose cycle which promotes gluconeogenesis (Yan et al., 2009).

Human urine samples were analysed by NMR pre- and post-exercise. Five metabolites of branched chain amino acids (BCAAs) were increased post-exercise and lactate and pyruvate were greatly increased. Increases were also observed in alanine, inosine, hypoxanthine, 2-oxoglutarate and fumarate. Glycine, tryptophan, histidine, trimethylamine N-oxide, citrate and formate were all decreased post-exercise. The observations on the purine metabolites reflected previous work. There was no change in the ketone bodies, acetoacetate and 3-hydroxybutyrate post-exercise and this suggested that there was no effect of exercise on the metabolism of fatty acid degradation. This as well reflects earlier work where it was observed that fat metabolism decreased with an increase in exercise intensity and there was an increase in reliance on glycogen to supply energy (Pechlivanis et al., 2010b). Additionally, it was proposed that the increase in branched- chain 2-oxo acids reflected their overproduction through transamination of BCAAs which exhibited increased turnover during exercise as a result of increased proteolysis.

An NMR-based study investigated the serum metabolic effect before and after two different training programmes (table 1-3 for details). The metabolites which separated pre- and post-exercise were as follows: lactate and pyruvate which were highly

elevated and leucine, valine, isoleucine as well as arginine/lysine which were lowered. Post 8 weeks of training the main metabolites discriminating the pre- and post-training samples were: methylguanidine, citrate, glucose, taurine, trimethylamine N-oxide, choline-containing compounds, histidine, 1-methylhistidine, 3-methylhistidine, and acetoacetate/acetone which were all elevated and lactate, pyruvate and lipids in general were all decreased post-training. The effect of exercise post-training was marked by increases in methylguanine and choline containing compounds and decreases in lactate and lipids. Thus the effects of training and exercise did not map strongly to particular pathways as was the case in the paper on urine by the same authors (Pechlivanis et al., 2013).

In an NMR study investigating the effects of high-intensity exercise, urine samples were collected 24 hours before an exercise session and for 24 hours post. The subjects were divided into trained and untrained groups. The products of glycolysis/ TCA cycle lactate, malonate and fumarate were all elevated post-exercise. As expected hypoxanthine was markedly elevated post-exercise. Acetate was elevated as well and its formation might be linked to the utilisation of pyruvate as an anti-oxidant (Mukherjee et al., 2014a). It is likely in this study that some of the acute effects of exercise were missed through the collection of 24 hour urine samples post-exercise.

NMR was used to profile metabolic changes in saliva post-exercise and link them to performance in soccer players. Tyrosine, inositol, creatine, lysine, citrate, glucose, acetate and arginine, lactate and glutamate were highlighted as being related to performance. The study indicated that salivary metabolomics using NMR was a viable technique for such studies (Santone et al., 2014). In another study metabolomic profiling was used in order to identify markers of aerobic capacity. Blood was sampled pre- exercise and then a $\dot{V}O_{2max}$ test was performed. The markers which correlated most closely with $\dot{V}O_{2max}$ were 4-ethylphenylsulfate, tryptophan, γ-tocopherol, and α-hydroxyisovalerate (Lustgarten et al., 2013). Elevated plasma tryptophan levels had been previously associated with lowered endurance however in this study they were positively correlated with increased endurance. Investigating the metabolite profiles (via NMR) of same sex twins where only one of the twins was physically active highlighted numerous differences. These differences between persistently physically

active and inactive individuals' metabolomes indicated better cardio-metabolic health in the physically active twin (Kujala et al., 2013).

A targeted MS/ MS approach (although methodological details were not provided) was used to monitor 60 metabolites in blood from subjects undergoing an exercise test. The data was evaluated by using a computational approach and lactate, alanine, glycine and acetyl and propionyl carnitines were identified as having the strongest correlation to the effects of exercise (Netzer et al., 2011a). An untargeted high resolution LC-MS was used to examine the effect of exercise on the plasma metabolome. The major effect observed was an elevation of C8:0, C10:0 and C12:0 carnitines. It was proposed that the elevation of these acyl-carnitines might be promoting beta-oxidation of palmitate through the transport of acyl-carnitines into muscle cells (Lehmann et al., 2010b).

The urinary metabolome of women divided into trained and untrained groups was examined pre- and post-exercise. The major changes were found to be in the following metabolites: lactate, pyruvate, alanine, β-hydroxybutyrate, succinate, acetate, and hypoxanthine. These metabolites were increased in both trained and untrained groups. The metabolites largely related to glycolysis and purine metabolism (Enea et al., 2010). Two different training regimens were investigated by using NMR to profile metabolites in the urine of trained road cyclists. Their metabolic profiles did not discriminate between the training regimens. The metabolites changed following training were hippuric acid (decreased) post threshold training distribution, creatinine (increased), dimethylamine (increased) 3-methylxanthine (increased) and hypoxanthine (decreased) (Neal et al., 2013).

A targeted GC-MS metabolomic analysis was used to assess changes in tricarboxylic acid (TCA) intermediates, fatty acids, and amino acids in a comparison of the effect of high-intensity interval exercise (HIIT) and continuous moderate-intensity exercise (MOD). Twenty nine metabolites were found to change significantly after exercise. Eleven of these changed after both HIIT and MOD and thirteen only changed after HIIT. The TCA cycle intermediates citric acid, succinic acid, aconitic acid and malonic acid all increased after HIIT. Leucine and isoleucine metabolites, 4-methyl-2-oxopentanoic acid and 3-methyl-2-oxopentanoic acid were increased after HIIT. Only

succinic acid increased significantly after MOD. Most amino acids did not change after HIIT or MOD apart from alanine, glutamate and tyrosine, which increased after HIIT. During recovery from exercise valine, isoleucine, methionine, alanine and proline all decreased below pre-exercise levels and this was most marked following MOD. A number of fatty acids and particularly non-essential fatty acids were also elevated post-exercise. The approach could be used to design exercise protocols for maximum fat oxidation (Peake et al., 2014).

The changes in the human serum metabolome produced by a 3 day period of intensified training were investigated by using GC-MS and LC-MS with compound identification based on 2800 authentic standards. Immediately following the 3-day exercise period, significant 2-fold or higher increases in 75 metabolites were observed with all but twenty two of these metabolites related to lipid/carnitine metabolism. Thirteen were related to amino acid/ peptide metabolism, four to haemoglobin/ porphyrin metabolism, and three to Krebs cycle intermediates. After a 14 hour overnight recovery period, 50 of the 75 metabolites remained elevated. Significant decreases were observed in 22 metabolites which were primarily related to lysolipid and bile acid metabolism and all but 4 of these were still decreased after 14 hour rest recovery (details in table 1-2). The multiple signatures of fatty acid beta-oxidation, omega oxidation, amino acid oxidation, and glycolysis indicate that during the 3 day exercise period the runners utilized multiple fuel types and oxidative pathways simultaneously. Novel findings from this study include the influence of exercise on 12,13 hydroxyoctadec-9-(Z)-enoate (HOME) and 9-hydroxy-10,12-octadedadienoic acid which were increased and are indicators or the formation of reactive oxygen species and also HOME acts as an inhibitor of neutrophil respiratory burst; enhanced omega oxidation causing an elevation in long chain dicarboxylic acids and decreases in phosphatidyl ethanolamine lipids as well as bile acids (Nieman et al., 2013b). Following on from this study, oxidation products of linoleic acid were investigated in athletes after a 75 kilometre cycling bout. A total of 80 metabolites had an increase of twofold or greater following exercise. The levels of several long chain fatty acids were increased in plasma and this study there was a particular interest in 9- and 13-hydroxyoctadecadienoic (HODE) acids. These compounds were elevated post-exercise along with several other hydroxy acids. The increase in HODE acids could be

correlated with an increase in F_2 isoprostanes, and they have potential as markers of oxidative stress during exercise (Nieman et al., 2014).

GC-TOF/MS was used to characterize plasma samples from 27 individuals divided into two groups based on physical fitness level. Multi- and univariate analysis between group comparisons based on 197 metabolites were carried out in samples collected at rest prior to any intervention and following exercise. Fitness was associated with decreased levels of γ-tocopherol and increased levels of α-tocopherol. In addition, a high fitness level was associated with elevated levels of docosahexanoic acid and decreased levels of C18 and C16 fatty acids. Following exercise there were increases in hypoxanthine and taurine and decreases in tryptophan, threonine, valine and isoleucine (Chorell et al., 2012). The effect of exercise on metabolites in plasma from healthy subjects was monitored by using GC- TOF/ MS. The main changes were elevation of fatty acids and hypoxanthine post-exercise and falls in levels of amino acids including BCAAs (Thysell et al., 2012).

The relationship between fitness and amino acid profile was investigated in two groups classified as being either fit or unfit. Exercise was used to classify the subjects but did not precede sampling. The group of fit subjects had lower levels of excreted amino acids and in particular higher levels of BCAAs were excreted in the unfit group. Finally, the group of fit subjects exhibited an increased rate of fat oxidation during exercise (Morris et al., 2013).

1.6.6. Exercise Studies Coupled with Supplementation

In healthy middle-aged males a ^{1}H-NMR study was used to examine the metabolomic changes involved with an 18 month training programme and with supplementation with calcium and vitamin D. The design was not set-up to measure the acute effects of exercise but rather measured the long term effects of an exercise programme combined with supplementation. There were no distinct changes in the urinary metabolome in response to calcium and vitamin-D3 supplementation however. Exercise produced marked changes in the urinary metabolites. Creatinine was reduced indicating perhaps greater conservation of creatinine as a result of training. Choline, dimethylamine,

guanidinoacetate, hypoxanthine, lactate, malonate, methylamine, methylmalonate, N,N-dimethylglycine, trigonelline, trimethylamine and tryptophan were all elevated after the 18 months training regimen. Of particular interest was the increase in urinary choline, guanidinoacetate and hypoxanthine, in conjunction with the decrease in the urinary creatinine observed in exercise-trained participants. These metabolites point to a metabolic adaptation for utilization of anaerobically derived ATP biochemical pathways (Sheedy et al., 2014).

Nieman et al. (Nieman et al., 2013a) investigated long-distance runners that were randomly assigned to either a placebo or an intervention group. The intervention group was given water soluble polyphenols from blueberry and green tea extracts which were captured onto a soy protein for 17 days. For the final 3 days of the supplementation they underwent a 3 day running exercise. The main effect of the polyphenol supplementation was on levels of microbiome metabolites e.g. hippurate, 4-hydroxyhippurate, 4-methylcatechol sulphate were all elevated. Fatty acid oxidation and ketogenesis were induced by exercise in both groups, with more ketones at 14 hour post- exercise in the group given the supplement. Established biomarkers for inflammation and oxidative stress did not differ between the groups. The main conclusions were that the polyphenol supplementation did not alter established biomarkers for inflammation and oxidation but was linked to an enhanced gut-derived phenolic signature and ketogenesis in runners during their recovery period (Nieman et al., 2013a). Another study by Nieman et al. (2012) studied the effect of ingesting bananas versus a carbohydrate drink (CHO) on cycling performance and post-exercise inflammation, oxidative stress and innate immune function. Analysis of blood indicated that glucose levels and performance did not differ between the two treatments. Fifty six metabolites were affected by exercise, and only one (dopamine) had a pattern of change that differed between bananas and CHO and this could be attributed to the presence of dopamine in bananas. Of the top 15 metabolites changed by exercise, five were related to liver glutathione production, eight to carbohydrate, lipid, and amino acid metabolism, and two were tricarboxylic acid cycle intermediates. Banana and CHO ingestion during 75 km cycling resulted in similar performance, blood glucose, inflammation, oxidative stress, and innate immune levels. Thus there

were no marked differences produced by the different supplements (Nieman et al., 2012).

Subjects underwent four identical exercise test-sessions, including ingestion of one of four beverages (water, low-carbohydrate beverage, high carbohydrate beverage, and low- carbohydrate- protein beverage (LCHO-P) immediately after cycling. Blood was collected at six time points, one pre- and five post- exercise. Extracted blood serum was analysed by GC-TOF/MS. Each of the four beverages taken after exercise produced different systemic patterns. In general, a metabolic marker pattern of a catabolic state, such as increased level of fatty acids and decreased levels of amino acids and sugars, was observed to be related to the intake of water in the recovery phase. In contrast, an apparent pattern associated to an anabolic state was detected following intake of LCHO-P which increased amino acid levels and also produced an enhanced insulin response. An interesting marker of the effect of the LCHO- P drink was elevated pseudouridine which was attributed to increased RNA turnover that was a consequence of an increase in anabolic processes triggered by the availability of the amino acids in the drink and the increased insulin response it provoked. In addition, the LCHO-P drink produced reduced levels of 3- methylhistidine which is a marker for myofibrillar catabolism. LCHO-P was shown to improve the metabolic status of less fit subjects in the recovery phase and the authors showed the potential for the methodology to be used for detection of early signs of insulin resistance (Chorell et al., 2009).

1.6.7. Discussion of Results from Metabolomics-based Studies

In the untargeted studies described above there are some clear metabolic themes which emerge, however there is also a mismatch in consistency and volume of observations across the selected studies. This makes it difficult to order the papers according to these clear themes and at the same time indicates that the exact study design may have an influence on what is being observed. For instance, the time of sampling post-exercise, the type of exercise, the fitness of the participants and the analytical method utilised will all influence the markers- some to a greater or lesser extent. Therefore, in

order to group these observations, generalised prominent metabolite responses have been targeted and explained below in detail.

The maximum rate of ATP re-synthesis from fatty acids is about 0.40 mol.min^{-1}, while anaerobic breakdown of glycogen can generate from 1.0 to 2.0 mol of ATP min^{-1} (Zajac et al., 2014). Thus during high-intensity exercise, the rate of ATP breakdown is too high to be matched by the rate of ATP synthesis from fatty acids. With prolonged exercise glycogen stores become depleted and there is a greater reliance on fatty acid metabolism to supply energy (Spriet and Watt, 2003). Interestingly, ATP level in the resting untrained human skeletal muscle is between 20- 25 mmol.kg^{-1} dry mass and this can be reduced by 20 % with intense sprint training (Stathis et al., 2006).

As might be expected increases in the end products of glycolysis, pyruvate, lactate and acetate following exercise are often observed and these are more likely to increase following intense exercise and in individuals who are not trained (Yan et al., 2009; Enea et al., 2010; Lewis et al., 2010; Pechlivanis et al., 2010a; Netzer et al., 2011b; Nieman et al., 2012; Kujala et al., 2013; Mukherjee et al., 2014b; Santone et al., 2014; Sheedy et al., 2014). Linked to glycolysis are the metabolites within the Krebs cycle and many studies found that these metabolites were elevated post-exercise (Enea et al., 2010; Lewis et al., 2010; Pechlivanis et al., 2010a, 2013; Chorell et al., 2012; Kujala et al., 2013; Peake et al., 2014; Santone et al., 2014; Sheedy et al., 2014). The generation of ATP from the Krebs cycle requires oxygen in the terminal respiratory chain hence during exercise there is, to a varying degree, a switch towards anaerobic glycolysis. Krebs cycle metabolites are more likely to be most elevated in trained individuals who have developed an increased aerobic capacity and who are able to oxidise pyruvate more efficiently. Another mechanism which is important in maintaining the glucose supply to muscles is gluconeogenesis, which takes place in the liver where lactate is converted to pyruvate which is then used to re-synthesise glucose. This process is energetically expensive but serves in the short term to maintain the glucose supply to muscles and thus the rapid energy supply from glycolysis can be prolonged, to some degree. It has been observed that endurance trained athletes have more efficient re- utilisation of lactate both for oxidative metabolism and gluconeogenesis (Emhoff et al., 2013). Levels of alanine and glutamine were found

to be elevated following exercise in several studies (Enea et al., 2010; Pechlivanis et al., 2010a; Netzer et al., 2011b; Chorell et al., 2012), however, in other studies they were found to be lowered following either exercise or training (Lewis et al., 2010; Nieman et al., 2014). Lewis *et al* (Lewis et al., 2010) observed elevated glutamine and alanine after acute exercise and lowered levels after endurance exercise, and it would also appear that glutamine and alanine levels fall after prolonged training (Yan et al., 2009; Lewis et al., 2010; Campbell et al., 2014). The release of large amounts of glutamine and alanine from skeletal muscle post-exercise has been known for a long time (Chang and Goldberg, 1978; Nurjhan et al., 1995). Alanine and glutamine are the most important gluconeogenic amino acids and alanine is formed in the alanine cycle via conversion of lactate to pyruvate followed by transamination with glutamine as the co-factor (Chang and Goldberg, 1978). Both glutamine and alanine are effective precursors for gluconeogenesis in the liver although the kidney can also synthesise glucose and in this case only glutamine is an effective precursor (Nurjhan et al., 1995). Lactate itself is used for gluconeogenesis and the advantages of releasing alanine to serve as a substrate in this pathway may be that it effectively transports both lactate and ammonia out of the muscle. Large amounts of ammonia are produced in muscle from the deamination of AMP (Sahlin et al., 1999) and glutamine and alanine formation might provide a mechanism for its removal. BCAAs are known to be the source of ammonia used for alanine and glutamine synthesis in muscle (Holecek, 2002). The lower level of alanine and glutamine release from muscle observed in endurance athletes is consistent with a lower requirement for gluconeogenesis resulting from a better balance between oxidative metabolism and glycolysis. However, it is also consistent with less build- up of ammonia from AMP deamination due to more efficient purine salvage in trained individuals (Sahlin et al., 1999; Hellsten et al., 2001; Stathis et al., 2006; Zieliński et al., 2009, 2011, 2013a, 2013b; Zielinski and Kusy, 2012). In addition, in trained athletes glutamine would be required to convert IMP back to AMP and would be thus less available for pyruvate transamination or for release from the muscle to promote gluconeogenesis.

A strong theme running through many papers is the metabolism of the branched chain amino acids valine, leucine and many papers either observed decreased levels of BCAAs or increased levels of the keto- or hydroxy acids resulting from their

transamination or both. Several papers observe changes in BCAAs and their metabolites (Lewis et al., 2010; Pechlivanis et al., 2010a; Chorell et al., 2012; Kujala et al., 2013; Lustgarten et al., 2013; Morris et al., 2013; Santone et al., 2014; Sheedy et al., 2014). This phenomenon has been attributed to the breakdown of protein in muscles followed by metabolism of the amino acids released (Pechlivanis et al., 2010a). However, essential amino acids, particularly leucine, are also involved in stimulating biosynthesis of muscle protein (Koopman et al., 2005; Sharp and Pearson, 2010) so there may be two processes occurring simultaneously, oxidation of BCAAs released by protein breakdown to keto- and hydroxyacids and uptake of BCAAs from plasma for muscle repair. Hydrocortisone and testosterone metabolism control of muscle proteins and the effects of the two steroids are to some extent antagonistic with hydrocortisone promoting muscle protein catabolism (Bergman, 2013) and testosterone promoting synthesis of muscle protein (Peckett et al., 2011). Previous work has observed that supplementation with BCAAs promotes the formation of muscle protein and increases plasma levels of testosterone relative to hydrocortisone (Sharp and Pearson, 2010). As discussed above both hydrocortisone and testosterone levels have been observed to increase post- exercise (Pritchard et al., 1999; Tremblay et al., 2004; Giraldo et al., 2009; Dovio et al., 2010) so it might be expected that both catabolism and anabolism would be observed. Other essential amino acids required for muscle protein biosynthesis such as phenylalanine, tyrosine, histidine, lysine and tryptophan have been observed to fall post-exercise as well (Lewis et al., 2010; Kujala et al., 2013; Campbell et al., 2014) and keto- and hydroxy acids derived from these amino acids have also been observed to increase (Lustgarten et al., 2013).

Several papers observed an increase in the levels of fatty acids post-exercise (Nieman et al., 2012; Thysell et al., 2012; Kujala et al., 2013; Morris et al., 2013; Peake et al., 2014) in addition elevated glycerol has also been observed in several studies which is consistent with increased lipolysis (Pohjanen et al., 2007; Yan et al., 2009). Increased lipolysis is consistent with the observation that hydrocortisone levels have been observed to increase post-exercise (Giraldo et al., 2009; Dovio et al., 2010) since it is widely accepted that hydrocortisone promotes the synthesis of lipases (Peckett et al., 2011; Bergman, 2013) which breakdown triglycerides. Fatty acid metabolism is promoted to a greater extent in endurance athletes (Lewis et al., 2010; Nieman et al.,

2013b) with depletion in glycogen reserves promoting a switch to fat metabolism and resulting in ketogenesis and oxidative metabolism. The observation of elevated fatty acid levels may again depend on exactly when post- exercise samples are taken. Romijn *et al* observed that fatty acid release into plasma decreased with increasing exercise intensity (Romijn et al., 1993) and fatty acid levels in plasma increased greatly during the minutes following a high-intensity exercise session. This is in line with the idea that an increase in oxidative metabolism occurs post- exercise where the NADH and flavin adenine dinucleotide produced by fatty acid β- oxidation can be used to produce ATP via the terminal respiratory chain. Tissues utilise ketone bodies (acetoacetate, β-hydroxybutyrate and acetone) as an energy source which are produced by partial metabolism of fatty acids in the liver (Fanelli et al., 1993). Several studies observed an increase in ketone bodies in plasma after exercise (Lewis et al., 2010; Nieman et al., 2012, 2013b; Morris et al., 2013; Pechlivanis et al., 2013). Unlike glycolysis oxidative metabolism requires a supply of CoA and two studies observed increased levels of the CoA precursor pantothenic acid in plasma following exercise and also observed that it was positively correlated with fitness (Lewis et al., 2010; Lustgarten et al., 2013). Pantothenate (vitamin B5) can be obtained from the diet but is also provided by intestinal bacteria. CoA is present in the mitochondria and in order for fatty acids to undergo oxidation they have to be transported into mitochondria in the form of their carnitine esters. It has been proposed that increased levels of pantothenate might indicate increased requirement for CoA but it is more likely that this indicates inhibition of CoA biosynthesis which is tightly regulated by pantothenate kinase (PK) activity (Dansie et al., 2014). Acetyl CoA inhibits PK by allosteric binding and an increase in pantothenate might indicate that in fitter individuals during exercise there is greater utilisation of oxidative metabolism via the Krebs cycle producing higher levels of acetyl CoA in comparison to individuals relying more on glycolysis for energy production. Related to observations on fatty acid metabolism there were a number of observations where acyl-carnitines were increased following exercise (Oberbach et al., 2011; Kujala et al., 2013; Lustgarten et al., 2013; Neal et al., 2013). The role of acyl-carnitines is ambiguous since on the one hand they transfer fatty acids into mitochondria for oxidation and on the other they remove fatty acids from the mitochondria. In addition to transferring fatty acids into the mitochondria for oxidation

the acyl-carnitines promote PK activity thus increasing CoA levels in the cytosol (Dansie et al., 2014). It is more likely that the range of acyl-carnitines which are elevated following exercise is due to removal of partially oxidised fatty acids from mitochondria so that efficient oxidation of pyruvate can occur. The preferred substrates for oxidation in mitochondria are long chain acids and, for instance, Lehmann et al (Lehmann et al., 2010b) report that medium to short chain acyl-carnitines are elevated following exercise and speculates that they act as signalling molecules stimulating β-oxidation. There might be greater likelihood that they indicate removal of partially oxidised acyl moieties from acyl CoAs, under conditions where the rate of glycolysis is high, in order to allow pyruvate to form acetyl CoA and enter the Krebs cycle (Lehmann et al., 2010b).

Increases in the levels of gut microbial metabolites following exercise are unexpected and have been reported by four papers although not exactly the same metabolites have been reported in each case (Lustgarten et al., 2013, 2014; Nieman et al., 2013a; Campbell et al., 2014). This in turn might have biological effects because for instance indoxyl sulphate and cresol sulphate are uremic toxins (Davila et al., 2013a). Effects on tryptophan metabolism are also less expected and were observed by two papers which reported elevation of metabolites in the kynurenine pathway increasing following endurance exercise (Lewis et al., 2010; Nieman et al., 2013b). The kyurenine pathway is a source of nicotinamide which is used for the synthesis of NADH for which there is a high demand particularly in the recovery phase of exercise where oxidative metabolism is restored. Thus this might be an explanation. However, another possibility is that mononuclear cells, which produce the tryptophan degrading enzyme indoleamine 2,3- dioxygenase, act to modulate the immune response of T-cells by depriving them of tryptophan thus preventing them from proliferating (Moffett and Namboodiri, 2003). Such a response could be aimed at modulating the inflammatory response to tissue damage occurring during exercise.

The major metabolic effects of exercise are discussed above but as can be seen in table 1-2 there are many other metabolites that have not been explained in the discussion above. One thing is clear exercise has a much larger impact on the metabolome than disease and must be normalised for in human metabolomics studies. The purine

pathway story is by far, however, the most well-cited and persistent finding which dates back over 40 years. These findings highlight that the adenine nucleotide metabolites which have been shown to change according to physical fitness as well as in combination with differing training loads have been reflected in predominantly athlete and well-trained population cohorts. This could highlight a potential for the application of these markers to (a) assess the effectiveness of a given training regime in already exercising populations (b) assess type of training load based on initial resting adenine nucleotide valuations as well as, (c) over-training/ loading in athlete groups. Perhaps, it would seem better to assess the acute effects of exercise rather than study the more general changes produced by training regimens over a long period since the metabolic disturbance is much greater. Thus fitness assessment and underlying health problems could be revealed by an acute exercise test and the same test could be applied after a training regimen aimed at improving health in order to evaluate the success of such a programme. Until now the observations on the effects of exercise have been rather diverse due to the reasons discussed above. By taking into consideration the scope of the analytical methods used and the optimal design for studies much greater insight into the effects of exercise will be obtained.

1.7. Aims

The detection of r-HuEPO's metabolic signature via metabolomics is a novel and relatively uncharted territory. There is a need to establish a systems biology approach to detecting performance enhancing drugs which focus on the holistic effect of a particular drug in question rather than simply detecting it. There is ample evidence to suggest the existence of EPO mimicking drugs, and it would simply not be cost-effective or practically viable to develop a test for each one.

The application of metabolomics to the series of WADA funded studies is an attempt to investigate a possible phenotypic response of r-HuEPO in a relatively homogenous Glasgow-based Caucasian cohort.

Therefore the main aim of this thesis is as follows:

1. Via an untargeted LC-MS approach examine whether there is a downstream metabolic effect of r-HuEPO in blood (plasma; chapter 3), urine (chapter 3) and RBCs (chapter 4).

Given that the primary study did not include a control group and the training frequency was not controlled either a secondary aim was included to observe the metabolite profile response of a one hour aerobic exercise session (section 1.6 of chapter 1 as well as chapter 5). This was devised in order to aid in the dissemination of the results from the primary study in an attempt to distinguish the metabolite profiles of r-HuEPO administration from training.

Chapter 2. Methods

2.1. WADA Study (08C19YP)

The official WADA funded study resulted in samples (whole blood and saliva) taken for gene- expression analysis which will not be included in this thesis. In brief, venous blood samples from an antecubital vein were obtained at baseline (over two weeks), during r-HuEPO administration (on days 2, 4, 8, 10, 14, 16, 20, 22, 26, 28) and for 4 weeks after r-HuEPO administration (on days 30, 35, 41, 43, 48, 50, 57). The blood samples in EDTA were homogenized using a roller mixer and then measured in triplicate using a Sysmex XT-2000i (Sysmex UK, Milton Keynes, UK) for haematological measurements. Total RNA was extracted from whole blood stabilized in Tempus Blood RNA tubes, amplified, labelled and randomly hybridized to the Illumina HumanHT-12 v4.0 Expression BeadChip. Resting blood pressure and heart rate were recorded three times on both arms in the supine position before blood sampling using an automated cuff oscillometric device (Boso-Medicus, Bosch & Sohn GmbH, Jungingen, Germany). No significant changes to blood pressure, resting heart rate or body mass were observed. Total haemoglobin mass was determined in triplicate prior to the first r-HuEPO injection and then weekly up to 4 weeks post r-HuEPO administration using the optimized carbon monoxide rebreathing method as previously described in Durussel et al. (2013). Two 3, 000 m time trials separated by at least one day rest were performed on a 200 m indoor athletic track (Kelvin Hall, Glasgow, UK) pre, post- r-HuEPO administration and at the end of the study. In addition to the time trial, $\dot{V}O_{2max}$ was determined pre, post- r-HuEPO administration and at the end of the study using an incremental test to exhaustion on a motorised treadmill. Gas exchange was measured breath by breath using an automated metabolic gas analysis system (Cosmed Quark b2, Cosmed, Rome, Italy). The methodological details of both performance tests as well as detailed results can be found in Durussel et al. (2013) where I am a co-author. I was involved in the sample collection, haematological analysis, total haemoglobin mass measurement, performance testing since the early inception of the WADA funded study (began as a summer intern in Summer 2009). I was not involved in the study design or the writing of the original proposal. From August 2011 to July 2012 I was one of the main research assistants responsible for the day-to-day running of all experiments, reporting and analysis of results, blood

collection and sample handling. As proof that the treatment was successful in all subjects tested, Table 2-1 outlines the haematological responses tested (Durussel et al., 2013).

Table 2-1. Haematocrit, haemoglobin concentration, reticulocytes, haemoglobin mass, blood volumes, carboxyhaemoglobin and body mass before, during and 4 weeks post r-HuEPO administration. Table taken from Durussel et al. (2013).

	Baseline	Week 2 of r-HuEPO	End of r-HuEPO	Week 2 post r-HuEPO	Week 4 post rHuEPO
Haematocrit (%)	41.9 (1.8)	44.7 (2.0)*	49.2 (2.0)*	47.7 (2.2)*	45.1 (1.7)*
Haemoglobin ($g \cdot dl^{-1}$)	14.4 (0.7)	15.2 (0.7)*	16.7 (0.9)*	16.1 (0.9)*	15.6 (0.7)*
Reticulocytes (%)	1.07 (0.31)	2.57 (0.44)*	1.46 (0.41)*	0.44 (0.13)*	0.55 (0.15)*
Hb_{mass} ($g \cdot kg^{-1}$)	12.7 (1.2)	13.4 (1.3)*	15.2 (1.5)*	15.1 (1.4)*	13.7 (1.1)*
Hb_{mass} (g)	947 (109)	1001 (127)*	1131 (131)*	1126 (136)*	1023 (132)*
Blood volume (l)	6.6 (0.9)	6.6 (1.0)	6.8 (0.8)	6.7 (0.9)	6.6 (0.8)
Red cell volume (l)	2.8 (0.3)	2.9 (0.3)*	3.3 (0.4)*	3.3 (0.4)*	3.0 (0.4)*
Plasma volume (l)	3.8 (0.6)	3.7 (0.7)	3.5 (0.4)*	3.4 (0.6)*	3.6 (0.5)
Carboxyhaemoglobin (%)	0.73 (0.15)	0.77 (0.16)	1.04 (0.19)*	1.01 (0.20)*	0.65 (0.15)
Body mass (kg)	75.1 (8.4)	74.7 (7.8)	75.0 (7.9)	74.6 (7.6)	74.6 (7.5)

N = 19. Values are means (SD). Significant differences compared to baseline values are indicated by * $p < 0.05$.

In brief, when compared to averaged baseline values, both haematocrit and haemoglobin concentration gradually increased throughout the treatment and reached a maximum approximately one week after the last injection. These haematological indices remained significantly elevated 4 weeks post administration, as with haemoglobin mass. Percentage of reticulocyte increased rapidly and significantly after

the first two weeks of injections. Blood volume remained unchanged, red cell volume increased significantly at 2 weeks during treatment, and at the end of treatment which remained elevated 2 weeks post administration. In comparison to baseline red cell volume remained elevated 4 weeks post administration. Finally, plasma volume decreased significantly at the end of treatment and 2 weeks post administration. These haematological results taken from Durussel et al. (2013) and presented here as proof of an effect of the r-HuEPO treatment.

2.1.1. Participants: Corresponding to Chapter 3 and 4

Twenty physically active men (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7, height: 179.5 ± 5.4 cm) participated in this study (Durussel et al., 2013). All subjects underwent medical assessment and gave written informed consent to participate. The subjects were regularly engaged in predominantly endurance- based activities, such as running, cycling, swimming, triathlon and team sports. They were requested to maintain their normal training and abstained from official sporting competition for the duration of the research protocol. One subject dropped out of the study in order to participate in the London marathon which was scheduled during the last week of the research protocol and, although three r-HuEPO injections were given, seven weeks of washout were deemed sufficient to return to competition at this event (his performance in this marathon was 2 h 57min 26 s; his best performance is 2 h 48 min 23 s). Subjects were questioned on each experimental visit about any nutritional supplements, medication, drugs or alcohol that they consumed leading up to their laboratory visit as well as illness. This study was approved by University of Glasgow Ethics Committee and conformed to the Declaration of Helsinki (Durussel et al., 2013).

2.1.2. Experimental Design and Blood/ Urine Samples Collection

Standardized phlebotomy procedures were applied in order to minimize the effects of diurnal variation and posture. Blood samples were drawn by trained phlebotomists, including myself, from an ante-cubital vein. Venous blood samples were obtained at

baseline (over 2 weeks prior to the first r-HuEPO administration, three of which were utilised for metabolomics processing), during r-HuEPO administration (on days **2**, 4, 8, 10, **14**, 16, 20, 22, 26, **28**) and for 4 weeks after r-HuEPO administration (on days **30**, **35**, 41, **43**, 48, 50, **57**); days in bold indicate additional metabolomics collection time- points. Each subject subcutaneously self- injected 50 IU $\cdot$ kg^{-1} body mass of rHuEPO (epoetin β, NeoRecormon, Roche, Welwyn Garden City, UK) which is in line with previous literature (Parisotto et al., 2000). Daily oral iron supplementation (~ 100 mg of elemental iron, Ferrous Sulphate Tablets, Almus, Barnstaple, UK) was given during the 4 weeks of rHuEPO administration.

All blood samples (i.e. 4 mL K3EDTA tube, Greiner Bio-One Ltd, Stonehouse, UK and 3.5 mL serum tube, Vacutainer SST II Advance, BD, Plymouth, UK) were taken after 10 min of rest in the supine position (Ahlgrim et al 2010). Blood samples in EDTA were homogenized using a roller mixer. Urine samples (in a 20 mL sterile clear tube) were collected prior to blood collection on each experimental day, unless the subject was unable to pass urine at the time in which case they were given water to drink and were allowed to do this at the end of the session. Plasma, serum (not presented in this doctorate thesis) and urine were then isolated, aliquoted into cryotubes and stored at – 80 °C until analysis. The remaining urine in the 20 mL tube was stored at - 20 °C until analysis. Resting blood pressure and heart rate were recorded three times on both arms in the supine position before blood sampling using an automated cuff oscillometric device (Boso-Medicus, Bosch & Sohn GmbH, Jungingen, Germany; data can be found in Durussel et al. (2013)). Figure 2-1 demonstrates how the blood collection time- points for the metabolomics analysis coincides with the original blood collection time- points as well as the administration of r-HuEPO. The sample collection period of this study took place between 2010 to the summer of 2012.

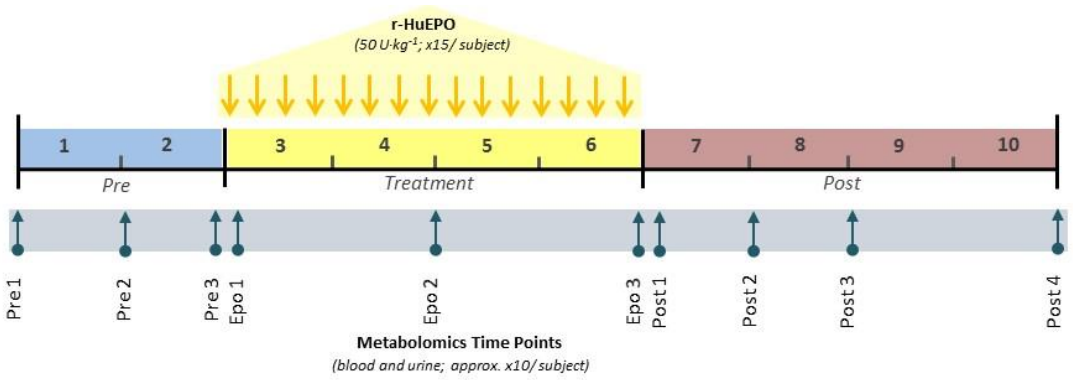


Figure 2-1. Venous blood (for RBC and plasma) and urine samples were obtained at baseline (three samples over 2 weeks prior to the first r-HuEPO administration), during r-HuEPO administration (on days 2, 14, and 28) and for 4 weeks after r-HuEPO administration (approx. on days 30, 35, 43, 57).

2.2. Metabolomics Approaches

Figure 2-2 below highlights the full spectrum of metabolomics- based approaches carried out for plasma (18 participants) and urine (18 participants plus 1 from initial pilot).

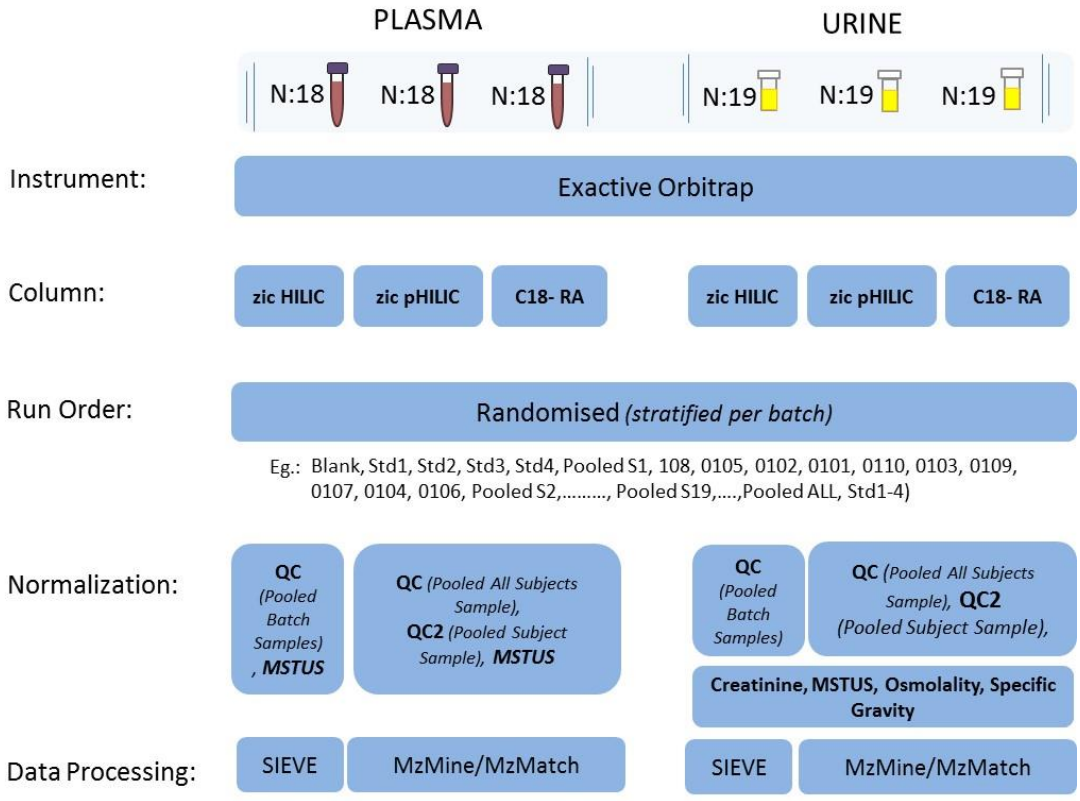


Figure 2-2. Metabolomics pipeline for plasma and urine analysis.

Based on the data output, plasma and urine from the ZIC-pHILIC method has been selected for discussion in the following doctorate thesis since it was previously found to give the best overall coverage of the urinary metabolome (Zhang et al., 2012). Data processing via MzMatch allowed for all the subjects to be processed in one go without the need to create separate batches as was the case with MZmine (open source) and Sieve (Thermo Fisher Scientific, UK; see appendix iii for details). RBC were also extracted for analysis from whole blood (3 subjects) and from the blood residue of centrifuged EDTA tubes (18 subjects).

2.3. Sample Preparation

2.3.1. Plasma and Pooled Samples

200 µL of plasma was thoroughly mixed with 800 µL of HPLC grade acetonitrile (ACN), followed by centrifugation at 10,000 revolutions per minute for 5 minutes; 800 µL of supernatant was then transferred to a LC auto-sampler vial (Thermo Fisher, UK). For quality control purposes a pooled sample representing all subjects as well as subject specific pooled samples were prepared (the latter was utilised as normalisation for instrumental variation). Pooled samples were prepared by removing 100 µL of plasma from each sample and subsequently treating as above.

2.3.2. Urine and Pooled Samples

As outline in *2.3.1. Plasma*.

2.3.2.1. Measurement of Creatinine

50 µL of diluted sample and prepared creatinine standard stock solutions for a calibration curve were thoroughly mixed with 100 µL of creatinine detection reagent (Enzo Life Sciences, Exeter, UK) in a 96- well plate. Absorbance was read at 490 nm via a Spectra Max M5 from Molecular Devices. The concentration of creatinine was determined via a seven point calibration curve and run in duplicate. This was conducted for a sub-set of the urine samples (6 participants).

2.3.2.2. Measurement of Osmolality

Post- calibration with specified standard solutions by Vitech Scientific Ltd. (West Sussex, UK), freezing- point depression measurement in duplicate for each sample was performed with an Advanced Micro Osmometer (Model 3300) in order to

determine average osmolality reading. This was performed for all eighteen participants to investigate status of the sample rather than hydration status of subject.

2.3.2.3. Measurement of Specific Gravity (g)

During urine sample preparation, each Eppendorf was weighed (Mettler-Toledo AG204, Beaumont Leys, Leicester, UK) empty and then with the added urine sample (200 µL) for each sample corresponding to the eighteen subjects. As a reference deionised water (H_2O) produced from a Direct- Q 3 Ultrapure Water System (Millipore, UK) was used. The temperature was tightly controlled between 18 to 19 ^oC. Subsequently equation 1 was used to calculate density (d), from mass (m) and volume (v) for reference and each urine sample, followed by equation 2 for g:

$$d = \frac{m}{v} \tag{1}$$

$$g = \frac{d(sample)}{d(H_2O)} \tag{2}$$

2.3.3. RBC from Whole Blood

EDTA tubes from the initial three subjects were allowed to thaw at room temperature prior to preparation. 500 µL of venous blood was removed and inserted into an Eppendorf containing 1, 000 µL of deionised water (Direct- Q 3 Ultrapure Water System from Millipore, UK). Eppendorfs were subsequently sonicated (Branson 1510, Emerson Industrial Automation, Genève, Switzerland) for 1 minute. Once completed, 200 µL of mixture was thoroughly mixed with 800 µL of ACN, followed by centrifugation at 10, 000 revolutions per minute for 5 minutes. 800 µL of supernatant was then transferred to a LC auto-sampler vial (Thermo Fisher, UK). For quality control purposes a pooled sample representing all the subjects as well as a subject specific pooled sample was prepared (the latter was utilised for normalisation of instrumental variation). Pooled samples were prepared by removing 100 µL of venous blood from each sample and subsequently treated as above.

2.3.4. RBC from Centrifuged Blood Residue

The blood residue of EDTA tubes, remaining from the WADA study that had previously been centrifuged prior to removal of plasma, were used for this analysis. After samples were allowed to thaw at room temperature, any remaining plasma residue was removed and discarded. 200 μL of residue was removed and inserted into an Eppendorf tube containing 200 μL of deionised water (Direct- Q 3 Ultrapure Water System from Millipore, UK). Eppendorfs were subsequently sonicated for 1 minute. Once completed, 200 μL of mixture was thoroughly mixed with 800 μL of ACN, followed by centrifugation at 10, 000 revolutions per minute for 5 minutes. 800 μL of supernatant was then transferred to a LC auto- sampler vial (Thermo Fisher, UK). For quality control purposes a pooled sample representing all subjects as well as a subject specific pooled sample was prepared. Pooled samples were prepared by removing 50 μL of blood residue from each sample and subsequently treated as above.

2.4. Chemicals and Reagents

HPLC grade acetonitrile was purchased from Fisher Scientific, UK and HPLC grade water was produced by a Direct- Q 3 Ultrapure Water System from Millipore, UK. AnalaR-grade formic acid (98%) was obtained from BDH-Merck (Poole, Dorset, UK). Authentic stock standards were prepared as stated previously in the literature (Creek et al., 2011) and diluted 4 times with ACN before LC-MS analysis. Ammonium carbonate was purchased from Sigma-Aldrich, UK.

2.4.1. r-HuEPO Injection

One vial of 50, 000 IU equates to 415 μg of epoietin β. One ampoule contains 10 mL solvent/ H_2O for injection. Excipients include phenylalanine (up to 5 mg/vial), sodium (less than 1 mmol per dose), benzyl alcohol (up to 40 mg per multidose solvent ampoule; preservative) as well as benzalkonium chloride (preservative) (Roche Products Ltd., Welwyn Garden City, UK).

2.5. LC-MS Method

LC-MS data were acquired on an Accela HPLC (Thermo Fisher Scientific, Hemel Hempstead, UK) coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in both positive and negative mode set at 50, 000 resolution (controlled by Xcalibur version 2.1.0; Thermo Fisher Corporation, Hemel Hempstead, UK). The mass scanning range was *m/z* 75–1200, the capillary temperature was 320 °C and the sheath and auxiliary gas flow rates were 50 and 17 arbitrary units, respectively. The separation was performed on a ZIC-pHILIC column (150 × 4.6 mm, 5 μm from HiChrom, Reading UK) in binary gradient mode. The mobile phase used was 20 mM ammonium carbonate buffer (pH 9.2) and pure ACN; the flow rate was 300 $\mu L.min^{-1}$. The gradient was programed as follows: 0 min 20 % A 80 % B to time 30 min 80 % A 20 % B. The injection volume was 10 μL and the sample tray temperature was controlled at 12 °C during the measurement. Samples were run in a stratified method with between subject samples placed in randomized order.

2.5.1. Run Order

A blank sample (ACN) and the four standard mixtures were placed at the beginning of each run followed by a pooled sample representing all subjects. All samples were then run in randomised order stratified by subject number and preceded by the subject specific pooled sample.

2.6. LC-MS Data Processing with MzMatch and IDEOM (version 18)

Raw LC-MS files were converted to mzXML (ProteoWizard) and separated into ESI positive and negative. Converted files were then processed with open source MzMatch (http://mzmatch.sourceforge.net/) and identification of putative metabolites was made via macro- enabled Excel file IDEOM (http://mzmatch.sourceforge.net/ideom.html). Example of the R script for data processing with MzMatch can be found in appendix viii.

2.6.1. Method of Sample Grouping for Data Processing

2.6.1.1. Plasma, Urine and RBC of Centrifuged Residue

Samples from the ZIC-pHILIC LC-MS analysis were grouped according to day order resulting in 16 time- points, with each participant sample acting as a technical replicate. There is a minimum of 3 samples per group, as per the guidelines of the MZMatch data processing software. Figure 2-3 outlines the number of samples per grouping in (A) plasma, urine and (B) RBC from centrifuged residue.

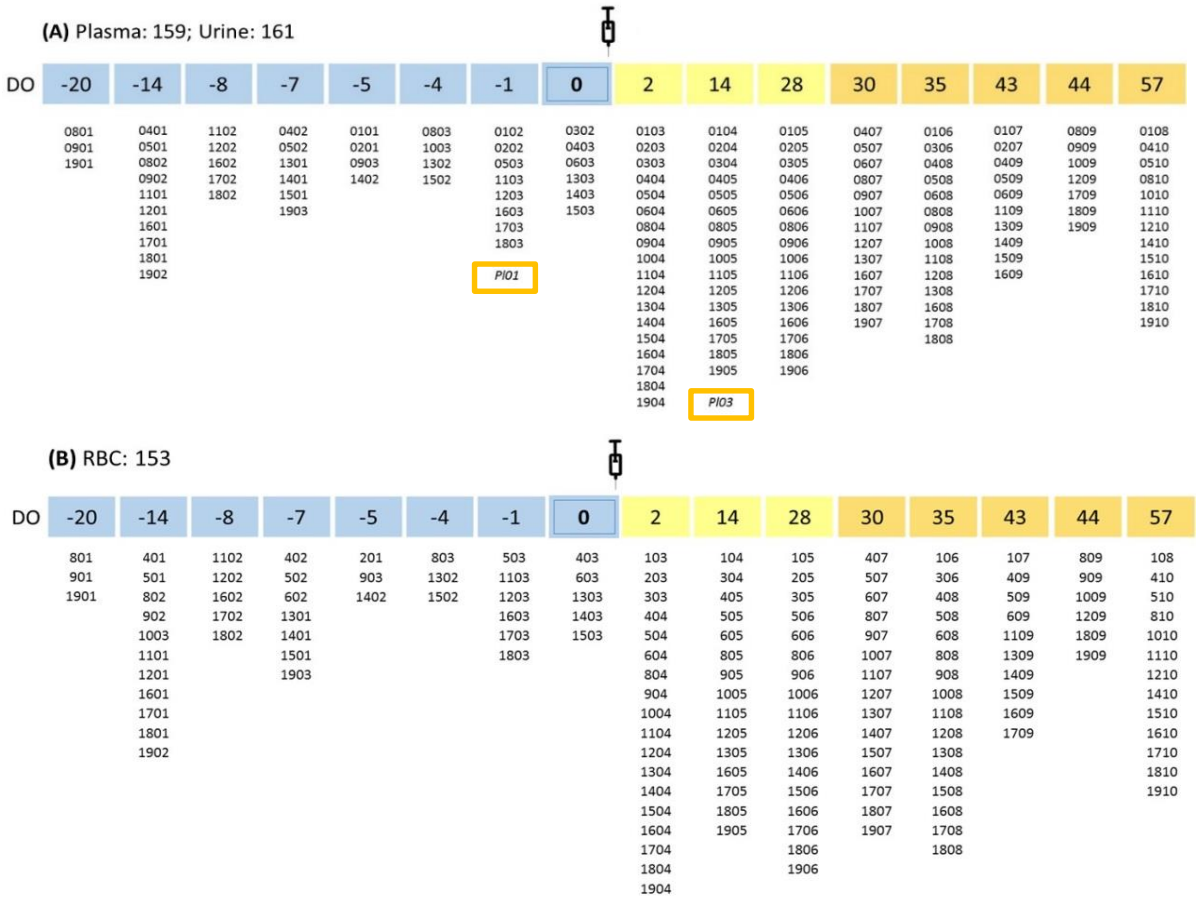


Figure 2-3. Timeline of day order (DO) groupings for (A) plasma, urine as well as (B) RBC from centrifuged blood. DO0 (control) vs. DO {-20, -14, -8, -7, -4, -1, 2, 14, 28, 30, 35, 43, 44, 57}. Pilot subject (PL) sample number 01 and 03 present in the urine cohort, were only utilised for general pie chart overview of metabolite classes.

2.6.1.2. RBC from Whole Blood

Samples were grouped according to phase pre- treatment (N:6), treatment (N:9) and post- treatment (N:8). The POST3 time-point sample of subject three could not be found in the storage at Glasgow University, thus could not be included in the analytical run.

2.7. Normalisation

Filtered raw urine data were subjected to a variety of normalisation techniques; osmolality, MS creatinine, specific gravity, subject-specific area percentage as well as average selected purine metabolite (hypoxanthine, xanthosine, inosine, deoxyinosine and guanine) response. For the subject-specific area percentage, the first step includes calculating the sum of the peak areas of each metabolite across the 10 week protocol for each subject individually. Secondly, each respective metabolite response from every time point is then divided by the subject specific sum and multiplied by 100. For plasma, filtered raw data was normalised to subject-specific area percentage as well as to the average for selected purine metabolite (hypoxanthine, and N2,N2-dimethylguanosine) response. For the RBC data sets, metabolite profiles were normalised to individual metabolic output. Unpaired t-tests were calculated on the new area percentage datasets (plasma, urine and RBCs) with all pre values averaged to give one baseline time-point.

2.8 Statistical and Multivariate Data Analysis

Graphical representations, tabular features and statistical analysis (*p*-value generation) were performed in Excel (Microsoft Office 2013). Pie charts of metabolite classes in plasma and urine were generated via IDEOM. Filtered raw and normalized data were subjected to unsupervised principal component analysis (PCA; scaled to unit variance) as well as partial least squares- discriminant analysis (PLS-DA) via SIMCA-P 13 (Umetrics, Sweden), in addition to the permutation analysis. Online server for

metabolite analysis MetaboAnalyst 3.0 (www.metaboanalyst.ca) (Xia et al., 2009, 2012) was utilized for correlation (with Pearson's *r* correlation analysis after being normalized with auto-scaling) as well as loading plots of urine and plasma metabolomes. The three PRE values of each subject were grouped into one averaged baseline value and subsequently compared to grouped EPO1, EPO2, EPO3, POST1, POST2, POST3 and POST4 time-points. Significant metabolites (via unpaired t-test) from plasma and urine were subjected to orthogonal partial least squares- discriminant analysis (OPLS-DA). All corresponding cumulative R^2X, Q^2 and R^2Y values were generated via SIMCA-P 13 (Umetrics, Sweden) in addition to Variable Importance for Projection (VIP) score. ROCCET (http://www.roccet.ca/ROCCET/) an online user-friendly Receiver Operator Characteristic (ROC) curve analysis explorer and tester (Xia et al., 2013), was subsequently used to measure the area under the curve (AUC) with corresponding 95% confidence intervals (CI) of the selected significant metabolites in urine, plasma and RBC data from centrifuged blood residue. CI via ROCCET are calculated via a non-parametric re-sampling based approach. For the predictive model generation of combined metabolite markers, in the plasma data set, the linear support vector machine (LSVM) algorithm was selected after comparison with PLS-DA and random forest algorithms.

Chapter 3. A Study of the Effects of r-HuEPO on the Plasma and Urine Metabolite Profiles of Caucasian Endurance-Trained Males

3.1. Introduction

Potent designer doping substances are being produced at an exponential rate with main aim to combat current doping control tests (Sottas et al., 2011). One of these designer substances is the recombinant form of EPO, an endogenous glycoprotein, which is favoured because of its well-cited ergogenic benefits (Durussel et al., 2013) and short half-life (Aachmann-Andersen et al., 2014). EPO was banned in the 1990s by the International Olympic Committee and subsequently by WADA. Endogenous EPO production is primarily stimulated by the kidneys and partly by the liver, via HIF-2 activation. The aim is to regulate red blood cell mass by stimulating the survival, proliferation and differentiation of erythrocytic progenitor cells (Durussel et al., 2013; Haase, 2013). Interestingly, the pleiotropic nature of EPO has been established in the past decade (Lundby and Olsen, 2011) showing tissue protective effects that are non-apoptotic and of a mitogenic nature, which is inadvertently carried over to malignant cells exhibiting a protective nature against chemotherapy (Debeljak et al., 2014). Thus, the dimeric EPO receptors (59 kDa; EPO-r) have been shown to be exhibited in non-haematopoietic cells, yet the functionality of those EPO cell surface receptors has yet to be fully understood (Jelkmann, 2010). Even so, the presence of such receptors on tumour and malignant cells has been, in recent years, raising questions regarding the role of ESA administration to cancer patient cohorts (Debeljak et al., 2014).

r-HuEPO administration in healthy subjects has been shown to increase haemoglobin concentration by producing a concomitant increase in red blood cell mass and a decrease in plasma volume (Lundby et al., 2007; Lundby and Olsen, 2011; Durussel et al., 2013). The decrease in plasma volume however, precedes the increase in red cell mass, appearing to be a rapid responding mechanism regulated by the renin-angiotensin-aldosterone system in order to control haematocrit (Lundby et al., 2007; Olsen et al., 2011). These associated blood markers (haemoglobin, haematocrit, and reticulocytes) are affected by altered erythropoiesis and form the basis of the ABP. However, due to the lack of specificity and sensitivity in the test (Lundby et al., 2008b) evidence has shown that micro-dosing with r-HuEPO is effective in evading the ABP (Ashenden et al., 2011). There may be, however, promise in the membrane-assisted isoform immunoassay that aims to quantify EPO isoforms as a percentage of migrated

isoforms (Aachmann-Andersen et al., 2014; Dehnes et al., 2014), which has been developed by L*ö*nnberg and Carlson. This method provides a quicker and cheaper technique in comparison to the official direct r-HuEPO detection method of isoelectric focusing (Lundby et al., 2012). There are other effects of r-HuEPO dosing that could be detected by investigating the ADMA–DDAH–NOS system. A targeted HPLC-ESI/MS analysis of urinary markers ADMA, SDMA, arginine and citrulline, which are involved in the nitric oxide (NO) generation pathways, showed an increase post-administration from a single bolus of IV Epocrine (2000IU) in comparison to pre-treatment levels in two male subjects (Appolonova et al., 2008, 2009). In fact the biosynthesis of the ubiquitous signalling molecule NO (Ashmore et al., 2014), via L-arginine metabolism by NO synthases (Levett et al., 2011) has been of recent interest due to the NO mediated benefits. This includes its antioxidant capacity, stimulation of increased mitochondrial energy production and blow flow regulation (Levett et al., 2011; Zafeiridis, 2014). Dietary nitrate (NO_3^-), is reduced to nitrite via bacteria in the gut as well as in the oral cavity and then further reduced to NO in the stomach, which has been shown to have various benefits on exercise performance (Muggeridge et al., 2014; Zafeiridis, 2014), in part due to the reduction of exercise related oxygen cost. Sea level dwelling individuals ascending to altitude, however, experience a decrease in NO production which could result from a combination of factors such as, oxidative stress incurred by hypoxia, as well as the deactivation of NO synthase (Muggeridge et al., 2014).

Ashmore et al. (2014) investigated the effect of a dose dependent dietary nitrate supplementation protocol in normoxic and hypoxic conditions on male Wistar rats. A significantly reduced plasma EPO level in both conditions post supplementation was associated with changes in hepatic EPO expression. The rise in oxygen tension mediated by production of NO from nitrate, resulted in a reduction in EPO expression that was closely linked to a fall in circulating Hb. With higher doses of nitrate these responses were partially reversed by an increase in renal EPO expression which prevented a potential detrimental fall of Hct (Ashmore et al., 2014). Following a 2 hour normobaric hypoxia exposure session (equivalent to 3000 m and 4500 m) the urine of six healthy male subjects was investigated via a LC-TOF/MS approach. This acute dose of hypoxia was shown to increase markers from the purine pathway

(hypoxanthine, xanthine, uric acid), energy metabolism (carnitines and acyl-carnitines) as well as the purine nucleosides 1-methyladenosine and 5-methylthioadenosine as well as a drop in amino acid 3-indoleacetic acid. A rise in urinary 1-methyladenosine has also been shown to be associated with malignant tumours in humans (Takahashi et al., 1993). Interestingly, moderate hypoxia (3000 m) resulted in higher inter-subject variability in terms of metabolite responses, which partly subsides at higher hypoxic exposure (Ge et al., 2002; Lou et al., 2014). It is also important to note that the purine pathway metabolite responses to normobaric hypoxia, are in line with responses seen in an hour of aerobic exercise examined in the urine of male subjects via an untargeted LC-MS approach (Daskalaki et al., 2015a) as well as previous literature dating back at least 40 years (Daskalaki et al., 2015b).

Taking a step-up the OMICs ladder, Christensen et al. (2011) applied a serum proteomic approach to investigate the serum protein biomarkers produced in response to r-HuEPO administration in healthy male volunteers highlighting the potential influences that altitude can have on key protein isoforms. Subjects were given r-HuEPO (5000 IU; SC) every second day for a total of 16 days. A hypoxia induced environment will result in enhanced EPO secretion that will encourage expression of certain acute phase proteins. The levels of four isoforms of haptoglobin, one isoform of hemopexin/albumin, and two isoforms of transferrin were significantly decreased on day 16. Both hemopexin and haptoglobin are produced in the liver in response to inflammation and hypoxia (Christensen et al., 2011). Interestingly, the changes induced by r-HuEPO on selected serum protein isoforms persisted post-administration; the authors were able to propose an increased window of detection from < 3 days (as current direct WADA protocols) to > 8 days due to the unchanged intensity changes of these biomarkers. However, the influence of key environmental stressors such as altitude was not taken into consideration neither were the changes due to endurance training. Both of these factors require further investigation in order to differentiate them from the effects of r-HuEPO administration (Christensen et al., 2011). A second study by Christensen et al. (2015) aimed to address the issue of aerobic training alone and training in combination with ESA (darbepoietin α; 1 SC injection/wk for 10 wks) in the serum proteome of healthy untrained male subjects. The results indicated an isoform of a haptoglobin/haptoglobin related protein, exhibited a lower intensity in

both the training and ESA group, during the r-HuEPO treatment period. The protein then increased in intensity during the washout period. The authors suggest this as an option for new doping control methods, with a potential to increase the window of detection post-administration to 1-2 weeks. However, this method was tested on an untrained cohort and it is as yet unclear how elite athletes would respond. The same study was also used to evaluate a potential whole body metabolic effect to the r-HuEPO treatment, details of which can be found in Chapter 1 Table 1-1. In brief, the authors highlighted an opposite lipolytic effect of r-HuEPO treatment and training that was neutralised when the two were combined. This highlights a more complex story in terms of metabolite profiling in comparison to proteomics. Nonetheless, it is vital to understand the downstream effects of the series of intrinsic mechanistic processes that potentially lead to a targeted physiological response. Perhaps if the effects of EPO were quantified in relation to its direct impact on the human phenotype this could provide further insight into the complex nature and functioning of EPO (Nicholson et al., 2008). As such, metabolomics-based approaches can be utilized for the identification of biological targets (such as novel drug targets), individual profiling (personalized healthcare, nutrition management) as well as population profiling (metabolome-wide association studies) (Gieger et al., 2008; Nicholson and Lindon, 2008; Nicholson et al., 2008). These impact areas can be used in the improvement of doping control by either increasing the detection window for specified drugs as well as for developing more sensitive and specific pre-screening tools.

The aims of this exploratory hypothesis generating untargeted LC-MS study were to a) discriminate between the experimental phases (pre-treatment, during treatment and post-treatment) in order to highlight potential trends within the data sets of the biological samples collected from the blood (plasma) and urine of Caucasian endurance trained males; and b) distinguish potential biomarkers that respond to r-HuEPO administration.

3.2. Results

The majority of the putatively identified urinary (N:~700 post filter) and plasma (N:~600 post filter) metabolites match metabolites in the human metabolome to within 2 ppm and thus are characterised to MSI level 2 where it is most likely that alternative metabolites would be isomers of the identity assigned. The major metabolite classes of the urine and plasma metabolome of the Caucasian subjects can be seen in Figures 3-1 and 3-2, respectively.

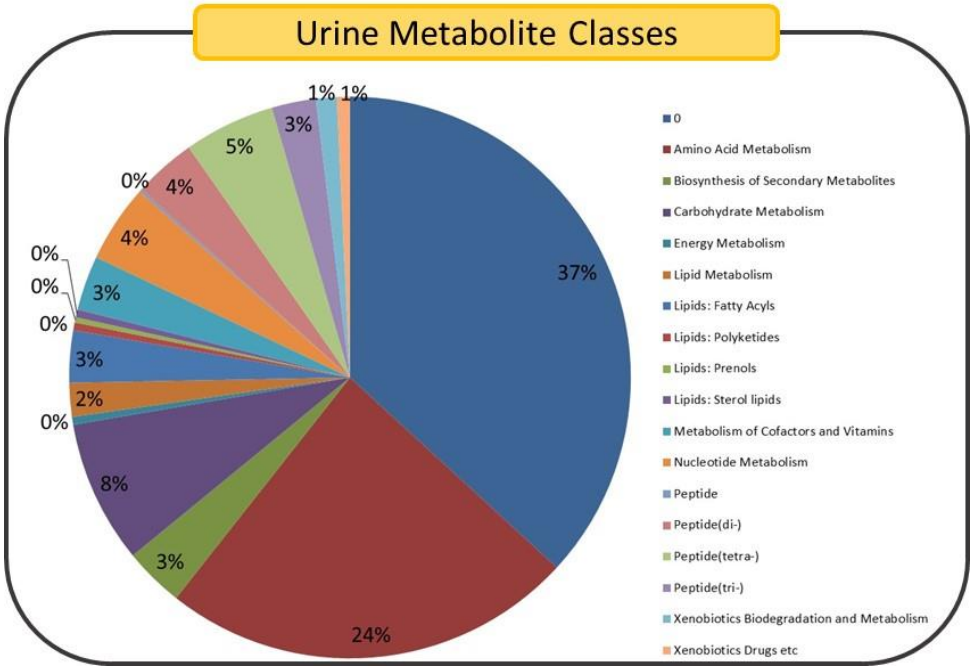


Figure 3-1. Pie chart of the major metabolite classes identified and generated via the macro-enabled Excel file IDEOM for the urinary metabolome of 19 subjects. In order of appearance: 37% of putatively identified metabolites were assigned to an unknown metabolite class (in figure shown as "0"), 24% to amino acid metabolism, 3% to the biosynthesis of secondary metabolites, 8% to carbohydrate metabolism, 2% to lipid metabolism, 3% to fatty acyl metabolism, 3% to the metabolism of cofactors and vitamins, 4% to nucleotide metabolism, 4% to peptides (di-), 5% to peptides (tetra-), 3% to peptides (tri-), 1% to xenobiotics biodegradation and metabolism and finally 1% to dietary xenobiotics or drugs. Remaining classes of 0% indicates a prevalence that is less than 1% but not null.

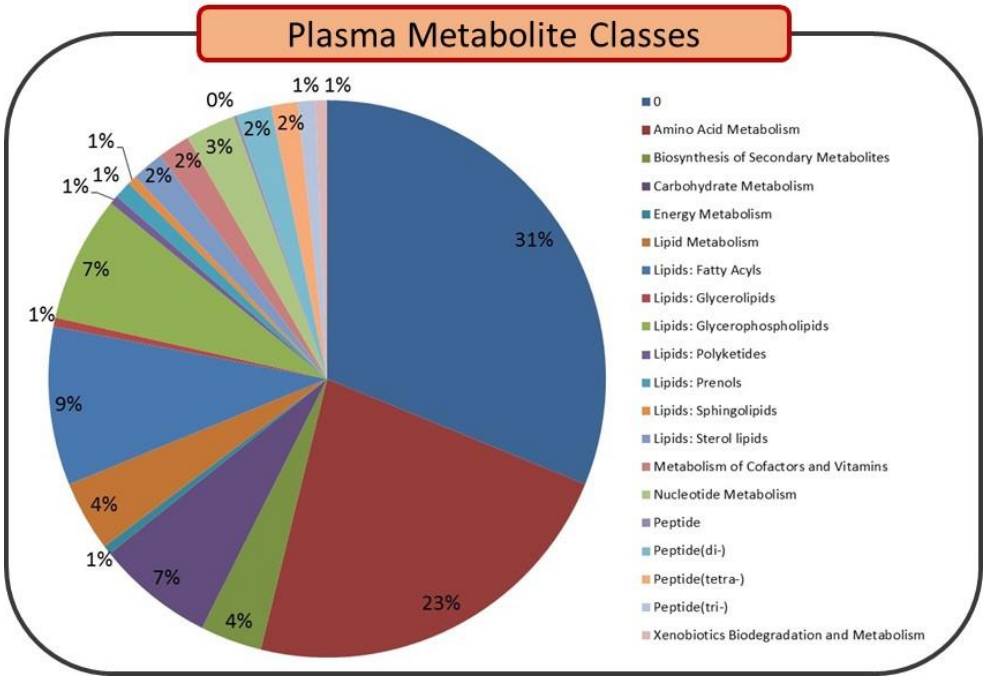


Figure 3-2. Pie chart of major metabolite classes identified and generated via the macro-enabled Excel file IDEOM for the plasma metabolome of 18 subjects. In order of appearance: 31% of putatively identified metabolites were assigned to an unknown metabolite class (in figure shown as "0"), 23% to amino acid metabolism, 4% to the biosynthesis of secondary metabolites, 7% to carbohydrate metabolism, 1% to energy metabolism, 4% to lipid metabolism, 9% to fatty acyl metabolism, 1% to glycerolipid metabolism, 7% to glycerophospholipid metabolism, 1% to polyketide metabolism, 1% to prenol metabolism, 1% to sphingolipid metabolism, 2% to sterol lipid metabolism, 2% to the metabolism of cofactors and vitamins, 3% to nucleotide metabolism, 2% to peptides (di-), 2% to peptides (tetra-), 1% to peptides (tri-), and finally 1% to xenobiotic biodegradation and metabolism. Remaining class of 0% indicates a prevalence that is less than 1% but not null.

3.2.1. Normalisation Strategies for the Urine Metabolome

The variation exhibited in the metabolome of each individual encompasses a combination of numerous endogenous and exogenous factors. Aside from the inter-individual variability the most concerning factors are those that the experimenter

introduces to the experimental data (also briefly discussed in Chapter 1 under *1.5.2. Source of Variance in Human Metabolomics Studies*). The samples collected from this WADA study extended across three year periods (2010, 2011 and 2012), unfortunately diet was not strictly controlled neither was the time-of-day of the sampling or the degree of recent physical activity prior to collection. However, given the length of the protocol and demand placed on the subjects certain controls had to be dismissed- so as to not discourage participation.

In order to observe the general pattern of the urinary metabolome PCA was performed on the raw data with either phase (PRE, EPO, POST) or year (2010, 2011, 2012) comparison (Figure 3-3). PCA was also performed on data normalised to specific gravity (Figure 3-6), osmolality (Figure 3-7), individual metabolic output (Figure 3-9) as well as to the average time-point response of selected purine metabolites (Figure 3-12). With each model year was also considered as a factor in the comparison as it is evident that it might be that, which separates the data rather than the actual phases of the intervention. This points to experimental error in the collection, and highlights an area which needs to be considered when observing metabolic patterns within data sets. In many cases, the only factor which was capable of separating the experimental phases of the data set, via the application of PLS-DA, was year (Figures 3-4, 8 and 14). When putative urinary metabolites were normalised to the internal creatinine MS value the data separation by PCA was not observed, which could be the result of several factors, such as x and y variables not being related or that there is underlying residual noise that is not effectively "removed" by the normalisation technique or potentially other simply unknown factors. As observed later in the pilot exercise study in Chapter 5, creatinine often behaves in a different pattern to the rest of the metabolites. Given also the nature of the subjects themselves, and their training behaviour it stands to reason that it would be, perhaps, inappropriate to normalise to a metabolite which is intrinsically involved in muscle activity (Daskalaki et al., 2015a).

The most interesting patterns emerge when data are normalised to both individual metabolic output as well as to average purine response. There is some justification in normalising to the selected average response of the purines given their well-cited role in exercise (Daskalaki et al., 2015b) as well as to normobaric hypoxia (Lou et al.,

2014). However, it is unclear whether the purines would respond in a similar way to r-HuEPO injections.

Previous attempts were made on the urine data set, analysed using a ZIC-HILIC method, to normalise to spectrophotometric creatinine, MSTUS as well as using log transformation (data not shown). These techniques did not alter the data to the same degree as the individual metabolic output strategy or the average purine response.

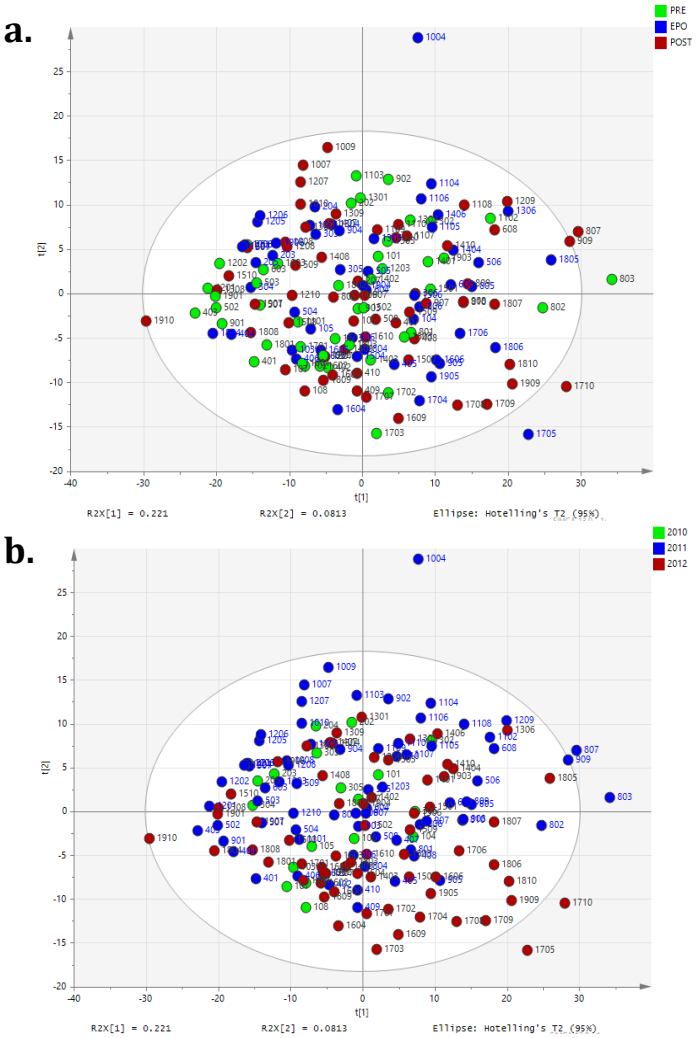


Figure 3-3. PCA of raw filtered urinary metabolite profile with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X:0.706 and Q^2: 0.403 for both models.

It is evident from the raw unfiltered urinary data, that PCA is not sufficient to reveal any trends between the phases (Figure 3-3 a) or year as a factor (Figure 3-3 b). This, however, is improved when applying PLS-DA, below (Figure 3-4). Year was the only factor which separated the data, and unfortunately samples from 2012 (red) and 2011 (blue) are distinguished in the analysis.

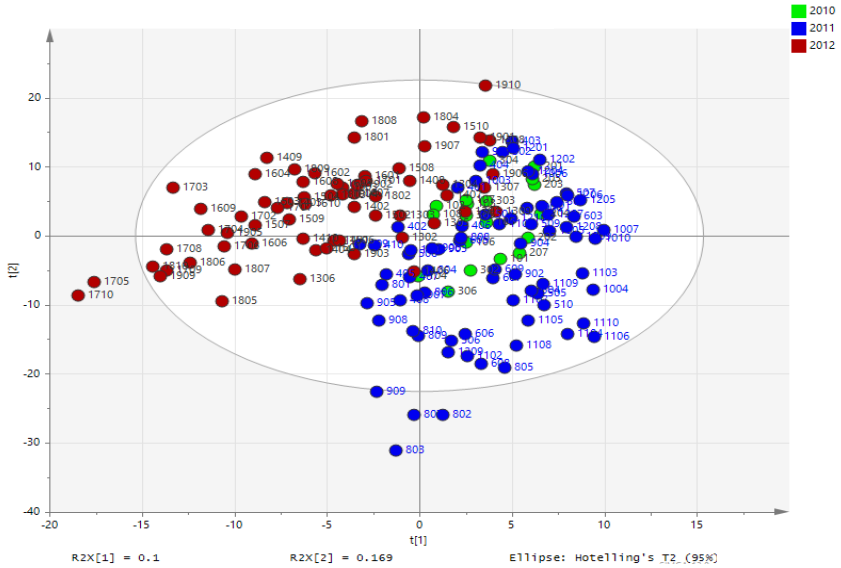


Figure 3-4. PLS-DA analysis of raw urine metabolite profile with year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.463, R^2Y: 0.914 and Q^2: 0.755.

Given the high Q^2 score of the model in Figure 3-4, permutation testing was performed to test the predictive and 'goodness of fit' value (Figure 3-5). In general, what is desirable is that the distribution of blue Q^2 values on the left are lower than the original point to the right (same for R^2 in green). Another good observation would be that the regression line of these values intersect at, or below zero. In this permutation test, for each factor comparison, the above requirements are met, however the green R^2 values do not exhibit the same reliability as the Q^2.

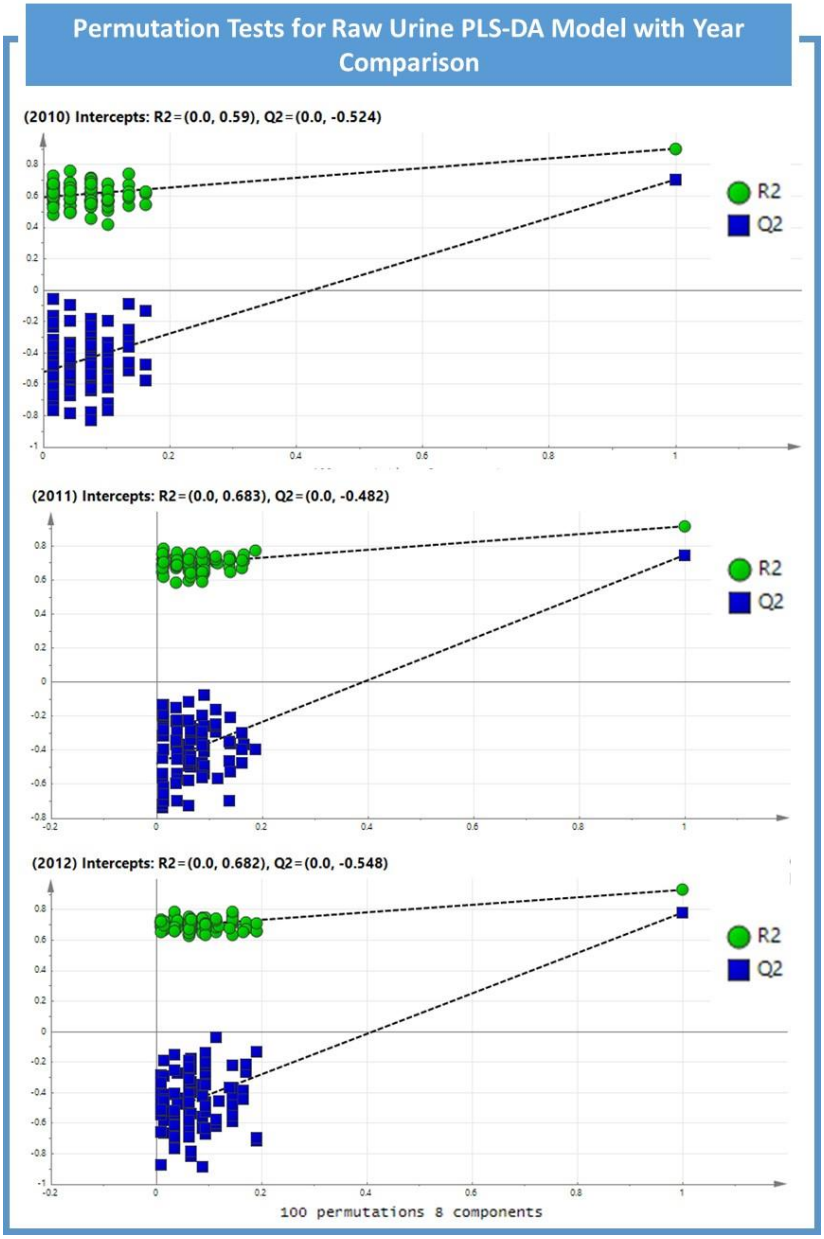


Figure 3-5. Permutation tests for raw urine PLS-DA model with year comparison (2010, 2011, 2012). Conducted with 100 permutations.

Normalising to specific gravity (Figure 3-6), or osmolality (Figure 3-7) does not improve distribution. In fact it is year, in the PCA (Figure 3-7 b) and PLS-DA (Figure 3-8) following osmolality normalisation that clearly, once again, further produces the separation.

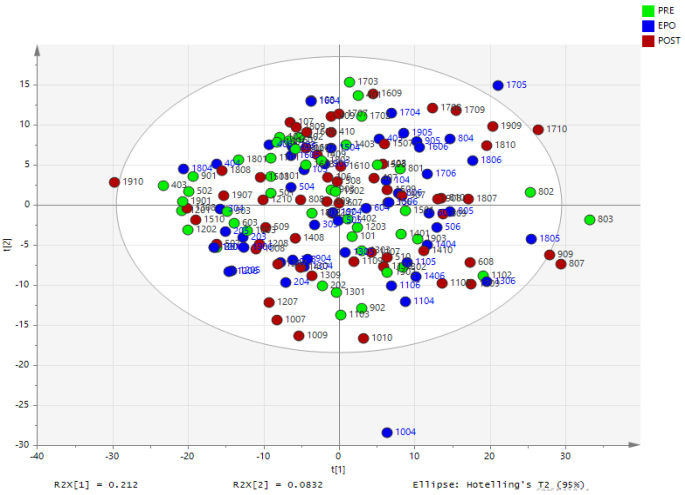


Figure 3-6. PCA of urinary metabolite data with normalisation to specific gravity with phase (PRE, EPO, POST) comparison. Cumulative R^2X: 0.693 and Q^2: 0.402.

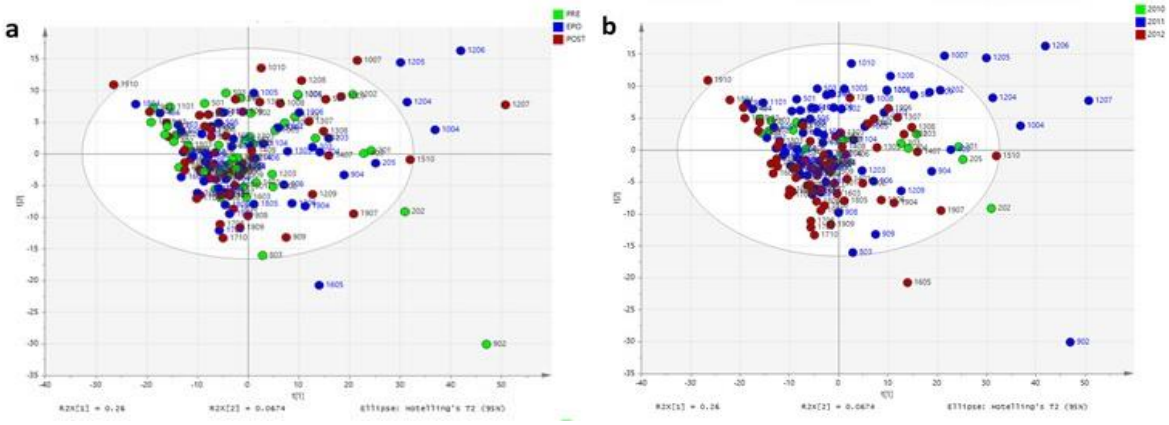


Figure 3-7. PCA of urinary metabolite profile normalised to osmolality with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X:0.719 and Q^2: 0.41 for both models.

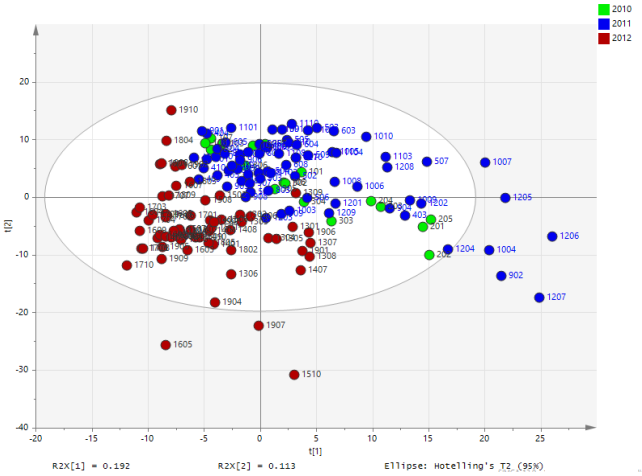


Figure 3-8. PLS-DA analysis of urine metabolite data normalised to osmolality with year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.472, R^2Y:883 and Q^2: 0.745.

Normalising the urinary data set to individual metabolic output (Figure 3-9), greatly improves the distribution of the samples and is not influenced by year (Figure 3-10 b). PLS-DA of phase, as a factor, begins to separate the PRE phase from the EPO and POST phases (Figure 3-10).

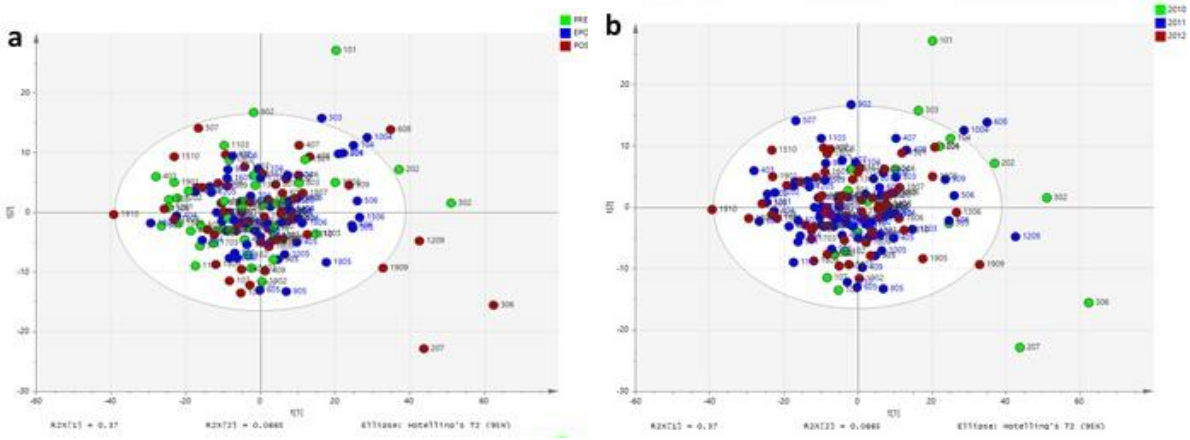


Figure 3-9. PCA of urinary metabolite profile normalised to individual metabolic output with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X:0.724 and Q^2: 0.473 for both models.

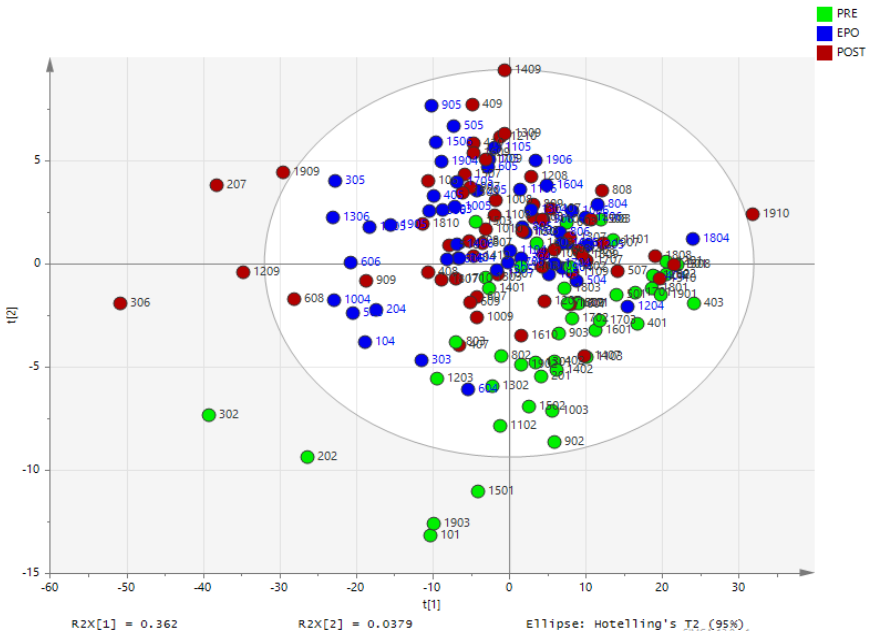


Figure 3-10. PLS-DA analysis of urine metabolite profile normalised to individual metabolic output with phase (PRE, EPO, POST) comparison. Cumulative R^2X: 0.4, R^2Y: 0.187 and Q^2: 0.0265.

In order to validate the low Q^2 score, permutation testing was carried out on each respective phase comparison (PRE, EPO, POST) and can be seen in Figure 3-11. These tests illustrate the low predictive and 'goodness of fit' value of the model generated in Figure 3-10.

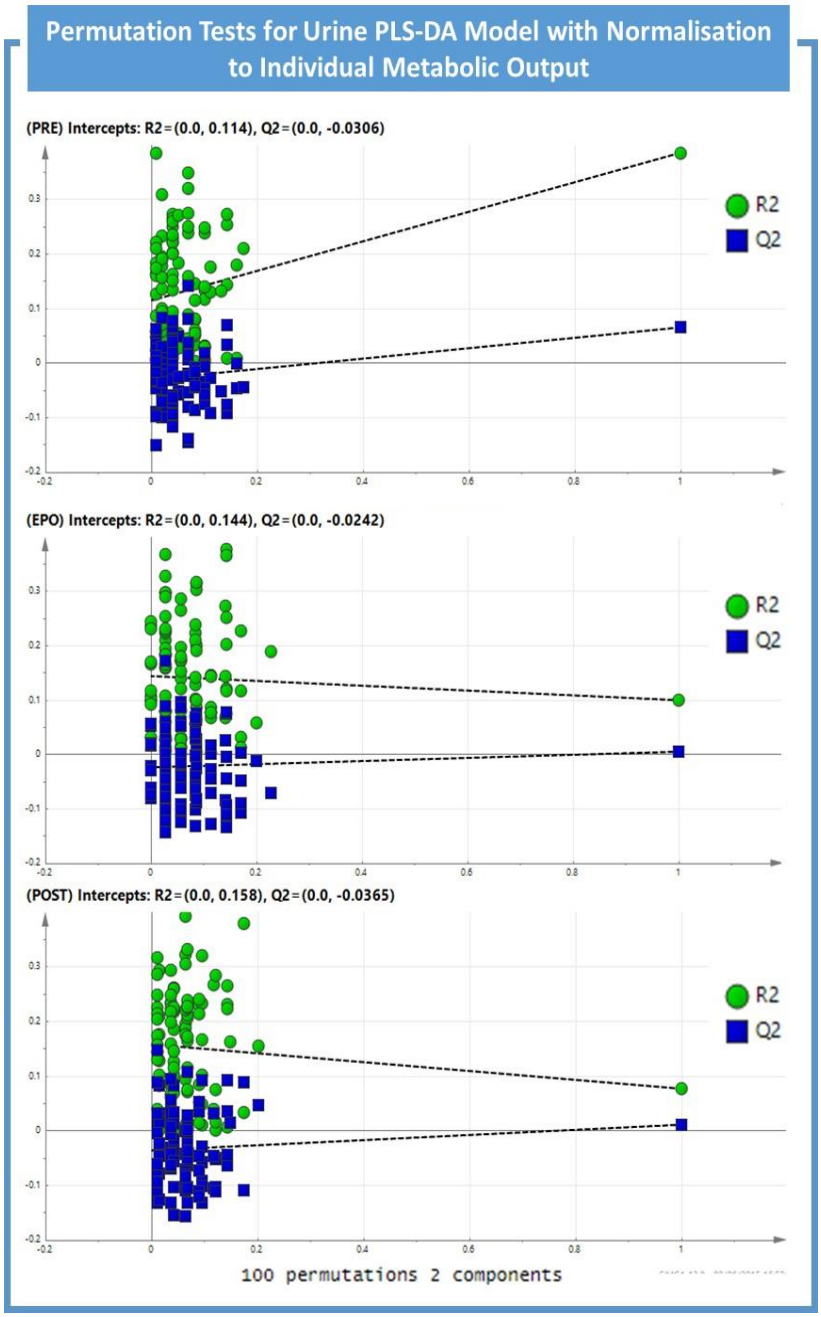


Figure 3-11. Permutation testing of urine PLS-DA model with normalisation to individual metabolic output of each phase comparison (PRE, EPO, POST). Conducted with 100 permutations.

Given the results of the pilot exercise study (Chapter 5) as well as Christensen et al. (2013), it stands to reason that exercise itself is a confounding variable. Some of the most prominent and well-cited effects are those elicited in the purine pathway, therefore they could, theoretically, be used to normalise the urinary metabolome. Figure 3-11 shows the response of the resulting output based on this technique, via PCA, both with phase (Figure 3-12 a) and year (Figure 3-12 b). However, this does not seem to be a superior method to utilising individual metabolic output, in spite of the improved R^2 and Q^2 scores, as once again year seems to be creating a separation within the data set.

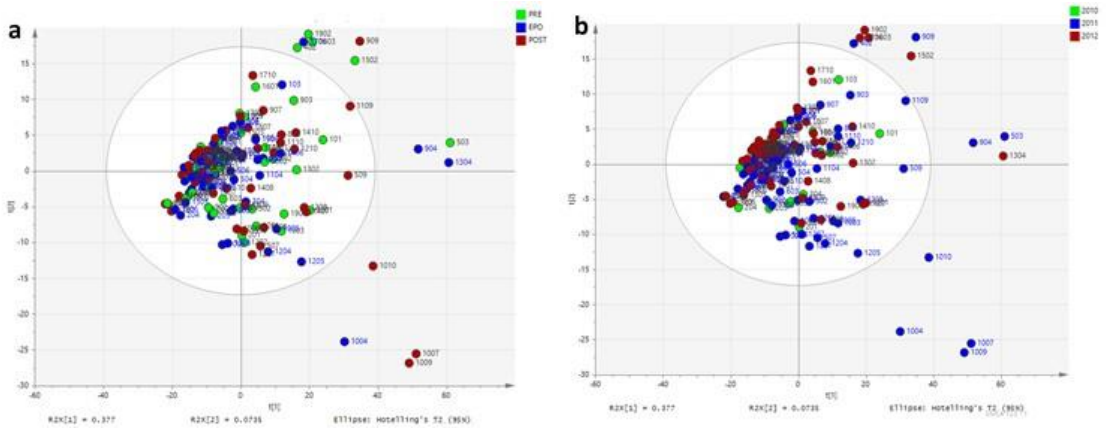


Figure 3-12. PCA of urinary metabolite profile normalised to selected purine metabolites (average time-point response of hypoxanthine, xanthosine, inosine, deoxyinosine and guanine) with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X:0.761 and Q^2: 0.499 for both models.

3.2.2. Normalisation Strategies for the Plasma Metabolome

The raw filtered plasma metabolite list was subjected to PCA in order to observe overall pattern of distribution (Figure 3-13) which was subsequently followed by PCA of normalised data to individual metabolic output (Figure 3-15) as well as the average time-point response of selected purine metabolites (hypoxanthine and N2-N2-dimethylguanosine; Figure 3-16). Comparisons were made with both phase (PRE, EPO, POST) as well as year (2010, 2011, 2012). Again the component of year becomes

a factor in the plasma metabolome which shows separation in the PLS-DA (Figure 3-14, 17). It is again difficult to say which normalisation strategy is superior however normalisation to individual metabolic output could potentially provide some valuable insight into the data as shown in the pilot exercise study, as it is uncertain what role the purines could play in response to the r-HuEPO injections.

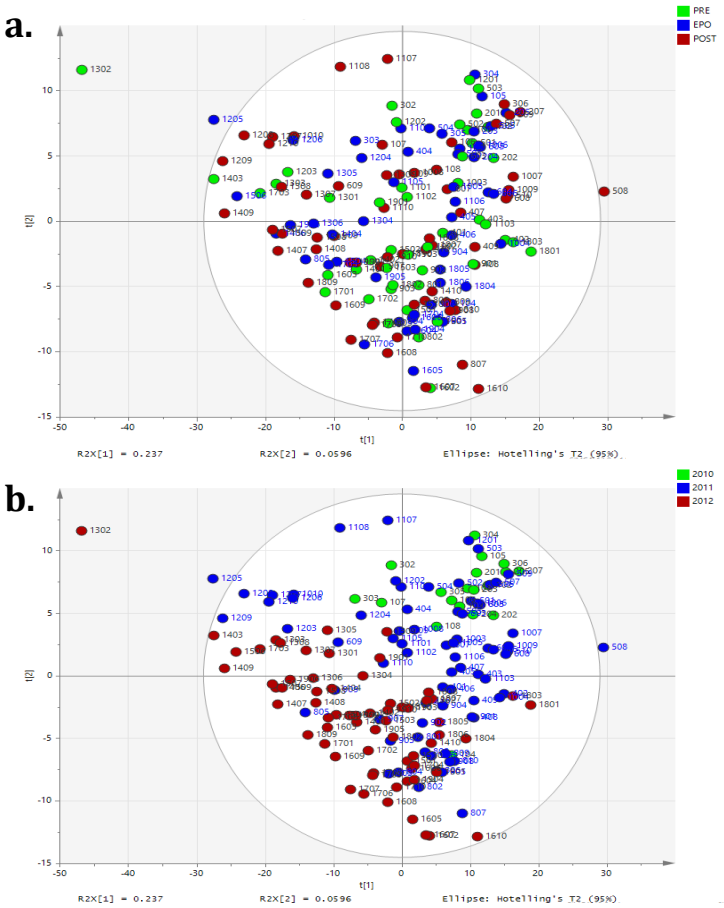


Figure 3-13. PCA of raw filtered plasma metabolite profile with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.754 and Q^2: 0.494 for both models.

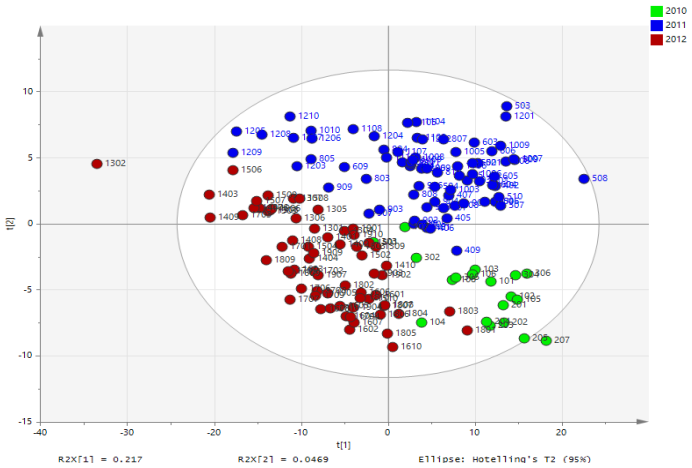


Figure 3-14. PLS-DA of raw filtered plasma metabolite profile with year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.391, R^2Y: 0.91, and Q^2: 0.861 for both models.

The plasma data, normalised to individual metabolic output, creates a similar model to urine as the samples seem to overlap well with no apparent effect of year (Figure 3-15 b). However, PCA of data normalised to selected purines (Figure 3-16) seems to centre the data into a directional line, with high R^2 and Q^2 scores. However, when PLS-DA is applied (Figure 3-17), the year as a factor creates a distinguishable pattern.

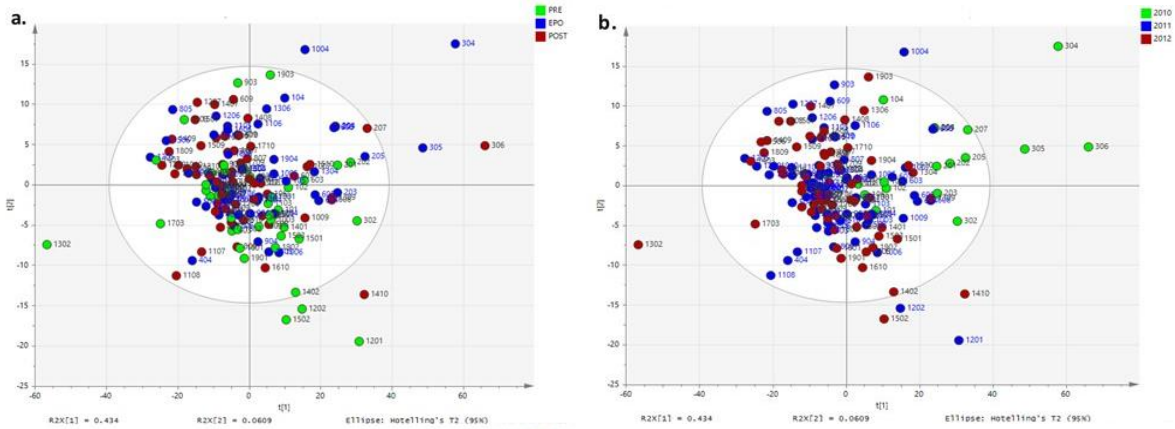


Figure 3-15. PCA of plasma metabolite profile normalised to individual metabolic output with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.729 and Q^2: 0.53 for both models.

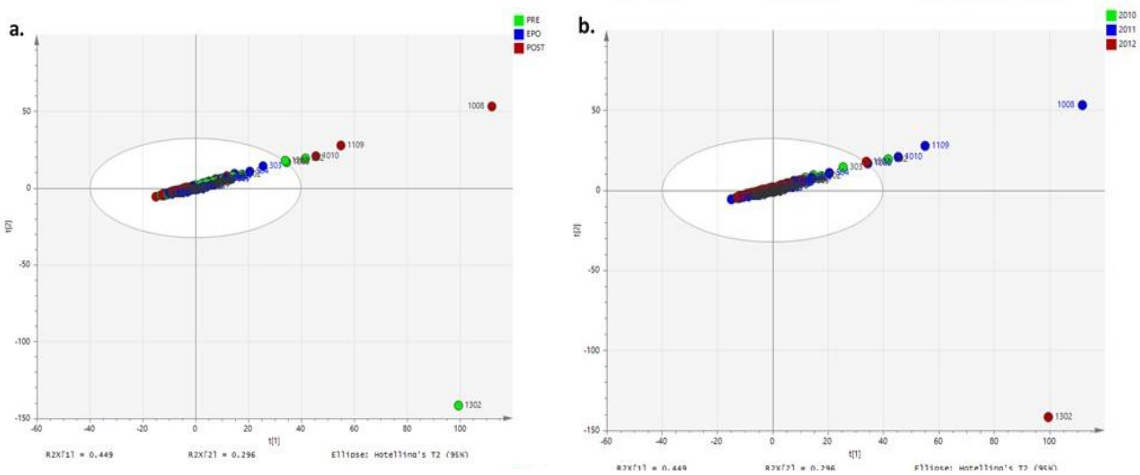


Figure 3-16. PCA of plasma metabolite profile normalised to selected purine metabolites (average time-point response of hypoxanthine and N2,N2-dimethylguanosine) with either **a.** phase (PRE, EPO, POST) and **b.** year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.918 and Q^2: 0.665 for both models. Distribution not affected by the removal of 1302 and 1008 outliers.

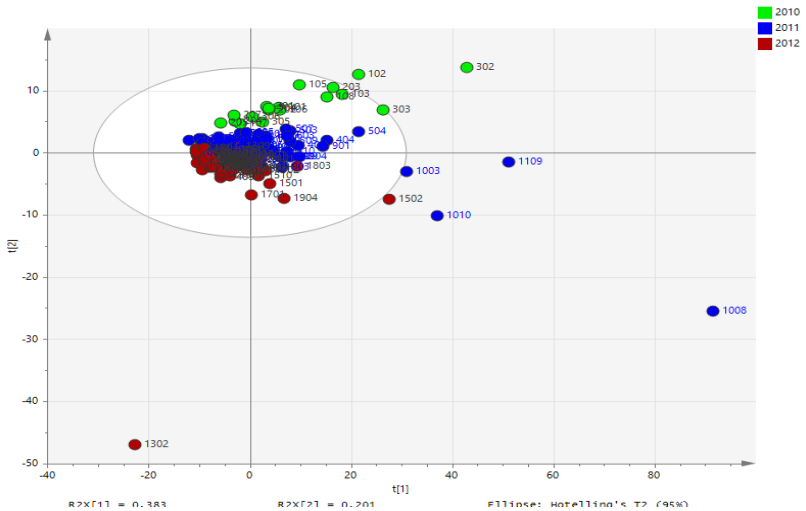


Figure 3-17. PLS-DA of plasma metabolite profile normalised to selected purine metabolites (average time-point response of hypoxanthine and N2,N2-dimethylguanosine) with year (2010, 2011, 2012) comparison. Cumulative R^2X: 0.827, R^2Y: 0.759, and Q^2: 0.658.

3.2.3. Preliminary Urine Correlation Analysis via MetaboAnalyst (Version 3.0)

The full set of urinary metabolite data normalised to individual metabolic output and auto-scaled, for normal distribution curve (Figure 3-18 a), was subjected to Pearson's *r* correlation analysis to observe potential patterns within the dataset. Metabolite markers for correlation were selected by observing the corresponding loading plot in Figure 3-18 b (adenine; Table 3-1), in addition to metabolites previously highlighted in the literature that could be affected by r-HuEPO administration (L-citrulline, L-arginine; Table 3-2). Hypoxanthine, a marker of oxidative stress and exercise (Table 3-3), was also selected in addition to uracil as it is a commonly occurring pyrimidine in RNA that base pairs with adenine (Table 3-1).

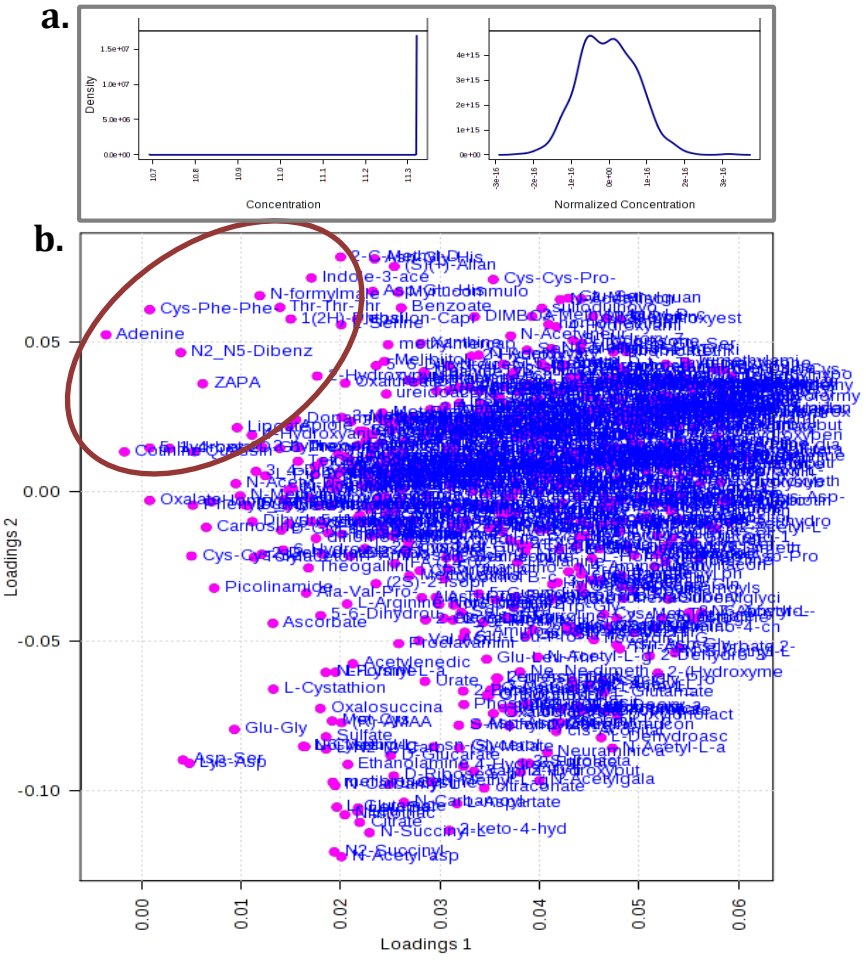


Figure 3-18. Representation of **a.** auto-scaling (mean-centered and divided by the standard deviation of each variable) with normalisation to individual metabolic output and **b.** scatter plot of loadings in normalised urine metabolite profile.

Table 3-1. Top 25 metabolites correlated to **a.** adenine and **b.** uracil with Pearson's *r* correlation analysis.

Adenine (HMDB00034)	Correlation Coefficient	**Uracil** (HMDB00300)	Correlation Coefficient
Cys-Phe-Phe-Phe	0.94	Uridine	0.86
N-formylmaleamate	0.67	Imidazol-5-yl-pyruvate	0.85
methylmercaptoethanol	0.58	Androsterone glucuronide	0.84
epsilon-Caprolactam	0.52	N4-Acetylcytidine	0.84
Indole-3-acetate	0.52	8-Oxocoformycin	0.83
ZAPA	0.51	NicotinamideN-oxide	0.83
1-4-beta-D-Glucan	0.47	4-Hydroxybutanoic acid	0.82
N2,N5-Dibenzoyl-L-ornithine	0.46	7-Aminomethyl-7-carbaguanine	0.82
Oxalate	0.46	sn-glycero-3-Phosphocholine	0.82
S-Sulfo-L-cysteine	0.46	7-Methyladenine	0.82
1(2H)-Phthalazinone	0.44	3_4-dihydroxybenzylamine	0.81
Oxalureate	0.43	5-Aminoimidazole	0.81
Cotinine	0.41	1-Methylhypoxanthine	0.81
Benzoate	0.40	Spermine dialdehyde	0.80
3-Methylguanine	0.39	S-formycinylhomocysteine	0.80
3-Methylcytosine	0.35	11-beta-hydroxyandrosterone-3-glucuronide	0.80
L-cysteine sulfinate	0.35	3',5'-Cyclic AMP	0.79
Xanthine	0.33	2-O-Methylcytosine	0.79
L-Histidine	0.32	Thymine	0.78
Asn-Gln-His	3.89	Decuroside III	0.78
Guanine	0.29	Pregnanediol-3-glucuronide	0.78
L-Serine	0.28	D-isoglutamine	0.77
Cys-Cys-Pro-Pro	0.27	Hexanesulfonate	0.77
S-5-methylthiopentylhydroximoyl-L-cysteine	0.27	Glu-Phe-Cys-Cys	0.77

Table 3-2. Top 25 metabolites correlated to **a.** L-citrulline and **b.** L-arginine with Pearson's *r* correlation analysis.

L-Citrulline (HMDB00904)	Correlation Coefficient	**L-Arginine** (HMDB00517)	Correlation Coefficient
Ala-Ser	0.85	L-Lysine	0.71
L-Albizziine	0.84	Ne_Ne dimethyllysine	0.61
L-Valine	0.83	N6-Methyl-L-lysine	0.59
D-Gluconic acid	0.83	3-Hydroxy-N6,N6,N6-trimethyl-L-lysine	0.59
7-Methyladenine	0.82	O-Carbamoyl-L-serine	0.58
Maleamate	0.82	L-Cystine	0.53
[FA trihydroxy(4:0)] 2_3_4-trihydroxy-butanoic acid	0.81	L-2-Aminoadipate	0.53
2-Oxoglutaramate	0.81	N6,N6,N6-Trimethyl-L-lysine	0.52
N-acetyl-(L)-arginine	0.80	Melibionate	0.52
Leu-Pro	0.80	N6-Methyl-2'-deoxyadenosine	0.50
Trimethylaminoacetone	0.80	Leu-Thr	0.49
1-Methylhypoxanthine	0.80	Leu-Ser	0.49
Slaframine	0.80	Neuraminic acid	0.49
Dethiobiotin	0.80	3-Mercaptolactate-cysteinedisulfide	0.49
sn-glycero-3-Phosphocholine	0.80	D-Gluconic acid	0.48
Iminodiacetate	0.79	NG_NG-Dimethyl-L-arginine	0.48
N6-Acetyl-L-lysine	0.79	L-Aspartate 4-semialdehyde	0.46
Urea-1-carboxylate	0.79	L-Glutamine	0.46
7-Aminomethyl-7-carbaguanine	0.79	Asp-Gly-Pro	0.45
Ala-Met-Cys-Pro	0.79	N2-Succinyl-L-ornithine	0.45
Uridine	0.79	N-Carbamoylsarcosine	0.44
5-Hydroxy-L-tryptophan	0.78	Ascorbate 2-sulfate	0.44
validamine	0.78	Homoarginine	0.43
8-Oxocoformycin	0.78	S-Methyl-1-thio-D-glycerate	0.42

Table 3-3. Top 25 metabolites correlated to hypoxanthine with Pearson's *r* correlation analysis.

Hypoxanthine (HMDB00157)	Correlation Coefficient
Deoxyinosine	0.96
Inosine	0.92
Xanthosine	0.83
Guanine	0.81
Glu-Cys-Cys-Pro	0.67
N2-(D-1-Carboxyethyl)-L-arginine	0.64
Glu-Phe-Cys-Cys	0.64
(S)-3-Methyl-2-oxopentanoic acid	0.61
NicotinamideN-oxide	0.59
1-Methylhypoxanthine	0.59
1-5-diazabicyclononane	0.57
7-Aminomethyl-7-carbaguanine	0.57
L-Allothreonine	0.57
8-Hydroxy-7-methylguanine	0.56
5-Hydroxy-L-tryptophan	0.56
L-Ala-L-Glu	0.56
Xanthine	0.56
sn-glycero-3-Phosphocholine	0.56
Xylitol	0.55
3-Methylguanine	0.55
7-Methyladenine	0.55
8-Oxocoformycin	0.55
Uridine	0.54
N-Acetyl-L-citrulline	0.54

Considering the data in tables 3.1-3.3, perhaps the most interesting set of data is for hypoxanthine (HMDB00157), a constituent of the purine pathway and a prominent maker of physical activity (Daskalaki et al., 2015b). Hypoxanthine is correlated to several metabolites including L-citrulline, adenine and uracil. However, the strongest

association is with the complimentary pathway metabolites deoxyinosine (HMDB00071), inosine (HMDB00195), xanthosine (HMDB00299) as well as guanine (HMDB00132) which are at the top-end of Table 3-3. In addition there are weaker associations with the related metabolites 1-methylhypoxanthine, xanthine (HMDB00292), as well as with modified nucleoside 8-hydroxy-7-methylguanine (HMDB06037) and methylated purine base 3-methylguanine (HMDB01566).

The highest correlation values linked to adenine (Table 3-1 a), support the distribution of metabolites in the loading plot (red circle in Figure 3-18 b). However, it is difficult to attribute a particular biological relevance to the correlated putative metabolites. The purine base adenine, found in RNA and DNA, is a pivotal adenine nucleotide (HMDB00034). If three phosphate groups are combined to adenosine (adenine plus ribose) ATP is formed, whereas the addition of deoxyribose results to deoxyadenosine. From the top-end of the table, indole-3-acetate (or indoleacetic acid; HMDB00197), seems to be one of the more interesting metabolites. Indoleacetic acid is a product of the breakdown in tryptophan metabolism and can also be formed by bacteria in the gut (HMDB00197). Finally, it has also been considered a uremic toxin (Volpe et al., 2014).

Uracil, in Table 3-1 b, forms thymine via methylation which has been highlighted through this analysis. Uracil combines with ribose and deoxyribose and phosphate in order to generate co-factors responsible for the integrity of cell function (HMDB00300). When uracil attaches to a ribose ring via a β-N1-glycosidic bond uridine is formed (HMDB00296), which is most highly correlated metabolite in Table 3-1. Uridine is also correlated with L-citrulline. 7-methyladenine, which may be released from the hydrolysis of alkylated DNA by DNA-3-methyladenine glycosylase II, which is a member of the base removal/repair pathway responsible for the repair of damaged DNA sequence errors which could otherwise result in potential mutations and/or lesions (HMDB11614). This is also correlated to L-citrulline.

Identified nicotinamide N-oxide (HMDB02730), is a precursor to nicotinamide, and the steroid conjugates: androsterone glucuronide (C19-steroid; HMDB02829), 11-beta-hydroxyandrosterone-3-glucuronide (HMDB10351) and pregnanediol-3-glucuronide (HMDB10318). 7-aminomethyl-7-carbaguanine, is involved in the queuine tRNA-ribosyltransferase activity, which forms tRNA (transfer RNA) 7-

aminomethyl-7-carbaguanine plus guanine (HMDB11690); which is also correlated to L-citrulline. The methylated form of hypoxanthine, 1-methylhypoxanthine, is a reaction intermediate to the formation of adenosine as well as nucleic acids (salvage pathway; HMDB13141); this is also correlated to L-citrulline.

L-citrulline, formed from ornithine and carbamoyl phosphate in the urea cycle, is an amino acid (HMDB00904). It is also involved in the release of NO as a by-product in the reaction involving L-arginine and nitric oxide synthase (HMDB00517). There were more metabolites correlated to L-citrulline (Table 3-2 a), such as L-valine (branched chain amino acid; HMDB00883) and trimethylaminoacetone (by-product of carnitine metabolized in the gut microbiome; HMDB12296). Another interesting finding is N6-acetyl-L-lysine (HMDB00206), an acetylated amino acid that can bind to specific locations of bromodomains of different proteins. In some instances this can influence various gene transcriptional complexes to bring about phenotypic alterations (Filippakopoulos and Knapp, 2014). Finally, the amino acid 5-hydroxy-L-tryptophan, is a precursor to the neurotransmitter serotonin (HMDB00472).

L-arginine (Table 3-2 b), which has several vital functions from generating NO, creatine, L-glutamate, L-proline (Luiking and Deutz, 2007) as well as stimulating protein synthesis via the mammalian target of rapamycin, seems to be correlated to several methylated lysine products (HMDB00517). Both lysine and arginine, compete for entry into the cell (Luiking and Deutz, 2007). In addition to L-lysine, other related products include, Ne,Ne dimethllysine (found in histone and related to gene regulation; HMDB13287), N6-methyl-L-lysine (HMDB02038), 3-hydroxy-N6,N6,N6-trimethyl-L-lysine (HMDB01422), N6,N6,N6-trimethyl-L-lysine (carnitine precursor and a co-enzyme of fatty acid oxidation; HMDB01325), as well as L-2-Aminoadipate (also a marker of oxidative stress; HMDB00510).

3.2.4. Preliminary Plasma Correlation Analysis via MetaboAnalyst (Version 3.0)

The full set of plasma metabolite data normalised to individual metabolic output and auto-scaled for normal distribution curve (Figure 3-19 a), was subjected to Pearson's *r* correlation analysis to observe potential patterns within the dataset. Specific metabolite marker for comparison was selected by observing the corresponding loading plot seen in Figure 3-19 b (bilirubin in red circle and Table 3-4 b). Additionally given the link to exercise hypoxanthine was also selected (Table 3-4 a).

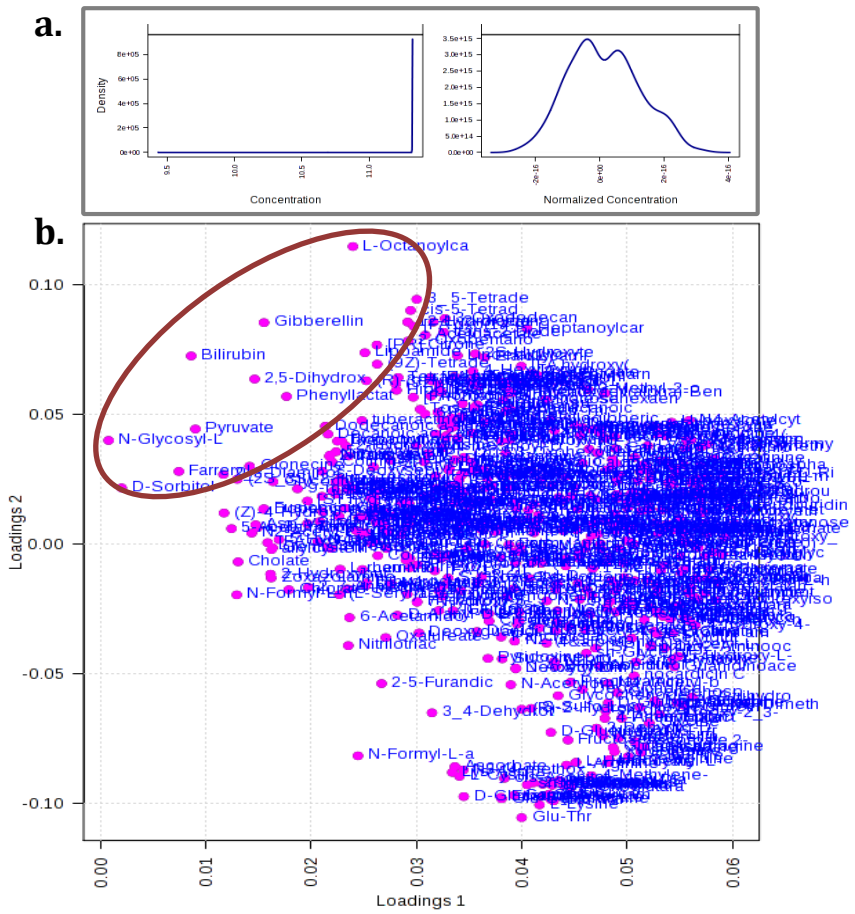


Figure 3-19. Representation of **a.** auto-scaling (mean-centered and divided by the standard deviation of each variable) with normalisation to individual metabolic output and **b.** scatter plot of loadings in normalised plasma metabolite profile.

Table 3-4. Top 25 metabolites correlated to **a.** hypoxanthine or **b.** bilirubin with Pearson's *r* correlation analysis.

Hypoxanthine (HMDB00157)	Correlation Coefficient	**Bilirubin** (HMDB00054)	Correlation Coefficient
6-methyltetrahydropterin	0.94	Pyruvate	0.66
5'-Dehydroadenosine	0.91	[PR] Vitamin E	0.57
Dihydrobiopterin	0.82	α-Ketoglutaricacidoxime	0.55
Xanthine	0.69	Cyclo(deltaAla-L-Val)	0.52
Linoelaidylcarnitine	0.65	2-Oxopentanoic acid	0.52
Elaidiccarnitine	0.65	3-Oxopropanoate	0.52
Leu-Ala	0.64	Acetoacetate	0.51
N2-(D-1-Carboxyethyl)-L-lysine	0.62	Lipoamide	0.51
8-Oxocoformycin	0.60	3-(4-Hydroxyphenyl)pyruvate	0.49
[FA] O-Palmitoyl-R-carnitine	0.59	[FA] O-Palmitoyl-R-carnitine	0.48
Glu-Asp-Phe	0.59	Elaidiccarnitine	0.47
(R)-Lactate	0.58	Succinate semialdehyde	0.49
N2,N2-Dimethylguanosine	0.58	Linoelaidylcarnitine	0.44
L-Glutamate	0.57	L-Octanoylcarnitine	0.44
N-Formyl-L-methionine.1	0.57	2_3-Diaminosalicylic acid	0.44
Pseudouridine	0.57	Dopaminequinone	0.42
2-Hydroxyethanesulfonate	0.57	cis-5-Tetradecenoylcarnitine	0.40
1-Methylhypoxanthine	0.56	3_ 5-Tetradecadiencarnitine	0.37
(S)-3-Methyl-2-oxopentanoic acid	0.56	6-methyltetrahydropterin	0.37
Uridine	0.55	3-Methyl-2-oxobutanoic acid	0.37
Hypoxylone	0.55	trans-2-Dodecenoylcarnitine	0.35
L-Phenylalanine	0.54	Hypoxanthine	0.34
Met-Cys-Gly	0.54	5-Hydroxyferulate	0.34
1-(beta-D-Ribofuranosyl)-1,4-dihydronicotinamide	0.54	[FA hydroxy(10:0)] N-(3S-hydroxydecanoyl)-L-serine	0.34

In plasma, hypoxanthine is strongly correlated to 6-methyltetrahydropterin, a cofactor of phenylalanine and tyrosine hydroxylase (HMDB02249) as well as dihydrobiopterin. The latter, is an oxidised product of tetrahydrobiopterin that is involved in the formation of dopamine and serotonin (neurotransmitters), tyrosine as well as NO from L-arginine and L-citrulline (HMDB00038). The association with the related metabolites such as inosine and xanthosine seems to be absent in this case.

Bilirubin, a degradation product of heme and bile pigment (HMDB00054), has been shown to have diurnal variation/ oscillation, as indicated in a recent review of metabolome-based circadian rhythms (Gooley, 2014). This observation was made through using an untargeted LC-MS analysis, across a 24-h time-period, where participants (8 males) were exposed to a sleep/wake cycle and light/dark stimuli (Ang et al., 2012). It would stand to reason, however, that due to the intervention and changes in red blood cell mass that bilirubin would be seen as an elevated excretory by-product. In spite of this, there are very few correlated findings above 0.5 (correlation coefficient) aside from pyruvate (HMDB00060) which is part of the glycolysis pathway.

3.3. Metabolites of Interest from Urine and Plasma

In order to create a shorter list of potential putative metabolites of interest, the PRE values of each individual were averaged, then this baseline value was subsequently used to compare (*p*-value and ratio) to the other grouped time-points EPO1 (number of significant findings in urine N:1, plasma N:5), EPO2 (urine N:34, plasma N:20) and EPO3 (urine N:16, plasma N:20) as well as POST1 (urine N:8, plasma N:78), POST2 (urine N:5, plasma N:10), POST3 (urine N:25, plasma N:60), and POST4 (urine N:8, plasma N:14). In urine this resulted in a total of 69 (Table 3-5) significant metabolites and in plasma 112 (Table 3-6), with the majority falling in the class of amino acids. It is vital, however, to consider the effect of training as we have previously identified a plethora of metabolites which respond strongly to a physical activity intervention (Daskalaki et al., 2015a, 2015b). In combination with an ESA, Christensen et al. (2013) highlighted the balancing act which could be occurring when training and EPO

are combined, that ultimately may obscure the down-stream metabolic signature of EPO doping. Hence, the metabolites featured in tables 3.5 and 3.6 represent an attempt to decrease the number of metabolites used in building a model, bearing in mind that inter-individual differences in response to the intervention would ultimately have to be considered. The next step would be to model the selected urine and plasma metabolites via OPLS-DA to identify specific patterns that may emerge between the subjects; possibly highlighting an area for metabotyping (section *3.3.1.1 Urine OPLS-DA Modelling*, as well as *3.3.2.1. Plasma OPLS-DA Modelling*). Selected PCA of individual subject profiles from plasma and urine can be seen in appendix x and xi, respectively.

Another valuable point, given that the application of these markers are for anti-doping control purposes, is to identify which findings are most likely to be false positives. In the absence of the false discovery rate (Sugimoto et al., 2012), which is a common tool in gene-expression analysis, a compatible alternative fitting to a metabolomics-based approach would be the ROC curve analysis (Xia et al., 2013). This might allow the possibility of measuring the predictability, based on specificity and sensitivity- both considered a 'must' in anti-doping control, in order to eliminate the chances of an athlete being wrongly accused of doping and that a truly negative test outcome is negative. The metabolites exhibiting a high AUC (above 0.8), would be considered to be strong markers that could also benefit from combined modelling to improve overall predictive value (section *3.4. ROC Curve Analysis*).

3.3.1. Urine Metabolites of Interest

Table 3-5. Normalised urinary metabolites (N:69) which are significantly changed by the r-HuEPO intervention. *p*-Value (*p*) for comparisons PRE (averaged pre-treatment values) *vs.* EPO1, EPO2, EPO3, POST1, POST2, POST3 and POST4. Arrows indicate up (↑) or down (↓) regulated in comparison to PRE.

Mass	RT (min)	Metabolite	HMDB	PRE *vs* EPO1		PRE *vs* EPO2		PRE *vs* EPO3		PRE *vs* POST1		PRE *vs* POST2		PRE *vs* POST3		PRE *vs* POST4	
Amino Acid Metabolites and/or Derivatives				*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*
176.0435	17.132	N-Carbamoyl-L-aspartate# (or Ureidosuccinic acid)	HMDB00828	0.6704	↑	0.2800	↑	**0.0281**	↑	**0.0118**	↑	0.7583	↓	0.0872	↓	**0.0072**	↓
155.070	14.507	L-Histidine*#	HMDB00177	0.6492	↓	0.8828	↓	**0.0339**	↓	**0.0340**	↓	0.6429	↓	0.8346	↓	**0.0078**	↓
105.043	15.709	L-Serine	HMDB00187	0.3091	↓	0.5323	↓	0.6757	↓	0.1543	↓	0.1270	↓	0.4392	↑	**0.0106**	↓
188.1273	25.388	Homoarginine (or Homo-L-arginine)	HMDB00670	0.4164	↓	0.9327	↓	0.7891	↑	0.5097	↑	0.7000	↑	0.3645	↑	**0.0221**	↓
226.107	15.504	Carnosine	HMDB00033	0.5189	↑	0.3130	↑	0.1594	↑	0.4754	↑	0.3612	↑	0.9741	↑	**0.0229**	↓
146.105	23.580	L-Lysine*	HMDB00182	0.6689	↑	0.9022	↑	0.5914	↑	0.1868	↑	0.8015	↓	0.5265	↓	**0.0233**	↓
146.069	14.988	L-Glutamine*	HMDB00641	0.7257	↓	0.3980	↑	0.7619	↑	0.6243	↓	0.8249	↓	0.5104	↓	**0.0467**	↓
240.024	16.272	L-Cystine*	HMDB00192	0.9305	↑	0.5277	↑	0.8250	↑	0.4009	↑	0.9219	↓	**0.0339**	↓	**0.0473**	↓

111.032	11.281	Pyrrole-2-carboxylate#	HMDB04230	0.2631	↑	**0.0227**	↑	**0.0188**	↑	0.2782	↑	0.2692	↑	**0.0330**	↑	0.0716	↑
240.122	13.906	Homocarnosine	HMDB00745	0.3558	↓	**0.0282**	↓	0.8065	↓	0.2850	↓	0.3232	↑	0.1926	↓	0.0799	↓
125.015	15.195	Taurine*	HMDB00251	0.7750	↑	0.3088	↑	0.0928	↑	0.7257	↑	**0.0267**	↑	0.0716	↑	0.0814	↑
156.0173	10.648	Orotate (or Orotic Acid)	HMDB00226	0.7436	↑	**<0.001**	↑	**<0.001**	↑	0.0705	↑	0.1719	↑	0.4865	↓	0.1009	↓
201.136	5.288	Capryloylglycine	HMDB00832	0.1483	↓	0.1253	↑	**0.0437**	↑	0.1476	↓	0.5143	↑	0.3775	↑	0.1388	↓
195.053	9.120	Dopaquinone	HMDB01229	0.2302	↑	0.2270	↑	0.1335	↑	0.6221	↓	0.6532	↑	**0.0233**	↑	0.2660	↓
89.048	14.649	L-Alanine	HMDB00161	0.9588	↑	0.3298	↑	**0.0409**	↑	0.7101	↑	0.5703	↑	0.3707	↑	0.3207	↓
103.0633	14.037	4-Aminobutanoate# (or Gamma-aminobutyric acid)	HMDB00112	0.7202	↑	**0.0022**	↑	**0.0026**	↑	**0.0091**	↑	**0.0296**	↑	0.2906	↑	0.5402	↑
103.0633	12.160	N,N-Dimethylglycine# (or Dimethylglycine)	HMDB00092	0.6086	↑	**0.0158**	↑	**0.0128**	↑	0.1358	↑	0.0703	↑	**0.0353**	↑	0.6568	↑
200.977	17.666	S-Sulfo-L-cysteine (or Cysteine-S-sulfate)	HMDB00731	0.6846	↓	0.0555	↑	0.1115	↑	**0.0193**	↓	0.2692	↑	0.2032	↓	0.7296	↓
246.101	6.753	N-Acetyl-D-tryptophan (or N-acetyltryptophan)	HMDB13713	0.6432	↑	**0.0087**	↑	**0.0345**	↑	0.4538	↑	0.7760	↑	0.2838	↑	0.7436	↑
189.043	6.789	Kynurenate (or Kynurenic acid)	HMDB00715	0.4427	↑	0.4921	↑	0.3762	↑	0.8645	↑	0.4852	↑	**0.0108**	↑	0.7687	↓
205.037	11.838	Xanthurenic acid	HMDB00881	0.6891	↑	0.8544	↓	0.1863	↑	0.5078	↓	0.1541	↑	**0.0273**	↑	0.7695	↑
117.079	12.466	L-Valine*	HMDB00883	0.7439	↓	0.3350	↑	0.5467	↑	0.2940	↓	0.3440	↑	**0.0309**	↑	0.9750	↓
Carbohydrate Conjugates																	
164.069	9.355	L-Rhamnose	HMDB00849	0.6590	↑	**0.0384**	↑	0.4181	↑	0.5080	↑	0.1519	↑	**0.0373**	↑	0.3422	↓
150.053	10.867	D-Xylulose	HMDB01644	0.6448	↑	0.1091	↑	0.1618	↑	0.1260	↓	0.2065	↑	**0.0368**	↑	0.5184	↓

106.026	16.504	D-Glycerate (or Glyceric acid)	HMDB00139	0.5797	↑	0.2109	↑	0.1446	↑	0.5410	↓	**0.0335**	↑	0.6092	↑	0.5545	↑
309.106	11.040	N-Acetylneuraminate (or N-Acetylneuraminic acid)	HMDB00230	0.7469	↓	0.4646	↑	0.0924	↑	0.6228	↑	0.8424	↑	**0.0249**	↑	0.6266	↑
344.132	12.203	Melibiitol	HMDB06791	0.7833	↓	**0.0376**	↑	0.2305	↑	0.1255	↑	0.8010	↑	0.2437	↑	0.7402	↑
134.058	7.617	1-Deoxy-D-xylulose	HMDB01292	0.7942	↓	0.4516	↑	0.3925	↑	**0.0218**	↓	0.8902	↑	0.9755	↓	0.8719	↑
Imidazopyrimidines																	
149.070	13.582	7-Methyladenine	HMDB11614	0.6795	↓	**0.0382**	↑	0.3147	↑	0.9675	↑	0.6238	↑	0.1547	↑	0.7162	↓
181.060	10.643	8-Hydroxy-7-methylguanine	HMDB06037	0.3689	↑	**0.0086**	↑	0.7295	↑	0.3783	↓	0.5637	↑	0.0972	↑	0.7995	↓
149.070	11.082	3-Methyladenine	HMDB11600	0.9616	↑	0.2212	↑	**0.0253**	↑	0.2227	↓	0.9671	↓	0.6510	↑	0.8364	↑
Indoles																	
205.074	8.072	Indolelactate (or Indolelactic acid)	HMDB00671	0.3460	↑	**0.0379**	↑	0.6719	↑	0.3739	↓	0.5015	↑	**0.0248**	↑	0.1234	↑
248.116	7.879	6-Hydroxymelatonin	HMDB04081	0.7700	↓	0.1542	↑	**0.0087**	↑	0.1154	↑	0.1274	↑	**0.0124**	↑	0.2025	↑
191.059	13.889	5-Hydroxyindoleacetate* (or 5-hydroxyindoleacetic acid)	HMDB00763	0.8452	↓	0.1768	↑	0.7841	↑	**0.0177**	↓	0.6930	↑	0.5249	↑	0.9082	↑
175.063	9.127	Indole-3-acetate (or Indoleacetic acid)	HMDB00197	0.4748	↓	**0.0064**	↑	0.8282	↑	0.0771	↓	0.3706	↑	0.4222	↑	0.9212	↓
218.106	6.889	N-Acetylserotonin	HMDB01238	0.9132	↓	**0.0336**	↑	**0.0211**	↑	0.7518	↓	0.4757	↑	0.0554	↑	0.9501	↓
Keto Acids																	
159.089	8.247	3-Dehydrocarnitine	HMDB12154	0.9266	↑	**0.0461**	↑	0.5076	↑	0.5830	↑	0.6085	↑	0.2007	↑	0.3146	↓
146.022	16.046	2-Oxoglutarate	HMDB00208	0.3966	↓	**0.0371**	↓	0.2846	↓	0.5862	↓	0.2121	↓	0.7933	↓	0.3539	↓

160.037	8.344	2-Oxoadipate (or 2-Oxoadipic acid)	HMDB00225	0.9297	↓	0.1002	↑	**0.0495**	↑	0.9064	↑	0.5303	↑	0.1678	↑	0.5043	↑
Microbiome																		
284.090	7.945	p-Cresol glucuronide#	HMDB11686	0.6067	↑	**0.0069**	↑	**0.0361**	↑	0.8014	↓	0.3084	↑	**0.0406**	↑	0.1611	↑
115.100	15.853	Trimethylaminoacetone	HMDB12296	0.5480	↓	0.1372	↑	0.2609	↑	0.5358	↓	0.4594	↑	**0.0357**	↑	0.4949	↑
244.085	7.060	Indolylacryloylglycine	HMDB06005	0.7595	↑	**0.0049**	↑	**0.0233**	↑	0.8310	↑	0.3261	↑	0.1200	↑	0.7443	↓
165.043	5.388	4-Pyridoxolactone	HMDB03454	0.2387	↓	0.0680	↑	0.0547	↑	0.9865	↑	0.3339	↑	**0.0286**	↑	0.7444	↑
213.010	7.921	Indoxyl sulfate	HMDB00682	0.4907	↓	**0.0192**	↑	0.0502	↑	0.7309	↓	0.3508	↑	0.0631	↑	0.7770	↑
Purine Metabolism and/or Derivative																		
152.034	11.773	Xanthine	HMDB00292	0.8723	↑	**0.0125**	↑	0.7499	↑	0.7176	↓	0.0817	↑	0.0753	↑	0.4394	↑
165.065	9.682	3-Methylguanine	HMDB01566	0.8245	↓	**0.0066**	↑	0.5044	↑	0.2048	↓	0.7456	↑	0.1604	↑	0.5750	↓
151.049	12.332	Guanine*	HMDB00132	0.3854	↓	**0.0048**	↑	0.6662	↑	0.5322	↑	0.2044	↑	0.6735	↑	0.6293	↑
166.049	8.505	7-Methylxanthine	HMDB01991	0.2938	↓	0.3742	↑	0.9405	↑	**0.0315**	↓	0.5277	↓	0.4946	↑	0.7095	↓
252.086	8.677	Deoxyinosine	HMDB00071	0.6201	↓	**0.0094**	↑	0.3150	↑	0.4074	↑	0.1827	↑	0.4510	↑	0.9224	↓
136.039	10.239	Hypoxanthine*	HMDB00157	0.7901	↓	**0.0137**	↑	0.2387	↑	0.3188	↑	0.1542	↑	0.3574	↑	0.9569	↓
329.053	9.724	3',5'-Cyclic AMP (or Cyclic AMP)	HMDB00058	0.7789	↑	**0.0156**	↑	0.4913	↑	0.9166	↓	0.3750	↑	**0.0337**	↑	0.9701	↑
Pyridine Metabolism and/or Derivative																		
136.064	23.396	1-Methylnicotinamide	HMDB00699	0.8458	↓	0.4321	↑	0.6715	↓	0.3612	↓	0.2632	↑	**0.0456**	↑	0.4072	↑

183.053	8.371	4-Pyridoxate (or 4-Pyridoxic acid)	HMDB00017	0.6171	↓	**0.0372**	↑	0.2521	↑	0.5829	↑	0.7608	↑	0.3491	↑	0.7277	↑
169.074	8.560	Pyridoxine	HMDB00239	0.4073	↓	0.2653	↓	0.8634	↓	0.8834	↓	**0.0453**	↓	0.8159	↑	0.8297	↓
Pyrimidine Pathway and/or Derivative																	
246.086	10.732	5-6-Dihydrouridine	HMDB00497	0.2358	↓	0.4761	↓	0.8370	↑	0.1751	↓	0.1229	↑	**0.0142**	↓	0.1116	↓
112.027	8.612	Uracil#	HMDB00300	0.7303	↓	**0.0329**	↑	0.0700	↑	0.6397	↑	**0.0436**	↑	**0.0026**	↑	0.2682	↑
125.059	10.542	5-Methylcytosine	HMDB02894	0.3929	↓	**0.0105**	↑	0.3008	↑	0.5379	↑	0.5493	↑	0.4509	↑	0.4978	↓
226.071	9.157	5-Acetylamino-6-formylamino-3-methyluracil	HMDB11105	**0.0303**	↓	0.8908	↓	0.0721	↑	**0.0229**	↓	0.9585	↓	0.4645	↑	0.8250	↓
126.043	7.103	Thymine	HMDB00262	0.6786	↑	**0.0330**	↑	0.2699	↑	0.8284	↑	0.5529	↑	0.0997	↑	0.8264	↓
128.058	14.710	5,6-Dihydrothymine	HMDB00079	0.9945	↓	0.1118	↑	0.1137	↑	0.9388	↑	0.0668	↑	**0.0458**	↑	0.9658	↓
Azoles																	
156.054	11.499	4-Imidazolone-5-propanoate	HMDB01014	0.6406	↑	0.0906	↑	0.3059	↑	0.9975	↓	0.1509	↑	**0.0427**	↑	0.2756	↓
124.064	9.552	Methylimidazole acetaldehyde	HMDB04181	0.6977	↑	**0.0409**	↑	0.5040	↑	0.6067	↑	0.5735	↑	0.2156	↑	0.6058	↓
Miscellaneous																	
390.107	9.004	Dopaxanthin	HMDB12221	0.1162	↑	0.8852	↑	0.1382	↑	0.1703	↓	0.3988	↑	**0.0349**	↑	0.3373	↓
257.102	9.169	sn-glycero-3-Phosphocholine (or Glycerophosphorylcholine)	HMDB00086	0.5559	↑	**0.0332**	↑	0.1294	↑	0.7223	↑	0.6422	↑	**0.0338**	↑	0.7344	↓
172.049	11.509	Hydantoin-5-propionate (or Hydantoin-5-propionic acid)	HMDB01212	0.4110	↓	**0.0302**	↓	0.1667	↓	0.4949	↓	0.4919	↑	0.2236	↑	0.5348	↓

75.069	10.917	Trimethylamine N-oxide[#]	HMDB00925	0.5017	↑	**0.0022**	↑	**0.0019**	↑	0.2450	↑	0.1082	↑	**0.0017**	↑	0.7187	↑
182.058	9.291	Homovanillate (or Homovanillic acid)	HMDB00118	0.3222	↑	0.4804	↑	0.1251	↑	0.9676	↓	0.6580	↑	**0.0435**	↑	0.3604	↓
179.081	8.530	7-Aminomethyl-7-carbaguanine	HMDB11690	0.8872	↓	**0.0432**	↑	0.2610	↑	0.6037	↓	0.7943	↑	0.1195	↑	0.8372	↓
312.674	7.923	Glycochenodeoxycholic acid 3-glucuronide (or Glycochenodeoxycholic acid)	HMDB02579	0.6155	↓	**0.0478**	↑	0.0995	↑	0.4045	↓	0.4888	↑	0.3376	↑	0.7359	↓

*MSI 1: finding matches the retention time of the authentic standard.
[#]Traces (ion chromatogram) generated due to multiple significant time-point comparisons (available in appendix xii).

3.3.1.1. Urine OPLS-DA Modelling

Figure 3-20 illustrates the distribution of subject samples modelled with OPLS-DA to the three phases as factors, and corresponding VIP score plot can be seen in Figure 3-22 a. The resulting outcome shows a clustering of the three groups, ultimately creating three different directional outcomes, however the R^2X, R^2Y and Q^2 scores are very low as there is still an evident centred overlap in the model.

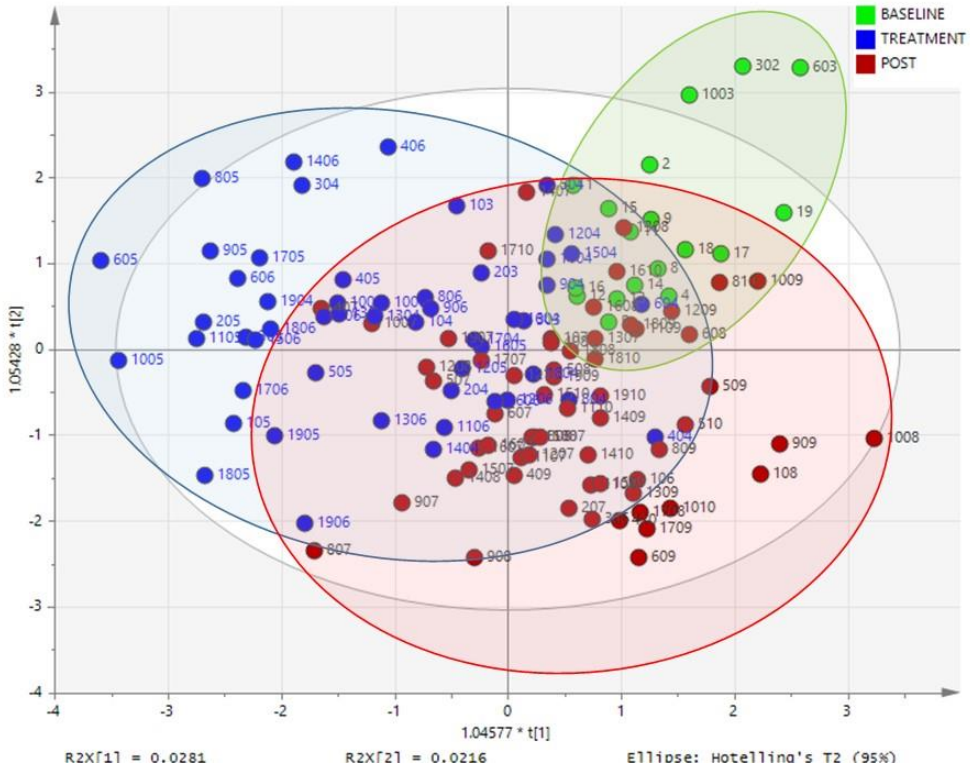


Figure 3-20. OPLS-DA of the 69 putative metabolites highlighted in Table 3-5 with averaged baseline and normalized to individual metabolic output. Cumulative R^2X: 0.55, R^2Y: 0.426 and Q^2: 0.0603.

To improve the model scores, and observe possible metabolites which influence this separation, and contribute to the selected variance, EPO2 and POST3 time-points were selected to compare to BASELINE- see Figure 3-21. The highest number of significant findings was found for these two time-points. What is surprising is the emergence of sub-groups within the phases that start to show clustering of subjects that potentially contribute to the variance in a slightly different way. Subjects 4, 13 and 10 have been

highlighted in the figure to show this variation in response. Figure 3-22 b illustrates VIP score plot for this OPLS-DA model. The R^2 and Q^2 scores are compatible with a fairly robust model.

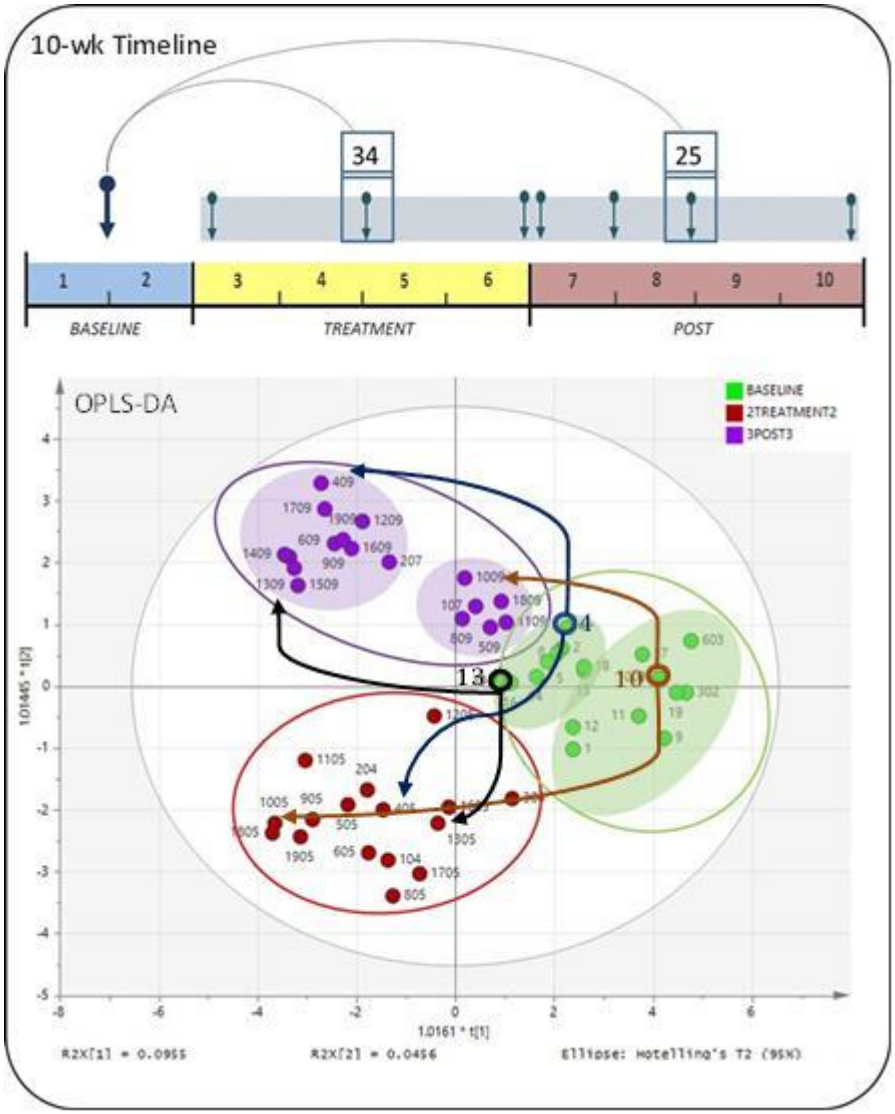


Figure 3-21. OPLS-DA of comparison groups BASELINE, TREATMENT (EPO) 2 as well as POST3 time-points. Two distinct metabotype responses start to emerge within the BASELINE and POST groups. When compared to baseline these two time-points yielded the highest number of significant (p-value < 0.05) putative metabolites as can be seen in the 10-wk timeline above. Cumulative R^2X: 0.589, R^2Y: 0.786 and Q^2: 0.397.

Both VIP score plots highlight the prominent score of orotate (VIP = > 2; HMDB00226), an intermediate in the synthesis of pyrimidine nucleotides. Orotate, was found to reach significance at EPO2 and 3 time-points (p-value < 0.001). Another intermediate product of pyrimidine biosynthesis is N-Carbamoyl-L-aspartate, which received a high score in both plots and reached significance at EPO3, POST1 and POST4 time-points (p-value <0.05). For the latter we observed a negative response in comparison to the other two time-points.

A drop in taurine significance was observed in the second score plot (Figure 3-22 b). In spite of maintaining a VIP score above 1, taurine was not found to be significantly different from BASELINE at POST3 time-point. Taurine is a sulfur amino acid with many biological functions, one being to act as a neurotransmitter in the brain and a drop in taurine levels in plasma has been linked to, for example, depression (HMDB00251).

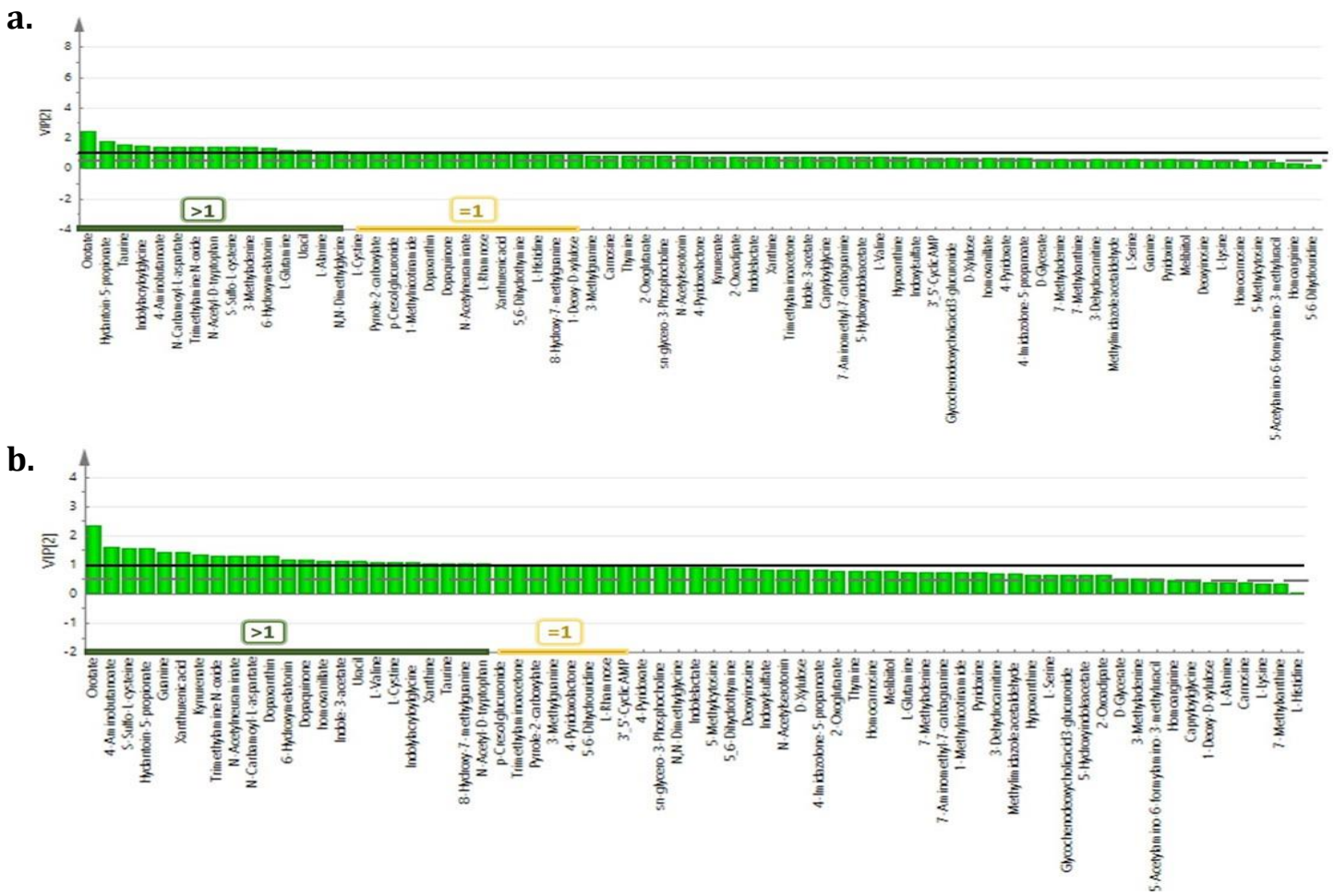


Figure 3-22. VIP score plots for **a.** BASELINE, EPO and POST phase comparison OPLS-DA model and **b.** BASELINE, TREATMENT (EPO) 2, and POST3 OPLS-DA model.

The second highest metabolite in the top VIP score plot is hydantoin-5-propanoate which is a histidine metabolite (HMDB01212). In replacement of this in the bottom score plot, 4-aminobutanoate becomes the second highest metabolite finding. 4-aminobutanoate was found to reach significance at EPO2 and 3 as well as POST1 and 2 time-points (*p*-value <0.05), which may warrant further investigation as a potential marker of either doping or as an aid towards alleviating symptoms of neurological pathologies. Uracil was also observed as a high scoring marker in both plots and reached significance at EPO2, POST2 and POST3 time-points (*p*-value <0.05).

3.3.2. Plasma Metabolites of Interest

Table 3-6. Normalised plasma metabolites (N:112) which are significantly changed by the r-HuEPO intervention. *p*-Value (*p*)for comparisons PRE (averaged pre-treatment values) *vs.* EPO1, EPO2, EPO3, POST1, POST2, POST3 and POST4. Arrows indicate up (↑) or down (↓) regulated in comparison to PRE.

Mass	RT (min)	Metabolite	HMDB	PRE *vs* EPO1		PRE *vs* EPO2		PRE *vs* EPO3		PRE *vs* POST1		PRE *vs* POST2		PRE *vs* POST3		PRE *vs* POST4	
Acyl- Carnitines				*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*
423.334	4.789	Linoelaidyl carnitine#	HMDB06461	**0.0227**	↑	**<0.001**	↑	**<0.001**	↑	**<0.001**	↑	**<0.001**	↑	0.7876	↑	0.1347	↓
425.350	4.745	Elaidic carnitine#	HMDB06464	0.1426	↑	**0.0042**	↑	**<0.001**	↑	**<0.001**	↑	**0.0022**	↑	0.6758	↓	0.1394	↓
275.137	13.529	Glutarylcarnitine	HMDB13130	0.6364	↓	0.1440	↓	0.1097	↓	**0.0115**	↓	0.4409	↓	**0.0209**	↓	0.4793	↓
Amino Acid Metabolites and/or Derivatives																	
240.024	16.969	L-Cystine*	HMDB00192	0.9308	↑	**0.0146**	↓	0.0976	↓	**0.0253**	↓	0.7722	↓	0.4154	↓	0.8291	↓
132.090	23.527	L-Ornithine (or Ornithine)	HMDB00214	0.2971	↓	0.0607	↓	0.2216	↓	0.1210	↓	0.6674	↓	**0.0115**	↓	0.9041	↓
275.112	16.061	Gamma-Glutamylglutamine	HMDB11738	0.2714	↓	**0.0384**	↓	0.1942	↓	**0.0104**	↓	0.6967	↓	**0.0152**	↓	0.9366	↓
337.094	15.353	S-(Hydroxymethyl)glutathione	HMDB04662	0.4015	↑	0.9601	↓	0.4435	↓	**0.0017**	↓	**0.0466**	↓	0.0513	↓	0.9872	↓
226.106	11.842	Carnosine	HMDB00033	**0.0095**	↓	0.1797	↓	0.8058	↓	0.1458	↓	0.8173	↑	0.3902	↓	**0.0003**	↓

156.017	10.932	Orotate	HMDB00226	0.1493	↑	0.0563	↑	**0.0137**	↑	**0.0445**	↑	0.4534	↓	0.1061	↓	**0.0175**	↓
173.105	10.048	N-Acetyl-L-leucine (or N-Acetylleucine)	HMDB11756	0.2948	↓	0.5782	↓	0.5235	↓	0.0864	↓	0.9168	↑	0.6230	↓	**0.0270**	↓
191.062	6.616	N-Acetylmethionine (or N-acetyl-L-methionine)	HMDB11745	0.2342	↑	0.6547	↑	0.8496	↑	0.4058	↓	0.5218	↓	0.1639	↓	**0.0311**	↓
285.132	9.377	Glycylprolylhydroxyproline	HMDB02171	0.3693	↑	0.3869	↓	0.7016	↓	**0.0170**	↓	0.4981	↑	0.1423	↓	**0.0321**	↓
89.048	15.525	L-Alanine	HMDB00161	0.9898	↑	0.2529	↓	0.2205	↓	**0.0044**	↓	0.2576	↓	**0.0147**	↓	**0.0383**	↓
160.085	11.386	D-Alanyl-D-alanine	HMDB03459	0.1608	↓	0.0506	↓	0.1331	↓	0.1099	↓	0.1483	↓	0.0797	↓	**0.0445**	↓
189.111	15.818	L-Homocitrulline (or Homocitrulline)	HMDB00679	0.4652	↓	0.2176	↓	0.4240	↓	**0.0257**	↓	0.8213	↓	**0.0498**	↓	0.0760	↓
165.046	14.072	L-Methionine S-oxide# (or Methionine sulfoxide)	HMDB02005	0.4780	↓	**0.0461**	↓	**0.0177**	↓	**0.0052**	↓	0.2181	↓	**0.0025**	↓	0.1093	↓
188.116	13.458	N2-Acetyl-L-lysine (or N-Alpha-acetyllysine)	HMDB00446	0.4096	↑	0.8002	↑	0.3108	↑	0.5464	↓	0.4244	↓	**0.0041**	↓	0.1449	↓
220.085	10.627	5-Hydroxy-L-tryptophan	HMDB00472	0.7431	↓	0.2062	↓	0.2135	↓	**0.0209**	↓	0.4371	↓	0.2250	↓	0.1821	↓
229.088	15.624	Ergothioneine	HMDB03045	0.1931	↑	0.5670	↓	0.2960	↓	**0.0005**	↓	0.2161	↓	0.2699	↓	0.1923	↑
174.137	21.898	Ne,Ne dimethyllysine	HMDB13287	0.6002	↑	0.6102	↑	0.9223	↓	0.6236	↓	0.8244	↓	**<0.001**	↓	0.1933	↓
117.079	13.129	L-Valine*	HMDB00883	0.7317	↓	0.1494	↓	0.4010	↓	**0.0406**	↓	0.8916	↑	0.3379	↓	0.2070	↓
133.038	15.521	L-Aspartate*# (or L-Aspartic acid)	HMDB00191	0.1554	↓	**0.0447**	↓	**0.0409**	↓	**0.0290**	↓	0.1242	↓	**0.0054**	↓	0.2216	↓
161.069	11.953	L-2-Aminoadipate (or 2-aminoadipate)	HMDB00510	0.6927	↓	0.7879	↓	0.4668	↑	0.1222	↓	0.9346	↑	**0.0338**	↓	0.2712	↓
119.058	15.198	L-Threonine*	HMDB00167	0.9840	↑	0.2426	↓	0.3968	↓	**0.0049**	↓	0.8451	↑	0.1119	↓	0.2954	↓

208.085	11.355	L-Kynurenine*	HMDB00684	0.7616	↑	0.4056	↓	0.4417	↓	**0.0365**	↓	0.8799	↑	0.3530	↓	0.2956	↓
173.069	10.230	N-Acetyl-L-glutamate 5-semialdehyde	HMDB06488	0.6311	↑	0.5786	↓	0.6539	↓	**0.0190**	↓	0.0915	↓	**0.0221**	↓	0.3235	↓
103.063	12.829	4-Aminobutanoate (or Gamma-aminobutyric acid)	HMDB00112	0.0837	↓	0.1062	↓	0.3428	↓	**0.0057**	↓	0.1688	↓	**0.0037**	↓	0.3354	↓
117.054	16.629	Guanidinoacetate (or Guanidoacetic acid)	HMDB00128	0.8095	↑	0.1691	↓	0.4829	↓	**0.0039**	↓	0.2396	↓	**0.0345**	↓	0.3418	↓
189.064	15.081	N-Acetyl-L-glutamate* (or N-Acetylglutamic acid)	HMDB01138	0.3420	↓	0.1750	↓	0.1752	↓	0.0919	↓	0.2009	↓	**0.0214**	↓	0.3477	↓
75.032	16.464	Glycine	HMDB00123	0.7887	↓	0.1156	↓	0.1076	↓	**<0.001**	↓	0.1243	↓	**0.0126**	↓	0.3663	↓
147.053	11.461	L-Glutamate* (or L-Glutamic Acid)	HMDB00148	0.3581	↑	0.5777	↑	0.9569	↑	0.4315	↓	0.4398	↓	**0.0475**	↓	0.4001	↓
188.152	22.692	N6,N6,N6-Trimethyl-L-lysine	HMDB01325	0.3953	↑	0.2394	↓	0.1613	↓	**0.0324**	↓	0.4692	↓	**0.0015**	↓	0.4022	↓
204.090	12.279	L-Tryptophan*	HMDB00929	0.9340	↓	0.4966	↓	0.4802	↓	**0.0131**	↓	0.6816	↑	0.1619	↓	0.4217	↓
132.053	16.015	L-Asparagine	HMDB00168	0.7740	↓	0.2524	↓	0.4654	↓	**0.0143**	↓	0.6761	↓	**0.0272**	↓	0.4522	↓
188.116	15.680	N6-Acetyl-L-lysine*	HMDB00206	0.7779	↓	0.4774	↓	0.4112	↓	**0.0069**	↓	0.5955	↓	**0.0363**	↓	0.4563	↓
188.127	27.083	Homoarginine (or Homo-L-arginine)	HMDB00670	0.7322	↓	0.0637	↓	**0.0445**	↓	**0.0050**	↓	0.9371	↑	**0.0143**	↓	0.4598	↓
174.112	26.679	L-Arginine*	HMDB00517	0.5407	↓	**0.0350**	↓	0.0663	↓	**0.0019**	↓	0.3617	↓	**0.0006**	↓	0.4742	↓
160.121	24.052	N6-Methyl-L-lysine	HMDB02038	0.8027	↓	0.4027	↓	0.2905	↓	0.1517	↓	0.9354	↑	**0.0059**	↓	0.4881	↓
169.085	13.681	N(pi)-Methyl-L-histidine* (or 3-Methylhistidine)	HMDB00479	0.9386	↑	0.3565	↓	0.1387	↓	**0.0033**	↓	0.5878	↓	0.1017	↓	0.5043	↑

202.143	22.092	NG,NG-Dimethyl-L-arginine (or Asymmetric dimethylarginine)	HMDB01539	0.8522	↓	0.6469	↓	0.3174	↓	**0.0422**	↓	0.7212	↓	**0.0365**	↓	0.5207	↓
155.069	16.039	L-Histidine*#	HMDB00177	0.5085	↓	**0.0311**	↓	**0.0444**	↓	**0.0011**	↓	0.3113	↓	**0.0157**	↓	0.5271	↓
175.096	16.661	L-Citrulline	HMDB00904	0.3928	↓	0.1862	↓	0.2209	↓	0.0586	↓	0.6463	↓	**0.0082**	↓	0.5348	↓
146.069	15.785	L-Glutamine	HMDB00641	0.7149	↓	0.2130	↓	0.3053	↓	**0.0147**	↓	0.4194	↓	**0.0184**	↓	0.5355	↓
105.043	16.532	L-Serine*	HMDB00187	0.4142	↓	0.0959	↓	0.1132	↓	**0.0025**	↓	0.4680	↓	**0.0188**	↓	0.5500	↓
264.111	6.830	alpha-N-Phenylacetyl-L-glutamine	HMDB06344	0.4143	↑	0.2221	↑	**0.0267**	↑	0.8411	↓	0.8866	↓	0.7213	↓	0.6213	↑
146.105	25.237	L-Lysine	HMDB00182	0.4304	↓	**0.0349**	↓	0.0543	↓	**0.0327**	↓	0.9007	↑	**0.0160**	↓	0.6493	↓
172.085	9.326	Glycylproline	HMDB00721	0.9400	↑	0.9085	↑	0.3575	↓	0.0681	↓	0.1118	↓	**0.0233**	↓	0.6909	↓
236.080	9.452	N-Formyl-D-kynurenine	HMDB01200	0.0777	↑	0.1863	↓	0.0526	↓	**0.0114**	↓	0.6560	↑	**0.0441**	↓	0.7255	↑
Carbohydrate Conjugates																	
172.014	15.102	sn-Glycerol 3-phosphate (or Glycerol 3-phosphate)	HMDB00126	0.1981	↓	0.1287	↓	0.0956	↓	**0.0317**	↓	0.5161	↓	**0.0212**	↓	0.0764	↓
106.027	12.858	D-Glycerate (or Glyceric acid)	HMDB00139	0.3061	↑	0.3493	↓	0.6351	↓	0.0656	↓	0.5663	↓	**0.0163**	↓	0.0928	↓
309.106	14.519	N-Acetylneuraminate (or N-Acetylneuraminic acid)	HMDB00230	0.3952	↓	0.7600	↓	0.4776	↓	0.5185	↓	0.5172	↓	**0.0174**	↓	0.1017	↓
179.079	15.238	D-Glucosamine* (or Glucosamine)	HMDB01514	0.7070	↓	0.4571	↓	0.2957	↓	**0.0007**	↓	0.5105	↓	**0.0153**	↓	0.1600	↓
293.112	12.639	N-Acetylmuramate	HMDB60493	0.9609	↓	0.5115	↓	0.7876	↓	**0.0220**	↓	0.2630	↓	0.1428	↓	0.1943	↓
150.053	15.637	D-Xylulose	HMDB01644	0.9991	↑	0.7479	↓	0.7359	↓	**0.0273**	↓	0.4800	↓	0.2044	↓	0.2583	↓

194.043	15.500	D-Glucuronate	HMDB00127	0.9206	↑	0.1446	↓	0.0733	↓	**0.0070**	↓	0.9070	↑	0.3400	↓	0.3006	↓
192.027	9.600	(4R,5S)-4,5,6-Trihydroxy-2,3-dioxohexanoate (or 2,3-Diketo-L-gulonate)	HMDB06511	0.8017	↑	0.2869	↓	0.0814	↓	0.0586	↓	**0.0325**	↓	**0.0088**	↓	0.3293	↓
267.096	15.056	Neuraminic acid	HMDB00830	0.6491	↓	0.8841	↓	0.1000	↓	**0.0140**	↓	0.5416	↓	0.8998	↓	0.3350	↓
180.064	15.643	D-Glucose*	HMDB00122	0.9144	↑	0.8871	↓	0.7028	↓	**0.0163**	↓	0.5376	↓	0.1773	↓	0.4172	↓
215.056	16.357	sn-glycero-3-Phosphoethanolamine	HMDB59660	0.1232	↓	**0.0475**	↓	0.0955	↓	**0.0246**	↓	0.0986	↓	**0.0010**	↓	0.4270	↓
196.059	15.082	L-Gulonate (or Gulonic Acid)	HMDB03290	0.6092	↓	0.2887	↓	0.0542	↓	**0.0372**	↓	0.0803	↓	**0.0148**	↓	0.5149	↓
150.053	10.949	D-Xylose	HMDB00098	0.5226	↑	0.4579	↓	0.6651	↓	**0.0497**	↓	0.5866	↓	0.1718	↓	0.7973	↓
Carboxylic Acid and Derivatives																	
161.033	16.445	N-Formyl-L-aspartate#	HMDB60495	**0.0159**	↓	**0.0054**	↓	**0.0087**	↓	**0.0152**	↓	**0.0245**	↓	**0.0051**	↓	**0.0152**	↓
206.043	11.026	2-Hydroxybutane-1,2,4-tricarboxylate (or Homocitric acid)	HMDB03518	0.3743	↓	0.9567	↑	0.3383	↑	0.3411	↑	0.8276	↓	**0.0088**	↓	0.0671	↓
134.022	17.113	(S)-Malate (or L-Malic Acid)	HMDB00156	0.8527	↓	0.1308	↓	0.1448	↓	**0.0257**	↓	0.7784	↓	0.0559	↓	0.6195	↓
192.027	19.146	Citrate* (or Citric Acid)	HMDB00094	0.7547	↓	0.4193	↓	0.0753	↓	**0.0490**	↓	0.2915	↓	**0.0266**	↓	0.7958	↓
Pteridine and Derivatives																	
328.102	9.543	7-Hydroxy-6-methyl-8-ribityl lumazine	HMDB04256	0.7424	↑	0.9399	↓	0.5801	↓	**0.0390**	↓	0.0631	↓	0.4979	↓	0.0794	↓
239.102	10.798	Dihydrobiopterin*	HMDB00038	0.4324	↑	**0.0249**	↑	**0.0089**	↑	**0.0489**	↑	0.0588	↑	0.5537	↑	0.9032	↑
181.096	10.784	6-methyltetrahydropterin	HMDB02249	0.4614	↑	**0.0032**	↑	**0.0114**	↑	**0.0199**	↑	0.0573	↑	0.3289	↑	0.9850	↑

Purine Pathway and/or Derivative																		
281.112	14.307	1-Methyladenosine	HMDB03331	0.1995	↓	0.3270	↓	0.3030	↓	**0.0145**	↓	0.7642	↓	**0.0149**	↓	**0.0052**	↓
297.089	7.043	5'-Methylthioadenosine*	HMDB01173	**0.0443**	↑	0.3323	↑	0.5469	↑	0.0748	↓	0.8065	↑	0.0748	↓	0.6438	↓
347.064	15.187	AMP*	HMDB00045	0.3367	↓	0.1194	↓	0.1067	↓	**0.0237**	↓	0.2964	↓	0.0623	↓	0.8496	↓
136.039	10.779	Hypoxanthine*#	HMDB00157	0.4871	↑	**0.0100**	↑	**0.0057**	↑	**0.0178**	↑	**0.0431**	↑	0.6267	↑	0.9399	↑
Pyrimidines and/or Derivative																		
228.075	8.607	Deoxyuridine#	HMDB00012	0.2074	↑	**0.0277**	↑	**<0.001**	↑	**0.0293**	↑	**0.0202**	↑	0.6862	↓	0.8106	↓
227.091	10.505	Deoxycytidine	HMDB00014	0.9781	↓	0.4017	↓	0.1792	↓	0.1254	↓	0.3505	↓	**0.0224**	↓	0.2746	↓
257.101	9.670	5-Methylcytidine	HMDB00982	0.6774	↑	0.1089	↑	**0.0098**	↑	0.5533	↑	0.7334	↓	0.8102	↓	0.6233	↑
226.070	9.280	5-Acetylamino-6-formylamino-3-methyluracil	HMDB11105	0.1883	↓	0.4921	↓	0.2443	↑	**0.0373**	↓	0.2147	↓	0.4167	↓	0.6354	↓
126.043	12.411	Thymine	HMDB00262	0.3369	↑	0.2337	↓	**0.0421**	↓	**0.0023**	↓	0.1688	↓	0.0529	↓	0.0745	↓
Fatty Acid and Conjugates																		
145.038	9.177	4-Oxoglutaramate (or 2-Keto-glutaramic acid)	HMDB01552	0.8333	↑	0.3050	↑	0.0976	↑	0.6783	↓	0.7886	↓	**0.0290**	↓	**0.0122**	↓
145.110	13.956	4-Trimethylammoniobutanoate (or 4-Trimethylammoniobutanoic acid)	HMDB01161	0.6797	↑	0.3868	↓	0.2643	↓	**0.0057**	↓	0.4813	↓	**0.0072**	↓	0.0671	↓
145.038	11.339	2-Oxoglutaramate (or 2-Keto-glutaramic acid)	HMDB01552	0.8503	↓	0.4936	↓	0.0614	↓	**0.0004**	↓	0.0902	↓	**0.0051**	↓	0.0767	↓

214.132	10.265	Dethiobiotin	HMDB03581	0.7503	↑	0.2466	↓	0.4718	↓	**0.0057**	↓	0.4110	↓	**0.0289**	↓	0.2008	↓
132.042	15.650	2-Acetolactate	HMDB06833	0.8637	↓	0.8159	↓	0.6955	↓	**0.0206**	↓	0.5190	↓	0.1193	↓	0.2964	↓
368.366	3.890	Tetracosanoic acid	HMDB02003	0.8823	↓	0.7638	↓	0.2398	↑	0.8411	↑	0.4577	↓	**0.0169**	↓	0.4658	↑
Glycerophospholipids																	
493.317	5.076	LysoPC(16:1(9Z))	HMDB10383	0.3776	↓	0.2118	↓	0.1551	↓	**0.0180**	↓	0.3043	↓	0.0848	↓	0.1216	↓
257.103	15.110	sn-glycero-3-Phosphocholine (or Glycerophosphorylcholine)	HMDB00086	0.2358	↓	0.0923	↓	0.0949	↓	**0.0055**	↓	0.2169	↓	**0.0171**	↓	0.2093	↓
Imidazolidines																	
172.049	11.938	Hydantoin-5-propionate (or Hydantoin-5-propionic acid)	HMDB01212	0.2384	↓	**0.0070**	↓	**0.0301**	↓	0.1565	↓	0.8574	↓	0.9261	↓	0.2270	↓
158.044	14.773	Allantoin*	HMDB00462	0.4510	↓	0.4702	↑	**0.0481**	↑	0.7184	↑	0.3887	↓	0.1787	↓	0.4716	↓
Microbial																	
117.058	10.606	Indole	HMDB00738	0.7111	↑	0.9914	↓	0.5393	↑	**0.0235**	↓	0.7878	↑	0.8220	↓	0.6201	↑
251.100	14.068	Muramic acid	HMDB03254	0.2866	↓	0.1073	↓	**0.0119**	↓	**0.0001**	↓	0.2560	↓	**0.0083**	↓	**0.0009**	↓
190.095	14.226	meso-2_6-Diaminoheptanedioate (or Diaminopimelic acid)	HMDB01370	0.6025	↓	0.0588	↓	0.3619	↓	**0.0288**	↓	0.4063	↓	0.1382	↓	0.4768	↓
147.053	15.546	O-Acetyl-L-serine	HMDB03011	0.0887	↓	0.0934	↓	0.0750	↓	**0.0081**	↓	**0.0491**	↓	**0.0083**	↓	0.7373	↓
Steroid Conjugates																	
625.346	8.164	Glycochenodeoxycholicacid 3-glucuronide	HMDB02579	0.7585	↓	0.2453	↓	0.2466	↓	**0.0352**	↓	0.0788	↓	**<0.001**	↓	**0.0305**	↓

264.636	5.079	Glycochenodeoxycholate 7-sulfate (or N-[(3a,5b,7a)-3-hydroxy-24-oxo-7-(sulfooxy)cholan-24-yl]-Glycine)	HMDB02496	0.5584	↑	0.9794	↑	0.9126	↓	0.3135	↓	0.0787	↓	**0.0084**	↓	0.3345	↓
284.162	8.234	Deoxycholicacid3-glucuronide	HMDB02596	0.3468	↓	0.1769	↓	0.3071	↓	**<0.001**	↓	0.1691	↓	**0.0381**	↓	0.5729	↓
Tetrapyrroles and Derivatives																	
584.264	4.145	Bilirubin	HMDB00054	0.5669	↓	0.2273	↑	0.1991	↑	0.1540	↑	**0.0175**	↑	**0.0498**	↑	0.0893	↓
582.248	4.583	Biliverdin	HMDB01008	0.0565	↓	0.7177	↑	0.5238	↑	0.2518	↑	**0.0133**	↑	0.7545	↑	0.2149	↓
Keto-Acids and Derivatives																	
88.016	7.024	Pyruvate (or Pyruvic Acid)	HMDB00243	0.0946	↑	0.3168	↑	0.0672	↑	0.1354	↑	0.1367	↑	**0.0120**	↑	0.6060	↑
159.090	7.638	3-Dehydrocarnitine	HMDB12154	0.2783	↑	**0.0389**	↑	0.1394	↑	0.1403	↑	0.1733	↑	0.4659	↑	0.7576	↓
187.084	12.399	6-Acetamido-2-oxohexanoate (or 2-Keto-6-acetamidocaproate)	HMDB12150	0.2717	↑	0.7102	↓	0.2693	↓	**0.0161**	↓	0.6822	↓	0.4649	↓	0.8578	↓
Miscellaneous																	
156.053	11.918	4-Imidazolone-5-propanoate	HMDB01014	0.9791	↓	0.1340	↓	0.2366	↓	**0.0033**	↓	0.2263	↓	0.2619	↓	0.1800	↓
165.043	8.001	Formylanthranilate (or Formylanthranilic acid)	HMDB04089	0.8294	↑	0.6122	↓	0.5031	↑	0.3003	↓	0.5349	↑	**0.0186**	↓	0.8664	↑
183.066	15.614	Choline phosphate	HMDB01565	0.1229	↓	**0.0492**	↓	0.0861	↓	**0.0067**	↓	0.0918	↓	**0.0127**	↓	0.6957	↓
235.012	16.531	1-Phosphatidyl-1D-myo-inositol3-phosphate	HMDB03850	0.8727	↓	0.5031	↓	0.7372	↓	**0.0261**	↓	0.4168	↓	0.1018	↓	0.0690	↓

180.064	17.984	myo-Inositol	HMDB00211	0.6524	↑	0.2056	↓	0.2129	↓	**0.0279**	↓	0.4087	↓	0.0665	↓	0.6117	↓
176.032	16.318	Ascorbate (or Ascorbic Acid)	HMDB00044	0.7099	↓	0.0979	↓	0.2454	↓	**0.0067**	↓	0.3055	↑	0.6642	↓	0.0847	↓
336.231	4.237	Leukotriene B4	HMDB01085	0.6867	↓	0.5849	↓	0.7888	↓	0.3114	↓	0.1176	↑	0.6364	↓	**0.0457**	↓
440.387	3.903	MG(0:0/24:1(15Z)/0:0)	HMDB11559	0.6788	↓	**0.0266**	↓	0.6646	↓	**0.0233**	↓	0.2516	↓	**0.0326**	↓	0.1367	↓
141.019	16.733	Ethanolamine phosphate* (or O-Phosphoethanolamine)	HMDB00224	0.2103	↓	**0.0402**	↓	0.0585	↓	0.0856	↓	0.1423	↓	**0.0136**	↓	0.8812	↓
110.037	5.790	p-Benzenediol (or Hydroquinone)	HMDB02434	0.1732	↑	0.4226	↑	**0.0272**	↑	0.3027	↑	0.3492	↑	0.6059	↑	0.0756	↑
300.209	4.298	[PR] Tretinoin/All-Trans Retinoic Acid	HMDB01852	0.7415	↓	0.2375	↓	**0.0488**	↓	**0.0005**	↓	0.1759	↓	0.1889	↓	**0.0039**	↓
129.043	10.942	L-1-Pyrroline-3-hydroxy-5-carboxylate (orPyrroline hydroxycarboxylic acid)	HMDB01369	0.5839	↓	0.5486	↓	0.7058	↓	**0.0163**	↓	0.5490	↓	**0.0350**	↓	0.0667	↓
674.536	4.552	SM(d18:1/14:0)	HMDB12097	0.8324	↑	0.4353	↓	0.5278	↓	**0.0200**	↓	0.3415	↓	0.2238	↓	0.2708	↓

*MSI 1: finding matches the retention time of the authentic standard.
#Traces (ion chromatogram) generated due to multiple significant time-point comparisons (available in appendix xiii).

3.3.2.1. Plasma OPLS-DA Modelling

Figure 3-23 illustrates the distribution of subject samples modelled with OPLS-DA to the three phases as factors which shows a similar pattern to that which was observed in urine emerging. Given the high number of significant findings in EPO2 and EPO3 time-points, modelling with BASELINE samples highlights their similar response and overlap (Figure 3-24).

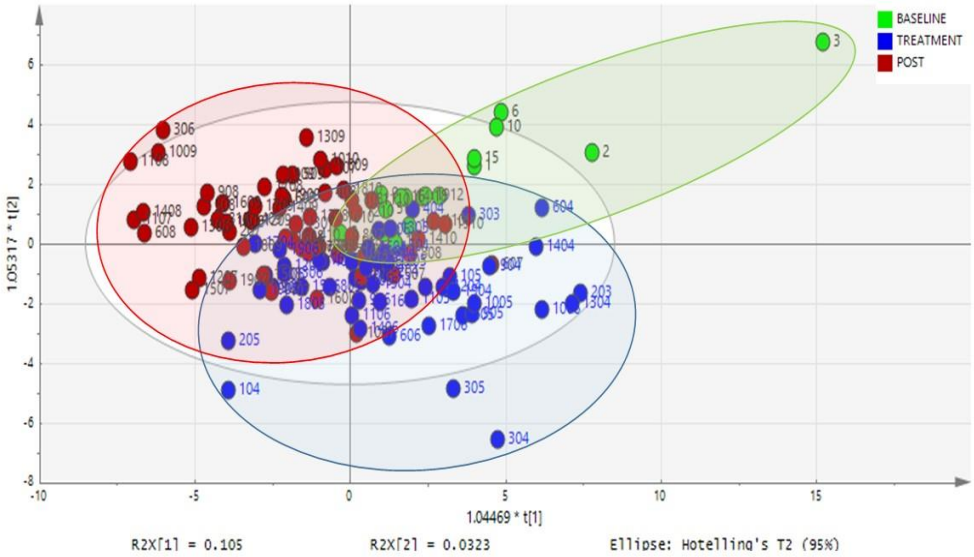


Figure 3-23. OPLS-DA of the 112 identified putative metabolites with averaged baseline. Cumulative R^2X: 0.672, R^2Y: 0.385 and Q^2: 0.217.

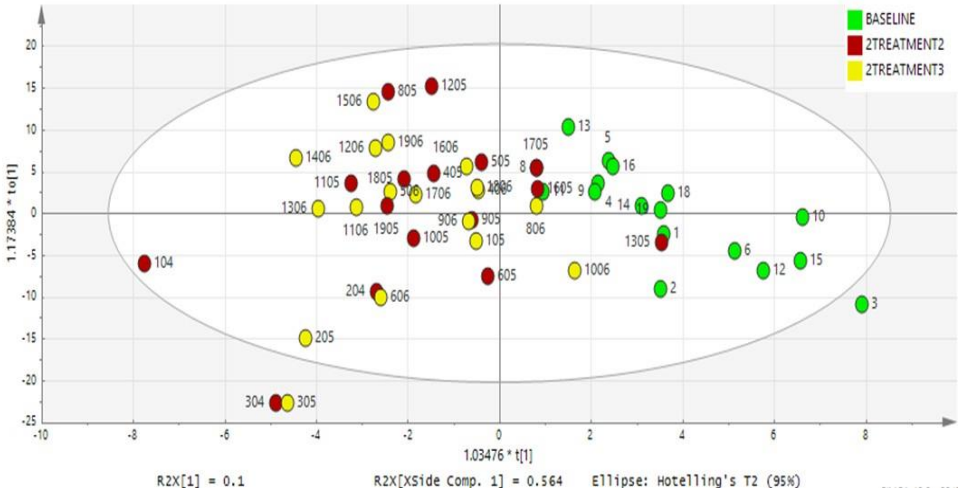


Figure 3-24. OPLS-DA of the 112 significant metabolites with averaged baseline compared to EPO2 and EPO3 time-points. Cumulative R^2X: 0.664, R^2Y: 0.302 and Q^2: 0.205.

The best two models were selected, in order to observe patterns between the subject samples. The first, shown in Figure 3-25, combines the EPO2 and EPO3 time-points, which improves the separation to BASELINE, as well as R^2X, R^2Y and Q^2 scores considerably. The second, shown in Figure 3-26, was created by comparing POST1 and POST3 with BASELINE. This particular model, once again, starts to exhibit individual subject sub-groups within the BASELINE phase. Corresponding VIP score plots for model one and two can be seen in Figure 3-27 a and b, respectively.

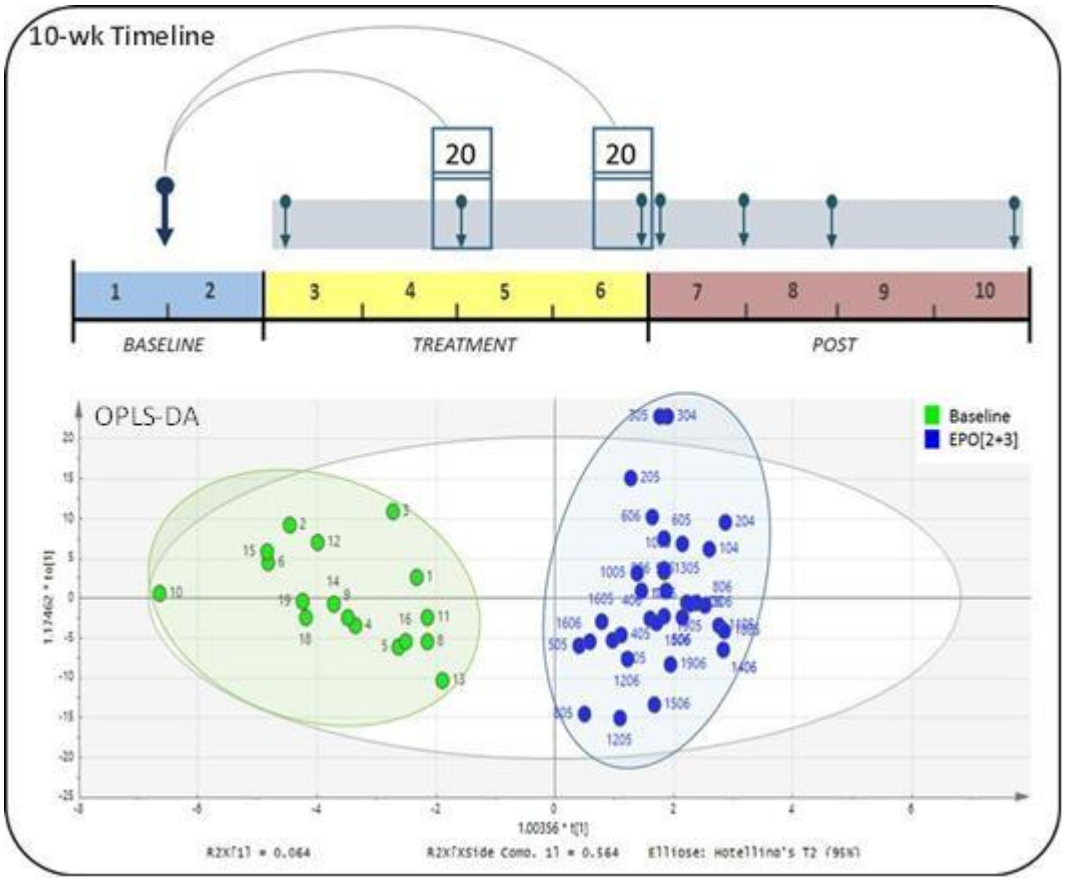


Figure 3-25. OPLS-DA of comparison groups BASELINE and combined TREATMENT (EPO) 2 and EPO3 time-points. This comparison improves modelling as well as separation. When compared to baseline these two time-points yielded a significant (*p*-value < 0.05) number of putative metabolites as can be seen in the 10-wk timeline above. Cumulative R^2X: 0.713, R^2Y: 0.886 and Q^2: 0.593.

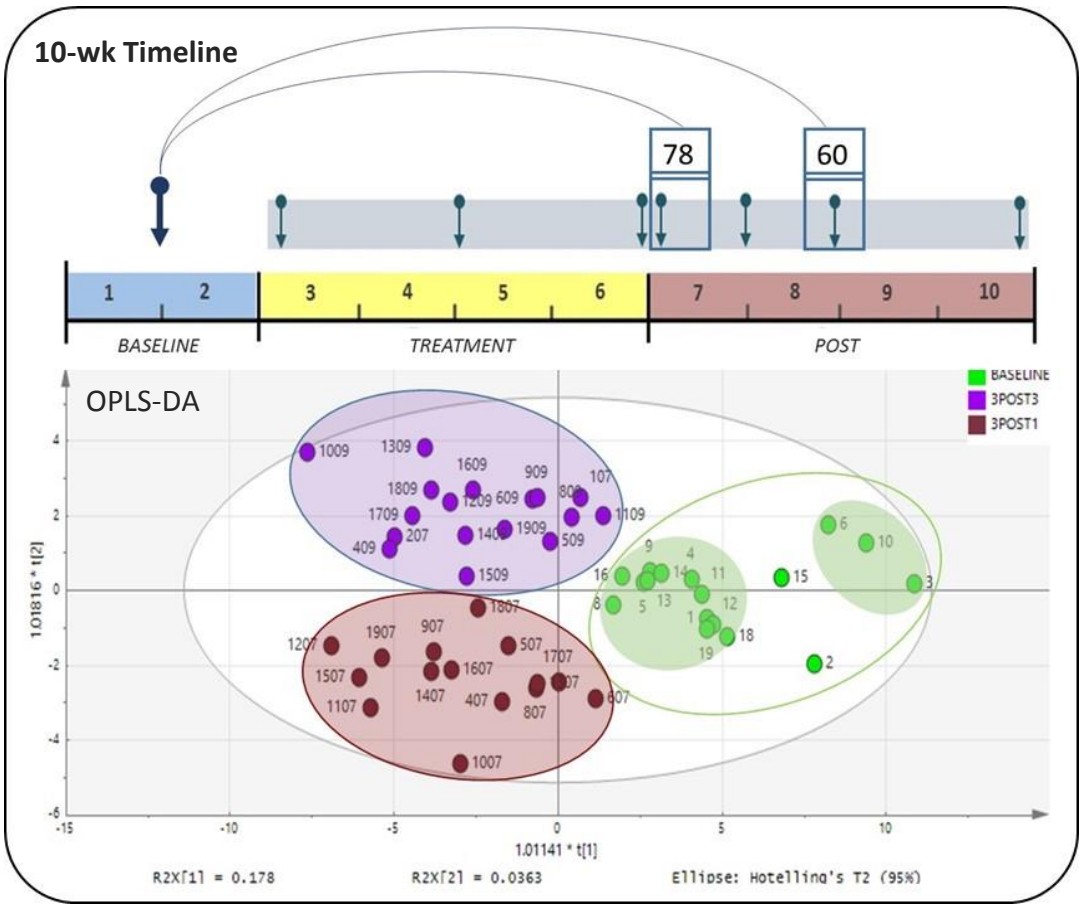


Figure 3-26. OPLS-DA of comparison groups BASELINE, POST1 and POST3 time-points. When compared to baseline these two time-points yielded the highest significant (*p*-value < 0.05) number of putative metabolites as can be seen in the 10-wk timeline above. Subjects 6, 10, 3 separate from the main BASELINE cluster. Cumulative R^2X: 0.698, R^2Y: 0.748 and Q^2: 0.434.

For the VIP score plots, model one (Figure 2-27 a), exhibited higher number of above 1 scores in comparison to two. The two persistently high VIP scores (> 2) in both models are assigned to linoelaidyl carnitine and elaidic carnitine. Linoelaidyl carnitine, which is an acyl- carnitine, was found to reach significance at EPO1 (*p*-value < 0.05), 2, 3 as well as POST 1 and 2 time-points (*p*-value < 0.001). This metabolite has several biological functions linked to cell signalling, fuel and energy storage/ source, and membrane integrity/ stability (HMDB06461). Elaidic carnitine, was found to reach significance at EPO2 (*p*-value < 0.05), 3 as well as POST 1 (*p*-value < 0.001) and 2 (*p*-value < 0.05) time-points. In model one the third highest VIP score was for N-formyl-L-aspartate, whereas in model two this was for orotate; similar to urine. In this

instance, orotate was found to be significant at the EPO3 (similar to urine), POST1 and POST4 time-points (p-value < 0.05), with the latter showing a negative response. Another interesting metabolite, ranked highly in model two, is Ne,Ne dimethyllysine, which is found in histones that contribute to gene regulation (HMDB13287). Furthermore, both L-citrulline and L-arginine are present in both models (≥ 1), as well as deoxyuridine (> 1), which is a nucleoside (HMDB00012).

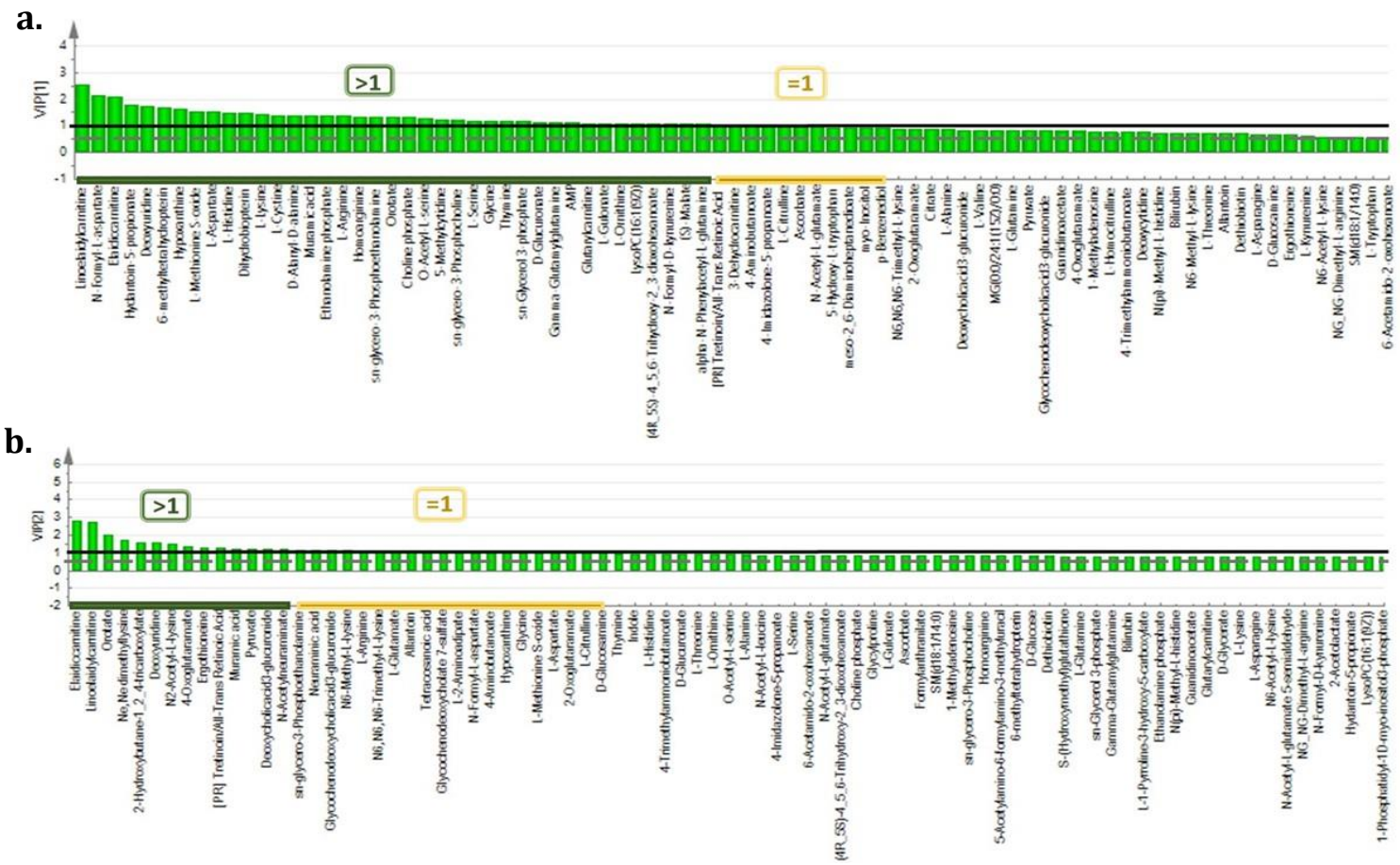


Figure 3-27. Corresponding VIP score plots for **a.** BASELINE and combined EPO2 + 3 phase comparison as well as **b.** BASELINE, POST1 and POST3 phase comparison.

3.4. ROC Curve Analysis

ROC curve analysis was conducted whilst taking into consideration all the significant urinary (N:69) and plasma (N:112) metabolites presented in Tables 3-5 and 3-6, respectively. Selectivity was based on achieving an AUC greater than 0.8. Due to the greater abundance of significant plasma metabolites, there were more promising predictive markers that could be attained from the plasma data set. This subsequently led to a grouped metabolite predictive model generation that improved, in some instances, the AUC value; mainly for POST1 time-point. The only urinary metabolite that achieved an AUC greater than 0.9 was orotate in the EPO2 *vs.* PRE comparison. It is vital, as is common practice in clinical research (Xia et al., 2012, 2013; Zhang et al., 2013; Jin et al., 2014) to establish a marker or a group of markers that have predictive potential. In this case, it would be to predict whether or not an individual has, or is using a banned substance.

What is of interest, is that the selected markers in both urine (i.e. orotate; for EPO2 and EPO3 *vs.* PRE comparison) and plasma (i.e. linoelaidyl carnitine, elaidic carnitine, deoxycholicacid3-glucuronide, muramic acid, [PR] tretinoin/all-trans retinoic acid, D-glucosamine, ergothioneine, glycine, 2-oxoglutarate, homoarginine, L-arginine, thymine, D-glucuronate, and 4-aminobutanoate; for POST1 *vs.* PRE combined model predictor) have been highlighted as promising markers in the VIP analysis (≥ 1) conducted in conjunction with the various OPLS-DA models (Figure 3-22 and 3-27).

3.4.1. Urinary Markers

For EPO2 *vs.* PRE comparison 6 metabolites were found to have an AUC above 0.8: orotate (AUC 0.924; CI 0.795- 0.997), indole-3-acetate (AUC 0.854; CI 0.698- 0.976), 4-aminobutanoate (AUC 0.847; CI 0.696- 0.965), trimethylamine N-oxide (AUC 0.837, CI 0.679- 0.958), guanine (AUC 0.811; CI 0.649- 0.936) and finally p-cresol glucuronide (AUC 0.806; CI 0.626- 0.938). The high AUC and high lower bound

confidence interval of orotate, indicate that this metabolite has greater predictive potential of this time-point in comparison to the other 5. For EPO3 *vs.* PRE comparison there were two metabolites: 4-aminobutanoate (AUC 0.846; CI 0.68- 0.969) and orotate (AUC 0.836; CI 0.683- 0.952). For POST1 *vs.* PRE only 4-aminobutanoate (AUC 0.833; CI 0.681- 0.954) reached the cut-off. POST3 *vs.* PRE comparison resulted in trimethylamine N-oxide (AUC 0.83; CI 0.686- 0.946) as well as uracil (AUC 0.801; CI 0.637- 0.925). Finally, POST4 *vs.* PRE yielded carnosine (AUC 0.829; CI 0.622- 0.962) as well as L-lysine (AUC 0.808; CI 0.603- 0.949). Furthermore, time-points EPO1 and POST2 did not yield any marker with an AUC greater than 0.8. All urinary ROC curve analysis can be found in appendix xiv. Figure 3-28 below highlights the pattern of orotate across the 10 weeks, indicating potential cut-off points based on the ROC curve analysis for EPO2 and EPO3 time-points.

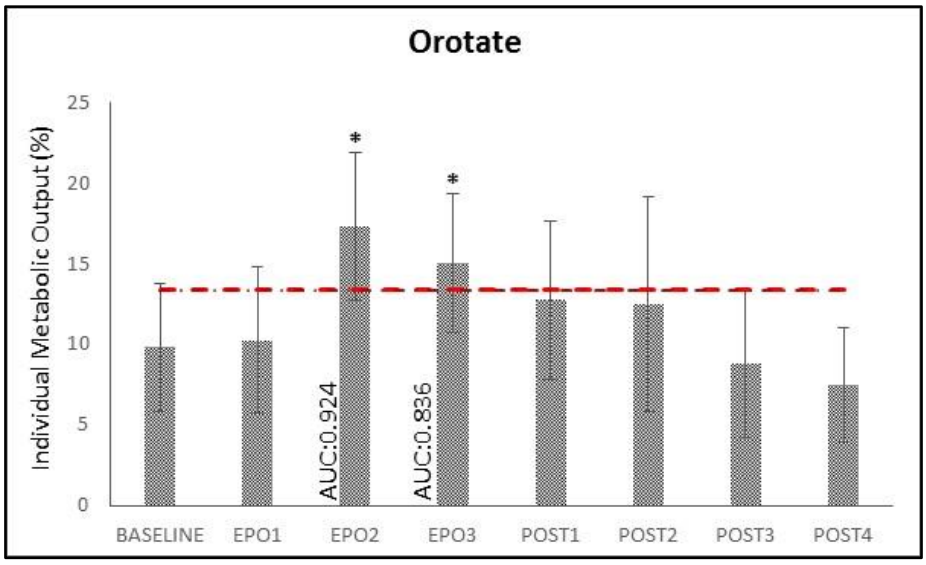


Figure 3-28. Bar graph representation of mean (± SD) values of orotate across the 8 time-points. ROC curve analysis indicated EPO2 and EPO3 as the time-points which yielded the highest AUC, with preference to EPO2. Red line indicates cut-off generated via ROC curve analysis. EPO2 *vs.* BASELINE has cut-off at 13.38 % with sensitivity at 88.9 % and specificity at 87.5 %. EPO3 *vs.* BASELINE has cut-off at 13.37 % with sensitivity at 88.9 % and specificity at 72.2 %. *Significantly different from baseline (p-value < 0.05).

3.4.2. Plasma Markers

For EPO2 *vs.* PRE comparison 5 metabolites were found to have an AUC above 0.8: linoelaidyl carnitine (AUC 0.941; CI 0.821- 1), N-formyl-L-aspartate (AUC 0.854; CI 0.687- 0.951), 6-methyltetrahydropterin (AUC 0.837; CI 0.677- 0.958), hydantoin-5-propionate (AUC 0.819; CI 0.627- 0.953), and elaidic carnitine (AUC 0.823; CI 0.649-0.95). For EPO3 *vs.* PRE comparison 4 metabolites were found to have an AUC above 0.8: linoelaidyl carnitine (AUC 0.969; CI 0.88- 1), elaidic carnitine (AUC 0.91; CI 0.79- 0.985), deoxyuridine (AUC 0.836; CI 0.659- 0.954), and dihydrobiopterin (AUC 0.812; CI 0.642- 0.926). For POST1 *vs.* PRE comparison 14 metabolites were found to have an AUC above 0.8: linoelaidyl carnitine (AUC 0.967; CI 0.88- 1), elaidic carnitine (AUC 0.926; CI 0.822- 0.991), deoxycholicacid 3-glucuronide (AUC 0.889; CI 0.748- 0.967), muramic acid (AUC 0.881; CI 0.739- 0.978), [PR] tretinoin/all-trans retinoic acid (AUC 0.852; CI 0.691- 0.956), D-glucosamine (AUC 0.841; CI 0.678-0.954), ergothioneine (AUC 0.883; CI 0.659- 0.972), glycine (AUC 0.841; CI 0.674-0.95), 2-oxoglutarate (AUC 0.841; CI 0.631- 0.944), homoarginine (AUC 0.819; CI 0.643- 0.948), L-arginine (AUC 0.822; CI 0.657- 0.939), thymine (AUC 0.815; CI 0.62- 0.944), D-glucuronate (AUC 0.807; CI 0.628- 0.941), and 4-aminobutanoate (AUC 0.807; CI 0.63- 0.933). For POST2 *vs.* PRE comparison 2 metabolites were found to have an AUC above 0.8: linoelaidyl carnitine (AUC 0.896; CI 0.733- 1) and elaidic carnitine (AUC 0.825; CI 0.644- 0.965). For POST3 *vs.* PRE comparison 8 metabolites were found to have an AUC above 0.8: N-formyl-L-aspartate (AUC 0.866; CI 0.717- 0.951), Ne,Ne-dimethllysine (AUC 0.833; CI 0.681- 0.954), L-arginine (AUC 0.827; CI 0.685- 0.94), glycochenodeoxycholicacid3-glucuronide (AUC 0.827; CI 0.668- 0.951), N6,N6,N6-trimethyl-L-lysine (AUC 0.817; CI 0.657- 0.925), (4R,5S)-4,5,6-trihydroxy-2,3-dioxohexanoate (AUC 0.812; CI 0.632- 0.925), L-methionine S-oxide (AUC 0.81; CI 0.619- 0.94), and sn-glycero-3-phosphoethanolamine (AUC 0.81; CI 0.647- 0.923). Finally, POST4 *vs.* PRE comparison yielded 4 metabolites with an AUC above 0.8: orotate (AUC 0.885; CI 0.733- 0.979), carnosine (AUC 0.861; CI 0.694- 0.966), muramic acid (AUC 0.859; CI 0.711- 0.974) and N-formyl-L-aspartate (AUC 0.821; CI 0.628- 0.947).

Furthermore, time-points EPO1 did not result in any marker with an AUC greater than 0.8. All plasma ROC curve analysis data can be found in appendix xv.

Ultimately, the most convincing markers, are those that are above an AUC of 0.9 which fluctuates across the time-points between linoelaidyl carnitine and elaidic carnitine. Conducting combined metabolite ROC curve analysis was only successful for the POST1 *vs.* PRE time-point. For POST1 all 14 metabolites combined resulted in a very strong curve (AUC) as well as predictive value, as can be seen in Figure 3-29 below.

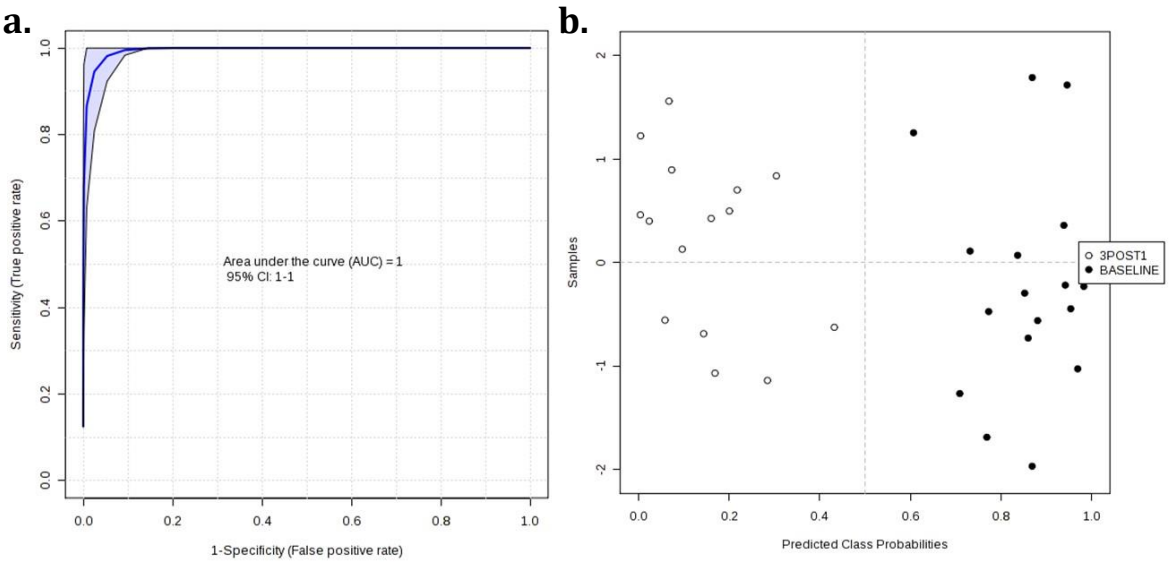


Figure 3-29. ROC curve analysis indicating **a.** AUC as well as **b.** predictive model, generated using the POST1 14 metabolite markers. The average accuracy based on 100 cross validations is an AUC of 0.99. The AUC when combining the 14 markers results in a strong predictive outcome which translates to all the samples being accurately assigned.

3.5. Discussion

The human metabolome can be considered to be a dynamic system encompassing multiple layers of complexity and finished off with seemingly infinite degrees of freedom. Patterns that subsequently emerge and disperse in space and time can often be preceded by unforeseen circumstances; as such each metabolome is unique with an inevitable innate variance. This variance across the participants requires attention in order to differentiate a universal trend that could be used in doping control as a potential screening tool. Given the diversity of performance enhancing drugs which mimic the endogenous forms of different biomolecules (Thevis et al., 2012) there needs to be a potential shift from targeting one specific drug to targeting the physiological (biomarker) response induced by drugs such as EPO. This is where the analysis becomes more complicated due to the non-linearity of intrinsic processes which span from the genome all the way down to the metabolome (Perkel, 2011). Changes observed in the metabolome have to be filtered to identify whether or not they are produced by the intervention or whether they are related to other natural challenges faced by the subject (Krug et al., 2012). Then, the next step involves backtracking in order to establish a link between these identified groups of metabolites and one or more complimentary gene-expression changes (Nicholson et al., 2008). As such, it might be possible that we are seeing effects elicited by ADIPOR1 (fatty acid oxidation and glucose uptake), ALAS2 (heme biosynthesis, porphyrin metabolism as well as glycine, threonine and serine metabolism) and DPYSL5 (beta alanine as well as thymine and uracil); details of which can be found in Table 1-2 of Chapter 1 as well as appendix v. The variation in the number of significant findings across the time-points is another interesting point to consider. The haematological parameters (haemoglobin, haematocrit, reticulocytes, haemoglobin mass (Durussel et al., 2013)) reached significance after two weeks of administration (i.e. EPO2), thus it stands to reason that there would not be an abundance of metabolites at the EPO1 time-point, as can be seen from the current results. Also, the same parameters were still elevated four weeks post-administration, which should result in downstream metabolic findings.

From the full list of urinary (Table 3-5) and plasma (Table 3-6) metabolites, there is difficulty discerning a concise story, aside from the fact that there are putative

metabolites which correspond to a diverse range of metabolic systems. There is, as previously mentioned, the chance that a certain amount of these findings are indeed false positives, hence why the ROC curve analysis can greatly decrease the volume of significant metabolites, yet at the same time, amplify the importance of specific metabolites that could warrant further investigation (Sugimoto et al., 2012; Xia et al., 2013). In the current case the study design itself, precludes a further in-depth investigation due to the lack of control in the training element. Having said that, the following markers, discussed in *3.5.1 Urinary Marker Orotate* and *3.5.2 14 Selected Plasma Markers*, should be taken into consideration in future EPO-related metabolomics investigations, along with the study by Christensen et al. (2013), and Appolonova et al. (2008).

3.5.1. Urinary Marker Orotate

Orotate, a natural precursor to uridine (Griffin et al., 2004), is characterised as being related to an inborn error relating to uridine 5'-monophosphate (UMP) synthase; resulting in orotic acid not being able to be converted to UMP, and subsequently to other pyrimidines (HMDB00226). Orotate has also been associated with inborn errors of the urea cycle and arginine metabolism (Brosnan and Brosnan, 2007).

In addition to orotate, we were able to observe related metabolites within the pyrimidine pathway and urea cycle. In the urine data set; N-carbamoyl-L-aspartate (aspartate + carbomoyl phosphate) was significantly elevated at EPO3, POST1 and decreased at POST4, as well as L-glutamine, which was significantly decreased at POST4. In the plasma set, orotate was also observed, and was shown to be significantly elevated at EPO3 and POST1 time-points which are lagging behind the urine data by one time-point (2 weeks). L-glutamine in plasma was down-regulated at POST1 and 3, which in contrast to urine, decreased significantly at the onset of the wash-out phase (only 2 days post last injection) and two weeks into wash-out (hence two weeks earlier). Moreover, L-ornithine (down POST3), L-arginine (up EPO2, POST1 and 3),

L-citrulline (down POST3) and L-aspartate (down EPO2, 3 and POST1 and 3) were all observed in the plasma set.

Taken from another angle, orotic acid supplementation, is known to induce fatty liver disease in rat models. Griffin et al. (2004) investigated the potential for a combined correlation between gene and metabolic indices (Griffin et al., 2004) in two strains of rats; Kyoto (outbred) and Wistar (inbred) exposed to 14 days of either a standard laboratory-based diet or supplemented with 1% orotic acid, *ad libitum*. ^{1}H-NMR spectroscopy was used for the evaluation of hepatic tissue extracts, intact liver tissue was investigated by a high-resolution magic angle spinning probe inserted into the spectrometer, and finally liver fatty acid profiles were investigated via GC-MS. Some of the main metabolic findings associated with orotic acid are as follows; a rise in triglycerides in the livers of both rats (8 fold in Wistar, 40 fold in Kyoto), a rise in trimethylamine oxide (TMAO), phosphocholine, and phosphatidylcholine (at 3 days), and increase in TMAO, uridine nucleotides, betaine, glycine and a drop in choline (which was shown to influence separation in the PCA of both rats from liver spectra). Additional increases in total cholesterol, unsaturated fatty acids were observed, as well as increase in methyl donors (Griffin et al., 2004).

Interestingly, it is possible to test for the pyrimidine degradation and *de novo* metabolites (N-carbamoyl-aspartate, dihydroorotate, orotate, orotidine, uridine, and uracil) via urine soaked filter paper strips detected with a HPLC-ESI MS/MS method in combination with stable isotope reference compounds for internal standards (van Kuilenburg, 2004). The method was tested in control patients (N:155) and 6 patients with an inborn error in the urea cycle or with dihydropyrimidine dehydrogenase deficiency. This could, if applied in field testing, aid in the transportation and storage of samples as well as associated costs (van Kuilenburg, 2004; Brosnan and Brosnan, 2007).

3.5.2. 14 Selected Plasma Markers

The most prominent markers in plasma, significantly increased at multiple time-points, during and post-administration are linoelaidyl carnitine and elaidic carnitine. Both being acyl- carnitines it is of interest to evaluate whether these are a response to diet or a direct effect of the treatment. Christensen et al. (2013) showed an increase in uncoupling protein 2, responsible for the proton leak of mitochondria and heat generation, in skeletal muscle in response to ESA treatment; in addition to opposing the lipolytic effect of training, when combined. Moreover, a single bolus of 400 IU/kg r-HuEPO, from a study of the same primary author, was shown to increase significantly resting energy expenditure and tended to increase fat oxidation in male subjects (Christensen et al., 2012). Ultimately, acyl- carnitines, removed from the mitochondria, are a by-product of the conversion of fatty acids being conjugated to CoA, the latter required for the maintenance of the Krebs cycle. This cycle is the central focus of cellular respiration, energy formation, source of precursors, and components that will aid in the formation of amino acids, nucleotide bases, as well as porphyrin (heme) (Berg et al., 2002). Given the aforementioned, it stands to reason that we are observing the by-product effects of an attempt to sustain this cycle that plays a role in the creation of several of the significant metabolites observed in plasma.

Interestingly, linoelaidyl carnitine has been reported in a variety of investigations. Firstly, a metabolomics-based approach was applied to evaluate the response to two drug therapies common to pancreatic cancer. Treatment of mitomycin C alone or in combination with rapamycin in a xenograft tumour was tested. The combined treatment in comparison to mitomycin C resulted in a 53% change in linoelaidyl carnitine, characterised via a LC-MS method (Navarrete et al., 2014). In another study, serum from hepatocellular carcinoma and cirrhosis patients was investigated via UPLC-QTOF MS in Egypt. Linoelaidyl carnitine was decreased in stage II and III of hepatocellular cancer patients in comparison to the patients with liver cirrhosis (Xiao et al., 2012).

In terms of diet, 210 participants in the greater Quebec city area, were selected to undertake a study involving the comparison of dietary patterns (via questionnaire), and

associated metabolic outcome. These were mainly composed of amino acids and acyl-carnitines, quantified via the Biocrates Absolute IDQ p150 (Biocrates Life Sciences AG, Austria) method. The two diets were: a) prudent diet, consisting of mainly high intake of vegetables, fruit, whole grain, non-hydrogenated fat as well as lower refined grain products; and b) western diet, consisting of processed meat, desserts, and increased intake of refined grain products (Bouchard-Mercier et al., 2013). Results indicated that linoelaidyl carnitine (C18:2) positively correlated with the prudent diet and more specifically with, fruit/ vegetable intake, non-hydrogenated fats, and was inversely associated with saturated fat (Bouchard-Mercier et al., 2013). In zebrafish, fed an ascorbic acid deficient diet, a metabolite that both humans and this animal model do not inherently produce on their own, resulted in a significant drop in C18:2 acyl- carnitine, and several amino acid derivatives (such as arginine and citrulline), with a rise in purine metabolites (IMP, GMP, AMP) (Kirkwood et al., 2012).

Muramic acid, seems to be associated with bacterial peptitoglycan, a cytoplasmic membrane, consisting of glycan chains and short peptides (Bal and Larsson, 2000). In plasma, this metabolite was significantly decreased at EPO3, POST1, 3 and 4 time-points. Tretinoin/all-trans retinoic acid (down EPO3, POST1 and 4; or otherwise known as 9-cis-retinoic acid) is responsible for the regulation of retinoid responsive gene regulation (HMDB02369), and is an active constituent of vitamin A. All-trans retinoic acid has been identified to have pleiotropic features, similar to EPO, influencing cell differentiation, proliferation as well as apoptosis, that has potential in the treatment of several different cancers (Schenk et al., 2014). The discovery of the retinoic acid receptors, has now led to its function as a “molecular switch”- exerting a molecular cascade in the bound as well as in the unbound form, ultimately generating the release of either histone acyltransferases or histone deacetylases, respectively (Schenk et al., 2014; Urvalek et al., 2014).

Glycine (down POST1, 3) is a non-essential amino acid, which aids in the co-production of DNA, porphyrin, collagen, phospholipids and energy release (HMDB00123), as well as purine nucleotides. Glycine in combination with L-arginine (up EPO2, POST1 and 3) via glycine amidinotransferase forms ornithine (down POST3) plus guanidoacetic acid (down POST1 and 3). Serine, from which glycine is

derived, was significantly reduced at the POST1 and 3 time-points as was glycine. L-threonine, an essential amino acid of the same metabolic pathway was significantly decreased at the POST1 time-point only. The relevance of the serine/glycine system and the concomitant one carbon metabolism is possibly linked to the ability to preserve the antioxidant response of the cancer cell (Amelio et al., 2014). An antioxidant response is also shown by ergothioneine, which is a naturally occurring histidine metabolite (HMDB03045). It is a key substrate of transporter solute carrier family 22 member 4. Polymorphisms of this transporter has been linked to rheumatoid arthritis and Crohn's disease, and its expression has been shown in bone marrow and fetal liver (Gründemann et al., 2005). In fact, Gründemann et al. via quantitative real time PCR (polymerase chain reaction) analysed the human ergothioneine transporter, which exhibited high levels in the $CD71^{+}$ cells (transferrin receptor in erythrocyte progenitor cells) of aforementioned bone marrow, fetal liver (main contributor to erythropoiesis) as well as cord blood (Gründemann et al., 2005). The main purpose of the transferrin receptor is to transport iron for haemoglobin production (Gründemann et al., 2005). Ergotheine was significantly decreased at the POST1 time-point, potentially exhibiting a marker of doping with a blood manipulation method- as it has been suggested that ergothioneine levels drop slowly throughout the entire life span of a red blood cell (Gründemann et al., 2005).

Homoarginine (down EPO3, POST1 and 3), is a guanidino compound (HMDB00670), closely related to L-arginine, that is also involved in the production of NO by NO synthase (Moali et al., 1998). The Krebs cycle generated precursor 2-oxoglutarate, which is a deaminated product of glutamine and was shown to be decreased at POST1 and 3 time-points (HMDB01552). It forms a part of the 2-oxocarboxylic acid metabolism pathway. The pyrimidine base (Azzazy, 2010), thymine, is also observed in the plasma date set to be decreased at the EPO3 time-point, whereas in urine, it is elevated at EPO2. In general, thymidine and phosphoric acid, via thymidine phosphorylase in a reversible reaction, can form thymine and deoxyribose 1-phosphate (HMDB00262). There might be a role here in the maintenance of blood vessels as well as angiogenic properties (HMDB00262).

Present in both plasma and urine, 4-aminobutanoate is an inhibitory neurotransmitter, an antiepileptic agent and similarly to taurine, lower circulating plasma levels have been linked to depressed patients (gamma-aminobutyric acid; HMDB00112). Interestingly, EPO as a neuroprotective agent, has shown to improve cognitive function and has been highlighted as a strong candidate for the improvement of mood and other related symptoms of depression (Miskowiak et al., 2011). In plasma, we observe a significant decrease at POST1 and 3 time-points which is in opposing pattern to urine which is elevate at EPO2, 3, POST1 and 2 time-points. The following metabolites were shown to the significantly decreased at the POST1 and 3 time-points: D-glucosamine (sugar residue involved in the glycosaminoglycan biosynthesis) and deoxycholic acid 3-glucuronide (metabolite of deoxycholic acid generated in the liver by UDP glucuronyltransferase), whereas D-glucuronate (involved in the glycosaminoglycan biosynthesis) only reached significance at the POST1 time-point. Deoxycholic acid 3-glucuronide is produced by the liver, by glucuronidation of the free acid. This compound allows for the break-down and excretion of toxic compounds, as well as the emulsification of fat components (HMDB02596).

3.5.3. Concluding Remarks

There seems to be a fundamental backstory to the 15 plasma and urine metabolites. With further investigation, bearing in mind that the findings correlate with the studies by Christensen et al. 2012 and 2013 as well as Appolonova et al. 2008, there might be, in future, the potential to finally understand what the downstream metabolic effects of of r-HuEPO are. In addition to what this would entail for patient groups as well as anti-doping control strategies. There seems to be a link between the markers of r-HuEPO and normobaric hypoxia (Lou et al., 2014) in addition to training, ultimately creating a tightly knit homeostatic control system. The next step would be to differentiate the ‘dose-response’ of each co-factor alone, and in combination, in order to effectively incorporate into the athlete’s biological passport. Ultimately, this will be achieved by improving the current study design, presented in this thesis, with the addition of a cross-over element.

Chapter 4. A Study of the Effects of r-HuEPO on the Red Blood Cell (RBC) Metabolite Profiles of Caucasian Endurance-Trained Males

4.1. Introduction

Erythropoiesis, leads to an increased production of red blood cells (RBCs) via the proliferation, differentiation as well as survival of erythroid progenitor cells. Some of the main functions of RBCs are as follows, protection of cellular proteins and iron source, shape preservation as well as the maintenance of a high K^+, low Ca^{2+} plus Na^+ environment (Barasa and Slijper, 2014). In order to achieve these functions, in the absence of mitochondria, glucose conversion via anaerobic means begins in the Embden–Meyerhof pathway, aided by the RBC Rapoport–Luebering shunt (Barasa and Slijper, 2014). Fundamentally, the series of intrinsic processes which occur are geared towards the production of NADH that is required to reduce methaemoglobin to haemoglobin. Interestingly, RBCs also limit the availability of NO in normoxic conditions (NO + oxyhaemoglobin = methaemoglobin + nitrate) (Cortese-Krott and Kelm, 2014). Whereas in hypoxic conditions or shear stress, NO is released via enzymatic mechanisms in order to induce vasorelaxation (NO_3^- → NO_2^- → NO) followed by a release of ATP (Cortese-Krott and Kelm, 2014).

In relation to the detection of r-HuEPO administration, it would stand to reason that investigating the RBC metabolome, given their direct relation to the physiological stimulus, could potentially lead to new markers of interest. One of the fundamental questions would be to differentiate between new and matured (aged) RBCs. In fact the lifespan of RBCs, in normal circulation, is approximately 120 days (± 10 %) (Bosman, 2013). During the aging process various morphological (> density, < size, membrane roughness) and biochemical (< hydration via potential > in intracellular Ca^{2+}, > IgG binding, < creatine, < intracellular ATP, < free radical scavengers, < acetylcholinesterase, > glycated Hb as well as < glutathione reductase (Franco et al., 2013; Lutz and Bogdanova, 2013)), changes occur which result to its removal via the reticulo-endothelial system; after specific senescence-related markers present on the cell surface (phosphatidylserine) (Girasole et al., 2012; Franco et al., 2013; Lutz and Bogdanova, 2013). There are various methods to determine the age of RBCs which include, but not limited to, labelled glycine, ^{51}Cr label, carbon monoxide (for heme turnover), or a biotin label followed by either multicolour flow cytometry or magnetic isolation (Franco, 2012). Another new method ‘reticulocyte-based estimation of

lifespan', aims to determine lifespan incorporating factors such RBC production rate (flow cytometry), RBC count, as well as the reticulocyte RNA degradation half-life (Krzyzanski et al., 2013).

Given the intricate nature of RBC aging as well as the abundance of downstream metabolic changes which occur as a result of this process, there are potentially obvious links to r-HuEPO administration that could be exploited. Thus, the aims of this exploratory hypothesis generating untargeted LC-MS study were to a) discriminate between the experimental phases (pre-treatment, during treatment and post-treatment) in order to highlight potential trends within the RBC metabolome collected from whole blood (samples from 3 subjects available) *vs.* centrifuged blood residue (18 subjects) of Caucasian endurance trained males; and b) distinguish potential biomarkers that respond to r-HuEPO administration.

4.2. Results

4.2.1. RBCs from Whole Blood

The majority of the putatively identified RBC metabolites (N:~700 post filter) match metabolites in the human metabolome to within 2 ppm and thus are characterized to MSI level 2 where it is most likely that alternative metabolites would be isomers of the identity assigned. Figure 4-1 illustrates PCA of the filtered full metabolite profile of the three subjects (a) followed by normalization to individual metabolic output (b).

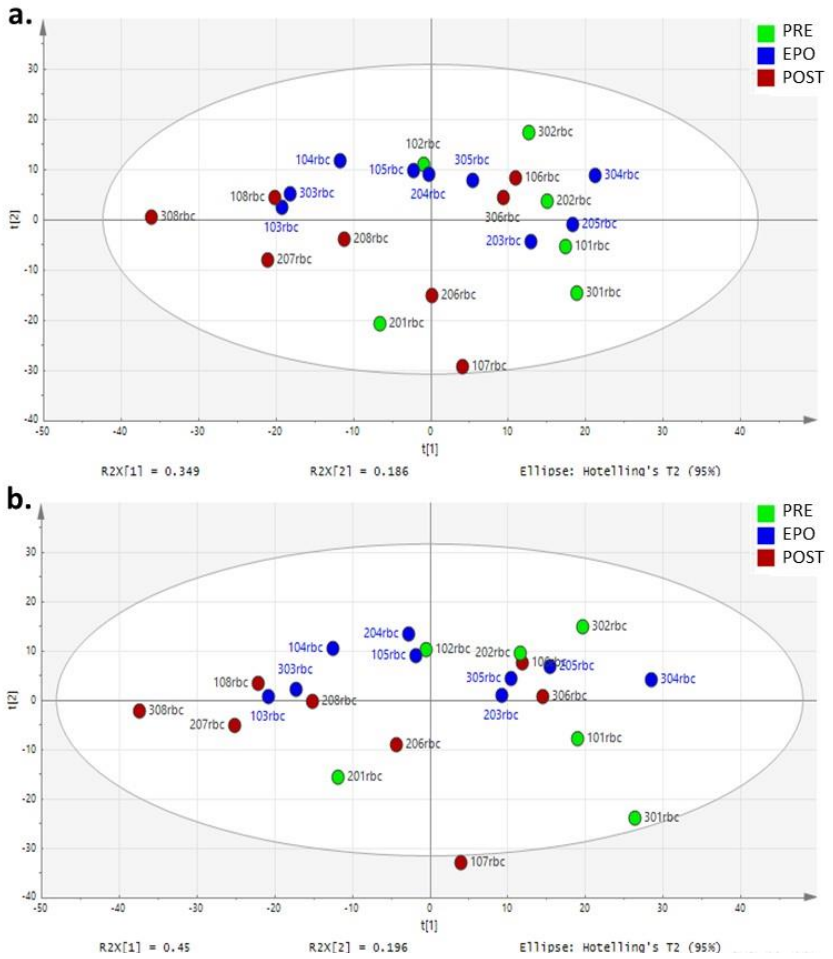


Figure 4-1. PCA of **a.** raw filtered RBC metabolite profile, and **b.** normalized to individual metabolic output with phase (PRE, EPO, POST) comparison. All samples from the three subjects were collected across the same time period in 2010. Cumulative R^2X:0.775 and Q^2: 0.577 for model a, and cumulative R^2X:0.728 and Q^2: 0.613 for model b.

From the raw filtered data, 303 metabolites were identified as being significant when compared to an averaged baseline of the three subjects. The greatest number of significant findings were in the POST4 *vs.* PRE comparison which yielded 246 metabolites (p-value < 0.05). Given the small number of subjects, and to avoid the possibility of false positives, metabolites which were significantly different at three or more time-points were chosen and are shown in Table 4-1. There is a diverse range of metabolic pathways in the 27 selected metabolites, with most abundant falling in the pyrimidine pathway. To observe whether separation and distribution is improved PCA was also performed in the reduced set, shown in Figure 4-2. Interestingly, the greatest difference can be seen in the PRE set showing a separation in the two collection time-points, in spite of the fact that only a few days separated these samples. The cumulative R^2X and Q^2 scores are also greatly improved.

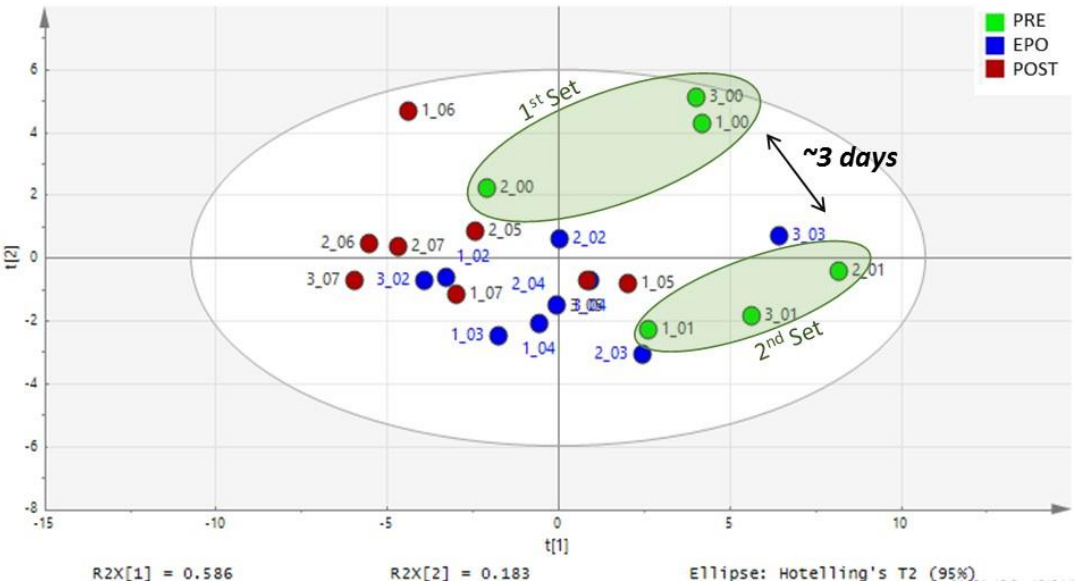


Figure 4-2. PCA of the 27 selected RBC metabolites normalized to individual metabolic output with phase (PRE, EPO, POST) comparison. Cumulative R^2X:0.931 and Q^2: 0.748. Samples 2_00, 1_00, and 3_00 were taken on 8/10/2010, sample 3_01 on the 11/10/2010, and finally samples 1_01 and 2_01 were taken on the 12/20/2010. Two baseline clusters are created, separating the subjects' primary and secondary sampling time-points, even though they are only approximately three days apart.

4.2.1.1. RBC Metabolites of Interest

Table 4-1. Normalised RBC metabolites (N:27) which are significantly changed by the r-HuEPO intervention at three or more time-points. *p*-Value (*p*) and ratio (*r*) for comparisons PRE (averaged pre-treatment values) *vs.* EPO1, EPO2, EPO3, POST2, POST3 and POST4.

Mass	RT (min)	Putative Metabolite	HMDB	PRE *vs* EPO1		PRE *vs* EPO2		PRE *vs* EPO3		PRE *vs* POST2		PRE *vs* POST3		PRE *vs* POST4	
				p	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*
Cysteine metabolism															
147.053	11.76	O-Acetyl-L-serine*	HMDB03011	**0.0051**	1.84	0.5765	1.13	**0.0123**	1.49	**0.0358**	1.42	**0.0027**	3.60	**0.0010**	2.38
153.010	10.88	L-cysteine sulfinate (or 3-Sulfinoalanine)	HMDB00996	**0.0430**	1.94	0.5883	1.25	**0.0224**	1.52	0.0757	1.28	**0.0059**	5.42	**0.0070**	2.40
Glutamate Metabolism															
145.038	11.62	2-Oxoglutaramate (or 2-Keto-glutaramic acid)	HMDB01552	0.2726	1.27	**0.0355**	0.55	0.0540	0.77	0.3855	0.76	**0.0157**	3.59	**0.0065**	2.49
113.048	16.26	(S)-1-Pyrroline-5-carboxylate (or 1-Pyrroline-5-carboxylic acid)	HMDB01301	**0.0324**	1.86	0.3983	1.17	0.0789	1.42	**0.0433**	1.89	**0.0102**	11.12	**0.0172**	2.08
250.062	11.67	gamma-L-Glutamyl-L-cysteine	HMDB01049	**0.0442**	1.58	0.7312	1.09	**0.0189**	1.51	0.1218	1.80	**0.0025**	13.57	**0.0232**	2.68

Lysine Biosynthesis																
190.095	15.17	meso-2,6-Diaminoheptanedioate (or Diaminopimelic acid)	HMDB01370	0.3265	1.31	0.0891	0.57	**0.0233**	0.59	0.3232	0.76	**0.0308**	3.26	**0.0134**	2.94
146.105	25.42	L-Lysine*	HMDB00182	**0.0024**	1.55	0.8069	1.04	**0.0464**	1.35	**0.0086**	1.64	**0.0015**	3.52	**0.0336**	1.72
188.116	13.78	N2-Acetyl-L-lysine	HMDB00446	**0.0135**	1.93	0.3392	1.25	**0.0316**	1.55	0.1245	1.40	**<0.001**	3.88	**0.0361**	2.01
Lysine Degradation																
174.137	22.12	Ne,Ne dimethyllysine	HMDB13287	**0.0154**	1.77	0.9160	0.98	0.5298	1.10	0.2923	1.25	**0.0063**	4.81	**0.0153**	2.11
Methionine Metabolism																
161.069	10.37	O-Acetyl-L-homoserine	HMDB29423	**0.0365**	1.88	0.8963	1.03	**0.0230**	1.42	0.2299	1.25	0.2001	2.82	**0.0033**	2.84
177.046	7.41	N-Formyl-L-methionine	HMDB01015	**0.0459**	1.50	0.4124	1.25	0.1161	1.22	0.3333	1.13	0.4872	1.88	**0.0106**	2.39
Purine Metabolism																
151.049	13.29	Guanine*	HMDB00132	0.4260	1.36	0.9479	1.02	**0.0243**	2.24	**0.0240**	2.40	0.0568	2.69	**0.0179**	2.27
152.033	12.46	Xanthine*	HMDB00292	**0.0427**	1.65	0.2179	1.36	0.0654	1.44	0.0845	1.39	**0.0243**	3.20	**0.0271**	2.04
Pyrimidine Metabolism																
183.076	11.69	2,6-Diamino-4-hydroxy-5-(N-methyl)formamidopyrimidine	HMDB11657	**0.0370**	2.49	0.0838	2.47	**0.0051**	2.84	**0.0220**	2.00	0.5460	2.15	**0.0108**	2.73
112.027	9.15	Uracil	HMDB00300	**0.0493**	1.69	0.4379	1.17	0.0585	1.54	**0.0370**	1.71	**0.0475**	7.50	**0.0112**	2.86
257.101	11.88	5-Methylcytidine	HMDB00982	**0.0008**	2.46	0.2328	1.47	**0.0037**	2.11	0.0532	1.85	**0.0430**	13.75	**0.0317**	2.31
176.043	17.87	N-Carbamoyl-L-aspartate (or Ureidosuccinic acid)	HMDB00828	0.2205	1.88	**0.0382**	0.53	**0.0363**	0.60	0.0871	0.61	0.9621	1.47	**0.0380**	1.94

Tyrosine Metabolism																
168.042	11.94	3,4-Dihydroxyphenylacetate	HMDB01336	0.1384	1.48	0.1224	1.91	**0.0221**	1.81	**0.0258**	1.69	0.9582	1.55	**0.0104**	2.22
209.069	6.68	4-Hydroxyphenylacetylglycine	HMDB00735	**0.0278**	1.51	0.1908	1.61	**0.0381**	1.58	0.1734	1.24	0.6993	1.20	**0.0139**	1.70
Valine, Leucine and Isoleucine Metabolism																
131.095	11.62	L-Leucine*	HMDB00687	**0.0387**	1.52	0.5052	1.14	**0.0251**	1.51	0.1366	1.34	0.1946	2.48	**0.0146**	1.81
Arginine and Proline Metabolism																
132.090	23.67	L-Ornithine*	HMDB00214	**0.0156**	1.28	0.8079	1.03	0.2190	1.11	0.0622	1.23	**0.0193**	2.70	**0.0397**	1.43
Taurine and Hypotaurine Metabolism																
125.015	16.09	Taurine*	HMDB00251	**0.0383**	1.54	0.2114	1.28	**0.0193**	1.40	0.0637	1.44	0.0774	3.63	**0.0071**	1.60
Tryptophan Metabolism																
08.085	11.62	L-Kynurenine*	HMDB00684	**0.0102**	1.96	0.2386	1.26	**0.0171**	1.40	0.7219	1.08	0.3351	2.76	**0.0121**	2.22
Glycine, Serine and Threonine Metabolism																
119.058	15.50	L-Threonine*	HMDB00167	**0.0247**	1.47	0.4337	1.11	**0.0484**	1.31	0.2950	1.23	0.1442	6.36	**0.0138**	1.83
Microbiome																
213.010	7.86	Indoxylsulfate	HMDB00682	0.1939	1.54	0.4040	1.18	**0.0269**	0.75	0.5414	0.79	**0.0058**	5.59	**0.0385**	2.12
Nitrilotriacetate Degradation																
133.037	12.93	Iminodiacetate	HMDB11753	**0.0143**	1.83	0.4625	1.31	0.1417	1.33	**0.0019**	1.18	0.5748	1.88	**0.0100**	2.34

Oxidation of Branched Fatty Acids																
217.131	10.51	O-Propanoylcarnitine	HMDB00824	0.2220	1.32	0.0028	0.55	0.0678	0.76	0.9470	1.01	0.0050	5.71	0.0023	4.38

*MSI 1: finding matches the retention time of the authentic standard.

4.2.2. RBCs from Centrifuged Blood Residue

The majority of the putatively identified RBC metabolites from the centrifuged blood residue (N:~800 post filter) match metabolites in the human metabolome to within 2 ppm and thus are characterized to MSI level 2 where it is most likely that alternative metabolites would be isomers of the identity assigned. PCA was performed on the raw filtered data with both phase and year comparison (Figure 4-3a and b, respectively). Separation of samples with PLS-DA (Figure 4-3c) was achieved with year, similar to the plasma and urine data sets in chapter 3.

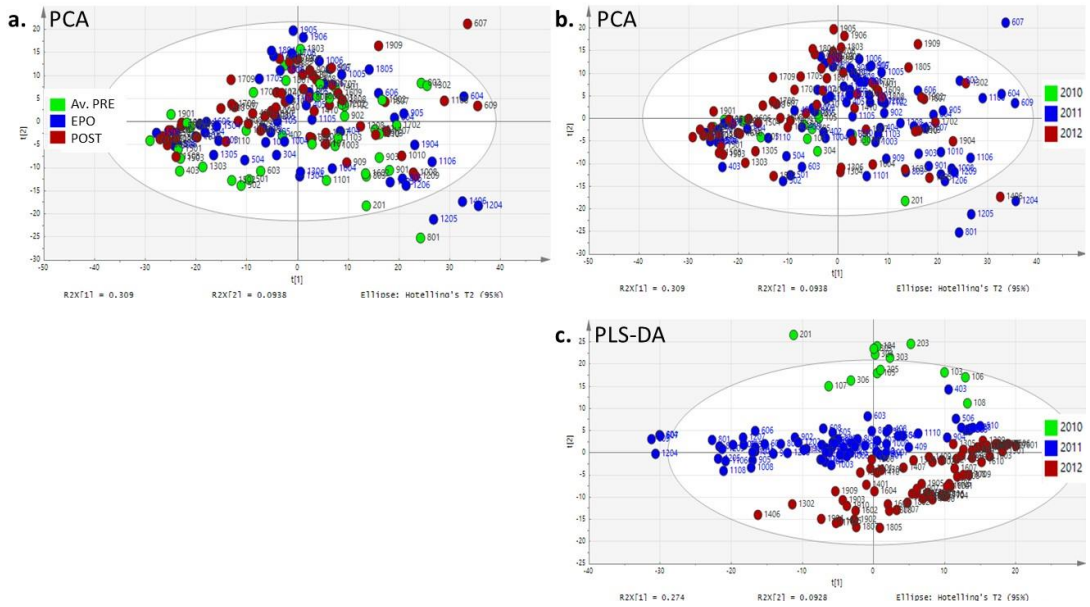


Figure 4-3. Raw RBC metabolite profiles, from centrifuged blood residue, subjected to PCA with either **a.** phase (averaged PRE values, EPO, POST) or **b.** year (2010, 2011, 2012). Separation with **c.** PLS-DA with year comparison was also performed which shows clear distribution of samples based on year of collection. Cumulative R^2X:0.784 and Q^2: 0.599 for models a as well as b, and cumulative R^2X: 0.599, R^2Y: 0.966, and Q^2: 0.893 for model c.

Normalizing the data set to individual metabolic output (Figure 4-4a) seems to cluster the samples more tightly in comparison to the raw data, with the exception to a few set of outliers. These outliers, in 4-4c, are from the 2010 cohort. With PLS-DA, the 2011 and 2012 sets merge without separation, yet once again, the 2010 group are showing a different trend response (Figure 4-4d).

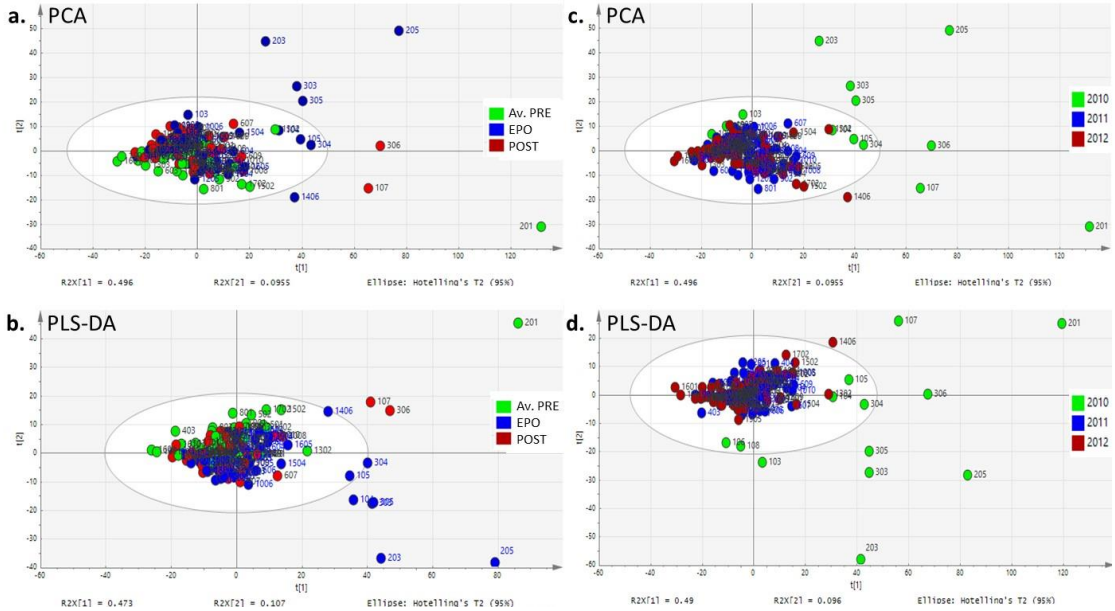


Figure 4-4. Normalized RBC metabolite profiles, from centrifuged blood residue, to individual metabolic output subjected to PCA with either **a.** phase (averaged PRE values, EPO, POST) or **c.** year (2010, 2011, 2012). Separation with PLS-DA with **b.** phase, and **d.** year comparison were also performed. The samples from 2010 seem to separate from the 2011 and 2012 samples. Cumulative R^2X:0.829 and Q^2: 0.651 for models a as well as c. Cumulative R^2X: 0.719, R^2Y: 0.546, and Q^2: 0.235 for model b and cumulative R^2X: 0.586, R^2Y: 0.235, and Q^2: 0.17 for model d.

From the raw filtered data, 309 metabolites were identified as being significant when compared to an averaged baseline of the eighteen subjects. The greatest number of significant metabolites was in the POST1 *vs.* PRE comparison which yielded 214 metabolites (p-value < 0.05). This is 2 days after the last r-HuEPO injection, in contrast to the RBCs from whole blood which exhibited the highest number of significant metabolites at the end of the wash-out period (approximately 4 weeks). Given the large

possibility of false positives, metabolites which scored an AUC above 0.9, via ROC curve analysis, were selected to include in Table 4-2 below. This amounted to 15 metabolites in total. The ROC curve analysis plots of the selected metabolites can be seen in appendix xvi. Interestingly, there is an overlap with the plasma data with regards to the presence of specific acyl- carnitines; mainly elaidic and linoelaidyl carnitine. This could indicate that the plasma from the EDTA tube was not sufficiently removed. However, there seems to be a more cohesive metabolite pattern emerging in the blood residue data set in contrast to whole blood. This would require replication to ensure that the results are attributed to the RBC metabolome, as the expectation was to see similar results to the pilot RBC study from whole blood. The effect on acyl-carnitines was particularly marked and must almost certainly be a real effect with 5 out of 7 time- points showing seven metabolites in this class, including carnitine itself, as altered with highly significant *p*-values.

4.2.2.1. Metabolites of Interest

Table 4-2. Normalised RBC metabolites from centrifuged blood residue (N:15) which are significantly changed by the r-HuEPO intervention and have an AUC above 0.9 (underlined). *p*-Value (*p*) and ratio (*r*) for comparisons PRE (averaged pre-treatment values) *vs.* EPO1, EPO2, EPO3, POST1, POST2, POST3 and POST4.

Mass	RT (min)	Metabolite	HMDB	PRE *vs.*EPO1		PRE *vs.*EPO2		PRE *vs.*EPO3		PRE *vs.* POST1		PRE *vs.* POST2		PRE *vs.* POST3		PRE *vs.* POST4	
				p	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*
Acyl- Carnitines																	
247.142	12.03	Hydroxybutyrylcarnitine	HMDB13127	0.952	0.99	**0.002**	0.53	**0.002**	0.43	**<0.001**	0.55	**0.012**	0.65	0.409	0.84	**<0.001**	1.95
413.350	4.81	Heptadecanoylcarnitine	HMDB06210	0.138	0.79	**0.003**	0.58	**<0.001**	0.46	**<0.001**	0.58	**0.023**	0.68	0.745	0.96	**0.004**	1.65
425.350	4.77	Elaidiccarnitine	HMDB06464	0.069	0.75	**0.001**	0.58	**<0.001**	0.44	**<0.001**	0.53	**0.008**	0.61	0.447	0.89	**0.005**	1.63
427.366	4.76	Stearoylcarnitine	HMDB00848	0.129	0.75	**<0.001**	0.59	**<0.001**	0.43	**<0.001**	0.54	**<0.001**	0.59	**0.033**	0.76	**0.017**	1.44
371.303	5.08	Tetradecanoylcarnitine	HMDB05066	0.172	0.81	**<0.001**	0.54	**<0.001**	0.48	**<0.001**	0.57	**0.039**	0.64	0.420	1.15	**0.020**	1.69
423.334	4.83	Linoelaidylcarnitine	HMDB06461	0.065	0.80	**<0.001**	0.57	**<0.001**	0.45	**<0.001**	0.56	**0.011**	0.62	0.837	1.03	**0.024**	1.59
Lysine Degradation																	
161.105	13.92	L-Carnitine	HMDB00062	0.805	0.96	**0.009**	0.56	**0.007**	0.50	**0.011**	0.70	0.089	0.76	0.54	1.46	**<0.001**	2.25

145.110	13.95	4-Trimethylammoniobutanoate	HMDB01161	0.451	1.13	0.086	0.70	**0.025**	0.55	**0.003**	0.73	0.164	0.78	0.061	0.73	0.589	1.09
Pyrimidine Metabolism																	
158.033	11.73	(S)-Dihydroorotate (or L-Dihydroorotic acid)	HMDB03349	0.981	1.00	**0.001**	0.50	**<0.001**	0.37	**<0.001**	0.41	**0.044**	0.64	0.959	1.02	**<0.001**	2.52
228.075	8.51	Deoxyuridine	HMDB00012	0.439	0.87	**0.008**	0.51	**0.014**	0.37	**0.001**	0.55	0.067	0.69	0.302	0.86	0.647	1.07
Fatty Acyl																	
441.345	5.21	3-Hydroxy-11Z-octadecenoylcarnitine	HMDB13339	0.118	0.79	**0.004**	0.62	**0.006**	0.52	**<0.001**	0.52	**0.032**	0.66	0.310	0.83	**0.008**	1.53
Arginine and Proline Metabolism																	
129.079	12.74	N4-Acetylaminobutanal	HMDB04226	**<0.001**	0.30	0.188	0.68	**0.002**	0.45	0.498	0.83	0.480	1.15	0.925	0.98	**0.018**	1.55
Aminosugar Metabolism																	
221.090	12.73	N-Acetyl-D-glucosamine*	HMDB00215	0.252	0.84	**0.001**	0.58	**0.001**	0.44	**<0.001**	0.55	**0.004**	0.66	0.139	0.78	**<0.001**	1.77
Glycine, Serine, Threonine Metabolism, Arginine and Proline Metabolism																	
131.069	15.30	Creatine*	HMDB00064	0.107	0.77	**0.005**	0.68	**0.010**	0.61	**<0.001**	0.67	**0.047**	0.75	0.121	0.84	0.436	1.11
Acyl Glycine																	
143.058	13.88	Vinylacetylglycine	HMDB00894	0.906	0.98	**0.002**	0.59	**0.009**	0.53	**<0.001**	0.67	**0.050**	0.75	**0.010**	1.56	**<0.001**	2.24

*MSI 1: finding matches the retention time of the authentic standard.

4.3. Discussion

The RBC metabolite profiles from both methods exhibited a large number of significant metabolite findings in comparison to the plasma and urine sets. Given the relationship between EPO and RBC production, this does not come as a surprise. However, on one hand the RBC data from whole blood is only from a small sub-group of the subjects, and the RBC from the blood residue has issues with regards to the integrity of the samples themselves, after such a long time in storage as well as handling which could have resulted in lesions. The latter being a common area of experimentation for its implications to stored RBCs in blood banks (Bosman, 2013). In the second set of samples, from the full eighteen subjects, the metabolite profiles seem to coincide better across the time-points providing a story behind the majority of the significantly identified findings with an AUC above 0.9. Again the lack of forethought with regard to collecting RBCs indicates a poor study design which, unfortunately, we could not control.

Hence some of the most notable changes include the acyl- carnitines of Table 4-2, which are elevated in contrast to baseline, during the EPO2, 3, POST1 and 2 time-points. This is followed by a significant drop, in all acyl- carnitines at the POST4 time-point. Via ROC curve analysis the majority of markers identified as having an AUC above 0.9 are located at EPO3 and POST1. There is an overlap with the plasma data set given the presence of linoelaidyl and elaidic carnitine, which were also elevated during the EPO and early POST phases. There could be a relationship between erythrocyte membrane regulation and RBC carnitine palmitoyltransferase, as the latter is required for acyl trafficking across the RBC membrane (Reuter et al., 2009). A drop in L-carnitine followed by an accumulation in medium- and long- chain acyl-carnitines results in a reduced activity of RBC carnitine palmitoyltransferase, and ultimately a destabilization of the RBC membrane that would have a knock-on effect on its lifespan (Reuter et al., 2009). In this instance, L-carnitine mirrors the response of the acyl- carnitines, with most dominant down-regulated response at POST4 (AUC > 0.9) which could coincide with the elimination of mature RBCs from circulation. L-carnitine aims to restore balance between acyl CoA pool and RBCs which results in

proper membrane preservation and repair (Pavese et al., 1997). This is further supported by creatine being elevated at EPO1, 2 as well as POST1 and 2 time-points. A rise in creatine is characteristic of younger RBCs (Lutz and Bogdanova, 2013). This similar pattern as the acyl- carnitines is followed by fatty acyl 3-hydroxy-11Z-octadecenoylcarnitine, the aminosugar N-acetyl-D-glucosamine as well as the acyl glycine vinylacetylglycine. N-acetyl-D-glucosamine. The latter is a residue of glucose, which is utilised in the Embden–Meyerhof pathway in a process to produce NADH. It is also more broadly involved in ATP binding via the enzyme Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase which allows erythrocyte cells to perform normal sialylation (HMDB00215). Additionally, the pyrimidines deoxyuridine and (S)-dihydroorotate, exhibit a similar pattern as well. Deoxyuridine is a nucleoside which is converted to deoxyuridine triphosphate during DNA synthesis (HMDB00012).

From Table 4-1, there might be related metabolites responsible for the generation of glutathione which is an essential component to RBCs, given its antioxidant capacity and the permeability of the RBC membrane to hydrogen peroxide. Glutathione, via the γ-glutamyl cycle, is synthesized from cysteine, glycine and glutamate; the latter required in a continuous supply, to maintain glutathione levels (Whillier et al., 2011). A substrate of glutamate, 2-oxoglutaramate (or 2-ketoglutaramic acid) was shown to be significantly elevated at EPO2 with subsequent drop at POST3 and 4. Other glutamate/ glutamine and cysteine related metabolites, were decreased across their significant time-points. We are also seeing a drop in the selected purines (guanine, xanthine) as well as a persistent drop in the pyrimidine uracil that's found in RNA. Guanine in the urine data, was significantly elevated at EPO2 whereas in the RBC data it decreased significantly at EPO3 and POST1 as well as increased at POST4. Dudzinska et al. (2010a) examined guanine nucleotides (GTP, GDP and GMP) post a standardized physical effort test with increasing intensity in erythrocytes and showed no change post- exercise or post 30 minutes of recovery. In chapter 5, we show that an hour of aerobic exercise results to increased guanine levels in urine. Hence, there might be a possibility of this being unique to the RBC metabolome and could identify r-HuEPO indirectly, in addition to xanthine which exhibits a similar response. On the other hand, N-carbamoyl-L-aspartate, shows almost the same significant trend

response as in the urine data set. In fact, in the RBC data, from whole blood, this metabolite is elevated approximately two weeks earlier (EPO2) in comparison to urine.

There is an overlap in the RBCs from whole blood to plasma and urine across several of the significant findings. Taurine, was elevated in urine at POST2, whereas in the RBCs it was decreased at EPO1, 3 and POST4. We are seeing a similar gut metabolite, indoxylsulphate, as well as O-acetyl-L-serine. In the RBC and plasma data (Table 4-1 and Table 3-6, respectively) we are observing a significant decrease in L-kynurenine, which is opposite to the reported effects of exercise (chapter 5). Threonine as well as ornithine, which were shown to decrease in the RBC and plasma data sets (as well as in urine for ornithine), have also been shown to decrease post-exercise. Whereas, leucine which was decreased in RBCs has generally been elevated post-exercise (Daskalaki et al., 2015b).

We are observing some cohesive patterns in both the RBCs of whole blood and blood residue, which do not appear in the plasma or urine sets. However, questions still remain with respect to the effect long-term storage has on sample integrity and ultimately resulting outcome. This not only affects the response, but the extent of that response across the subjects. Perhaps, it would be advisable to replicate these conditions bearing in mind the storage, transportation and handling of the RBC samples.

Chapter 5. A Study of the Effects of Exercise on the Urinary Metabolome Using Normalization to Individual Metabolic Output

5.1. Introduction

From the smell and colour of urine as a tool for disease diagnosis (Bouatra et al., 2013) to the elusive promise of an "exercise pill" as a preventative measure of disease (Goodyear, 2008; Woldt et al., 2013)- could this be the future of health prescription? How could exercise, with its multi-factorial and multi-organ health benefits (Goodyear, 2008; Lehmann et al., 2010a; Lewis et al., 2010; Clouse et al., 2013; Kujala et al., 2013) be successfully administrated as an oral medication? If this theoretical pill existed, it would have to replicate the downstream metabolic effects of a single type of exercise tailored specifically to each unique metabolome or even metabotype (Krug et al., 2012; Dumas et al., 2014). Exercise, as an external challenge to the human metabolome, creates an immediate response (turn-over rate in seconds (Sweedler and James, 2011)) across this biological matrix which, unlike studies investigating the effects of fasting, have been shown to exhibit large inter-subject variability (Nicholson et al., 2008; Krug et al., 2012). Therefore, the question of dosing specific exercise regimes to varying population cohorts (sedentary *vs.* regularly active *vs.* athletes) in order to maximize efficacy of the intervention remains to be answered. So how can we, by utilizing evidence-based markers, predict the intensity and training modality that would ensure a quicker adaptation and improved outcome in these subgroups? The answer could lie within the plethora of human metabolism which, in spite of its vast number of constituents, still is the most sensitive measure to investigate cellular and human phenotype (Sweedler and James, 2011; Bouatra et al., 2013; Dumas et al., 2014).

As such, metabolomics the rapidly developing omics technology, allows for hundreds of metabolites (generally with a mass < 1500 Da, at ≤ 5 ppm mass deviation) to be investigated within each metabolome at any given time-frame - creating a 'snapshot' of the biological state of an organism (Ellis et al., 2007). A number of metabolomics-based studies have provided evidence to suggest that there is a clear effect of exercise on the human metabolome (Yan et al., 2009; Kujala et al., 2013; Nieman et al., 2013b; Mukherjee et al., 2014b). The most persistent observations concern effects on the purine pathway, highlighting increases in adenine nucleotides in both acute and

prolonged exercise regimes (Stathis et al., 2006; Zieliński et al., 2009, 2013b; Pechlivanis et al., 2010a, 2013; Zielinski and Kusy, 2012; Neal et al., 2013; Sheedy et al., 2014), with reduced excretion exhibited following an adaptive response (Zielinski and Kusy, 2012) that is accommodated by a reduced level of resting hypoxanthine (Bianchi et al., 1999; Sahlin et al., 1999; Stathis et al., 2006; Dudzinska et al., 2010a; Pechlivanis et al., 2010a; Zieliński et al., 2011). Moreover, Hellsten et al. showed that muscle urate as well as allantoin levels, the latter being a product of urate oxidation, were increased in habitually active male subjects following exhaustive exercise (Hellsten et al., 2001). Allantoin can only be formed non-enzymatically in humans and it was concluded that uric acid was acting as an antioxidant against reactive oxygen species generated during exercise. Hence this could be a useful tool in examining levels of oxidative stress. As previously mentioned, plasma levels of hypoxanthine were also increased after exercise and this may result from xanthine dehydrogenase being a rate limiting enzyme in urate formation (Sahlin et al., 1999). In muscle, ATP is degraded to hypoxanthine which is lost from the muscle but may be salvaged by hypoxanthine-guanine phosphoribosyl transferase. Several papers have observed that hypoxanthine salvage tends to be more efficient in trained individuals and that along with other purine metabolites can provide an indication of the effectiveness of a training regime (Zieliński et al., 2011). A targeted metabolomics study examining approximately 200 plasma metabolites in relation to exercise in individuals from a longitudinal cohort study concluded that metabolic profiles obtained during exercise gave a signature of exercise performance as well as cardiovascular disease susceptibility (Lewis et al., 2010). Important marker metabolites for the effect of exercise included purine metabolites, tryptophan metabolites, citrulline (marker of nitric oxide formation and present to a lesser extent in the plasma of fitter individuals) and finally nicotinamide (tryptophan metabolite), which is known to enhance insulin release [5]. Lustgarten et al. (2013) noted a positive correlation between $\dot{V}O_{2max}$ with tryptophan, and an increase in tryptophan related metabolites such as kynurenate was exhibited in a study of individuals after running a 26.2 mile Boston marathon (Lewis et al., 2010). A ^{1}H-NMR investigation into same sex twins looked into the effect of prolonged physical activity adherence to metabolic and gene-expression links (Kujala et al., 2013). Numerous differences were found between persistently physically active

and inactive individuals in the circulating metabolome and the results reflected a better cardio-metabolic health in the physically active twin (Kujala et al., 2013).

Apart from the aforementioned studies there have been very few comprehensive LC-MS based investigations utilizing an untargeted metabolomics method. The research conducted by Lewis et al. (2010) and the Nieman et al. exercise studies (Nieman et al., 2013b) have set a standard for future work however there are new prospects available in metabolite coverage and understanding due to the rise of high resolution and high throughput monitoring systems. Therefore, we conducted an exploratory, hypothesis generating pilot study utilizing an untargeted LC-MS method in order to explore the effects of a one hour aerobic exercise challenge in the urine metabolome of three physically active males. We utilized our well established hydrophilic interaction chromatography method to carry out the analysis (Creek et al., 2011; Zhang et al., 2013, 2014). The use of urine provides an ideal non-invasive method that results in a large overview of the metabolite matrix. As serialized time-points, each individual urine sample provides an averaged response of the metabolic output of a particular individual and in doing so provides a unique overview of the daily metabolite variation. Hence, we devised a continuous 37-h protocol with sampling post-exercise accounting for the majority of the time-points (across 31-h) in order to generate an understanding of the acute effects of exercise and where possible the duration of that response in the metabolite profiles.

5. 2. Materials and Methods

5.2.1. Ethics Statement (UEC 14/28, Watson/Daskalaki: Pilot Exercise Trial)

This pilot study was approved by the Ethics Committee of the University of Strathclyde (Glasgow, UK) and conformed to the Declaration of Helsinki. Prior to successful recruitment, all subjects completed a physical activity readiness questionnaire as well as a health questionnaire, in order to assess physical activity levels and to ensure that they had no history of the following: anaemia, diabetes, epilepsy as well as any underlying respiratory or cardiovascular complications. All subjects gave written informed consent to participate.

5.2.2. Subjects and Experimental Design

Three physically active non-smoking males (age range: 32–38 years) participated in the pilot study. The subjects were regularly engaged in predominantly running, long-distance walking and cycling. For at least two weeks prior to study commencement subjects were asked to abstain from taking any sport or nutritional supplements and had no sign of illness. Subjects underwent a two-day (37 hour) trial, whereby urine samples were collected at regular intervals across this timeline: Day 1, ~08:00 Pre-exercise (P) 1 , first pass urine), 11:00 (P2), 14:00 (Post-exercise (PT) 1; 17:00 (PT2) and 21:00 (PT3); and Day 2, ~8:00 (PT4; first pass urine), 12:00 (PT5), 14:00 (PT6), 17:00 (PT7) and 21:00 (PT8)). Aerobic exercise was performed at the Strathclyde Sport and Recreational Centre (Glasgow, UK) on Day 1 between 12:00 and 13:00 (referred to as A.E. session in this diagram) on either a treadmill, bicycle ergometer or in a combination. Subjects were permitted to control their own pace and drink water *ad libitum* but had to be engaged in activity for at least 50 min. After the exercise session and for the remainder of the sample collection timeline subjects were asked to refrain from any physical activity. Figure 5-1 provides a full illustration of the two day protocol.

Figure 5-1. Illustration of experimental design and sample collection.

5.2.3. Sample Collection and Preparation

Coded pre-labelled sterilized urine containers were distributed to the subjects along with cool bags (Sistema Plastics, available from Amazon Co., Slough, UK) in order to store and transport to the laboratory located at the Strathclyde Institute of Pharmacy and Biomedical Sciences. A designated drop-off section (at a temperature of −35 °C) was created for the samples. The unopened urine containers were stored for a maximum of two weeks prior to being thawed at room temperature and prepared for LC-MS analysis. For the analysis, using ZIC-pHILIC column, 200 µL of urine was thoroughly mixed with 800 µL of ACN, followed by centrifugation at 7840 relative centrifuges force for 5 min; 800 µL of supernatant was then transferred to a LC auto-sampler vial (Thermo Fisher, UK). For quality control purposes a pooled sample representing all subjects as well as a subject specific pooled sample were prepared (the latter utilized as a normalization technique). For the pooled samples 100 µL of urine were gathered from each sample and then treated as above. Four standard mixtures containing 150 authentic standards were run also after the samples.

5.2.4. Measurement of Creatinine

Fifty microlitres of diluted samples and prepared creatinine standard stock solutions were thoroughly mixed with 100 μL of creatinine detection reagent (Enzo Life Sciences, Exeter, UK) in a 96-well plate. Absorbance was read at 490 nm via a Spectra Max M5 from Molecular Devices. The concentrations of creatinine in the test samples were calculated as stated previously in the literature (Zhang et al., 2013).

5.2.5. Chemicals and Solvents

HPLC-grade acetonitrile (ACN) was purchased from Fisher Scientific, UK and HPLC-grade water was produced by a Direct-Q 3 Ultrapure Water System (Millipore, UK). AnalaR-grade formic acid (98%) was obtained from BDH-Merck (Poole, Dorset, UK). Authentic stock standards were prepared as stated previously in the literature (Creek et al., 2011) and diluted 4 times with ACN before LC-MS analysis. Ammonium carbonate was purchased from Sigma-Aldrich, UK.

5.2.6. LC-MS Method

LC-MS data were acquired on an Accela HPLC (Thermo Fisher Scientific, Hemel Hempstead, UK) coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in both positive and negative mode set at 50,000 resolution (controlled by Xcalibur version 2.1.0; Thermo Fisher Corporation, Hemel Hempstead, UK). The mass scanning range was *m/z* 75–1200, the capillary temperature was 320 °C and the sheath and auxiliary gas flow rates were 50 and 17 arbitrary units, respectively. The separation was performed on a ZIC-pHILIC column (150 × 4.6 mm, 5 μm from HiChrom, Reading UK) in binary gradient mode. The mobile phase used was 20 mM ammonium carbonate buffer (pH 9.2) and pure ACN; the flow rate was 300 $\mu L.min^{-1}$. The gradient was programed as follows: 0 min 20% A/ 80% B to time 30 min 80% A/ 20% B. The injection volume was 10 μL and the sample tray temperature was controlled at 12°C during the measurement. Samples were run in a stratified method with between-subject samples placed in randomized order.

5.2.7. LC-MS Data Processing with MzMatch and IDEOM (Version 19)

Raw LC-MS files were converted to mzXML (ProteoWizard) and separated into ESI positive and negative. Converted files were then processed with open source MzMatch (http://mzmatch.sourceforge.net/) and identification of putative metabolites was made via macro-enabled Excel file IDEOM (http://mzmatch.sourceforge.net/ideom.html). Details regarding the data processing, metabolite identification as well as databases available through IDEOM can be found in previous literature (Creek et al., 2012). Details of the R script for data processing with MzMatch and IDEOM configurations can be found in appendix viii, R script, and appendix ix, IDEOM settings, respectively.

5.2.8. Statistical Analysis

Graphical representations, tabular features and statistical analysis (*p*-value generation) were performed in Excel (Microsoft Office 2013). Raw data was subjected to a variety of normalization techniques; pooled subject specific MS creatinine samples, MS and spectrophotometric creatinine as well as a subject-specific area percentage. For the latter, the first step includes calculating the sum of the peak areas of each metabolite across the 37-h protocol for each subject individually. Secondly, each respective metabolite response from every time-point is then divided by the subject-specific sum and multiplied by 100. Paired t-tests and fold changes were calculated on the new area percentage data set. In order to observe the effect of the various normalization strategies, data were also subjected to unsupervised PCA (scaled to unit variance) via SIMCA-P 13 (Umetrics, Sweden).

5.3. Results

5.3.1. Normalisation

Since the strength of urine can vary, a major question in urinary metabolomics is how does one normalise to allow for variation in strength? Creatinine normalisation is often used yet the reliability of such a method is uncertain; this is, in fact discussed at length in the review (Ryan et al., 2011a) on urinary normalization techniques by Ryan et al.. It is clear from a number of metabolomics studies examining differences between individuals that each person presents a unique metabolic profile (Nicholson et al., 2008; Krug et al., 2012). These inter-individual variations can be seen between the clustering patterns of the PCA where data have been normalised to either (1) MS creatinine; (2) pooled subject specific MS creatinine; or (3) spectrophotometric creatinine (Figure 5-2). The result of normalizing to MS response for creatinine (Figure 5-2a) does not vary from the original raw data set and does not improve the observed clustering between the subjects. However, when attempting to normalize to pooled subject- specific (Figure 5-2b) and spectrophotometric (Figure 5-2c) creatinine the results vary considerably, especially for the latter. Having previously seen the clustering of each subject uniquely, the results of normalization via the assay kit aid in the potential further differentiation of two metabotypes within our results. In Figure 5-2c all of subject 3 samples are separated from subject 1 and 2. Given that all our subjects were healthy, non-smoking and regularly active, this could be an additional factor in determining relative fitness prior to any form of exercise challenge due to the relationship of creatinine clearance and muscle activity. The most effective strategy, however, can be seen in Figure 5-2d as the data are normalized to the subject-specific area percentage.

This approach has not been widely used but there is some justification for doing this as an almost complete recording for each metabolite was collected over the designated time-frame of the study. There are very few studies which have collected urine samples separately over long periods so there is no information variability of individual metabolites with time. Table 5-1 shows a range of metabolites in urine with their

relative standard deviation (RSD) over 30 samples taken from our three subjects. Many of the amino acids have only small variations over time indicating that they are sufficiently abundant in the body. However, this may suggest that they are not affected to such a great extent by this particular form of exercise; ultimately maintaining a relatively constant output. On the other hand, there are some metabolites shown in Table 5-1 that are very variable over time.

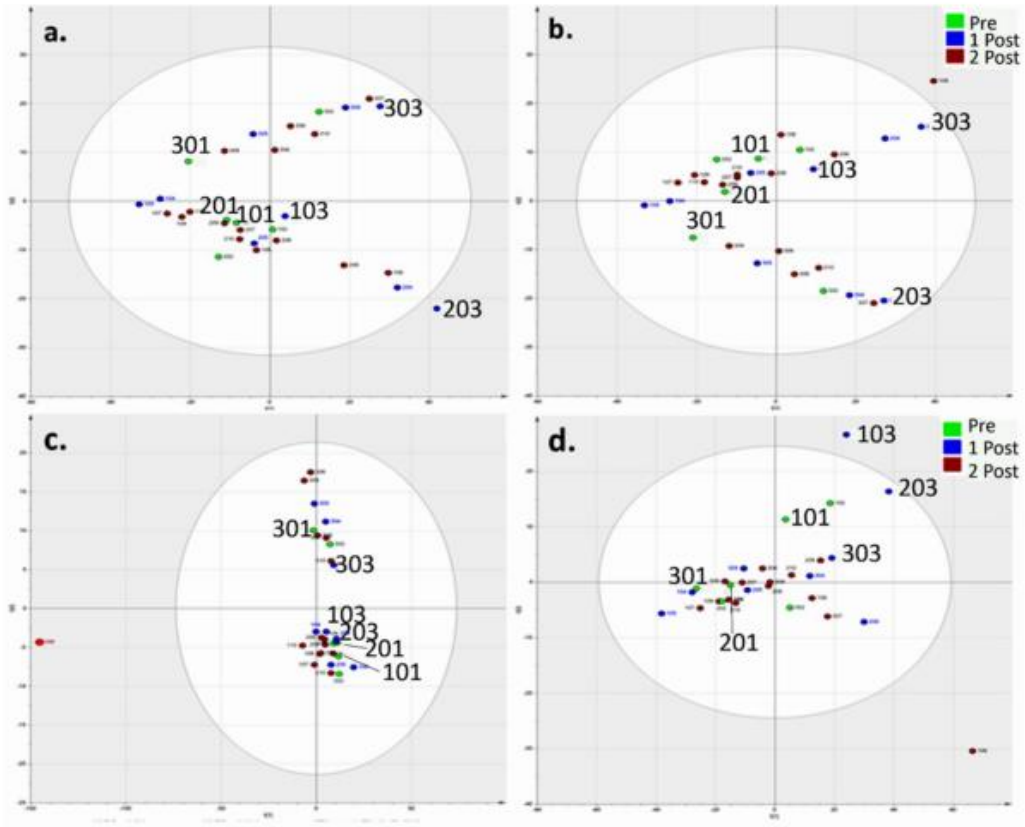


Figure 5-2. Normalization strategies applied to the urine metabolome of three subjects (~1000 putative metabolites). PCA of data normalized to (**a**) MS creatinine value of each unique sample detected exhibiting similar pattern to raw data (R^2X: 0.721 and Q^2:0.432); (**b**) MS creatinine value of subject specific pooled samples (*i.e.*, pooled subject 1 creatinine value utilized to normalize all of subject 1 raw data and so on; R^2X:0.767 and Q^2:0.489); (**c**) spectrophotometric creatinine value (the normalized data are closely clustered in the PCA model with the exception of all sampling time-points from subject3; R^2X:0.883 and Q^2:0.468); and (**d**) subject-specific area percentage (R^2X:0.704 and Q^2:0.447 showing smallest difference between goodness of fit and predicted fit). PRE group includes P1 and P2; POST1 group includes PT1, 2, and 3; POST2 includes PT4, 5, 6, 7, and 8.

Table 5-1. RSD values for metabolites across 30 urine samples collected over 37 hours.

Compound	Mass	Retention Time (RT in min)	RSD (%)
* Creatinine	113.050	10.1	16.3
* Threonine	119.058	14.9	25.4
Imidazolone propanoate	156.053	11.5	25.5
* Glutamine	146.069	15.5	27.2
* Serine	105.043	16.1	27.7
Dihydrothymine	128.058	15.2	31.2
N-acetylhistidine	197.080	10.6	31.5
Dimethylarginine	202.143	22.0	31.9
Dihydrouridine	246.085	10.8	32.0
* Betaine	117.079	11.7	32.3
* N-acetylglucosamine	221.090	12.2	33.2
N-acetylarginine	216.122	15.3	34.9
* N6-acetyllysine	188.116	15.5	35.6
* Adenine	135.055	9.5	37.9
* Methylthioadenosine	297.089	7.0	38.1
* Citrulline	175.096	15.9	39.0
* Proline	115.063	13.2	40.0
Methylimidazole acetic acid	140.058	9.7	41.0
* Alanine	89.048	15.2	41.3
* Methylhistidine	169.085	13.3	41.9
Thymine	126.043	12.0	42.3
* Isoleucine	131.095	11.6	42.5
Butenyl carnitine	229.131	9.8	42.8
Methylcytosine	125.059	11.0	43.2
* N-acetylglutamine	188.080	11.0	44.2
* Adenosine	267.097	9.3	44.9
* Phenylalanine	165.079	10.5	46.8
* Histidine	155.069	15.0	47.9
* Kynurenine	208.085	11.2	51.2
* Cytosine	111.043	11.6	51.1
* Ornithine	132.090	22.3	56.6
* Tryptophan	204.090	12.0	55.6
Carnitine	161.105	13.8	60.4
* Arginine	174.112	26.4	62.6
Creatinine (assay kit)	-	-	73.0
* Pantothenate	219.111	8.9	82.4

Tetrahydro aldosterone glucuronide	542.273	7.4	84.8
* Lysine	146.105	25.0	100.6
* Pyruvate	88.016	8.3	106.3
Cresol glucuronide	284.090	7.8	113.1
* Urate	168.028	13.0	121.6
* Xanthosine	284.075	12.7	141.3
* Tyrosine	181.074	13.3	133.7
* Inosine	268.081	11.2	180.3
* Hypoxanthine	136.038	10.5	186.1
Deoxyinosine	252.086	9.0	210.6

* Matches retention time of standard from one of the standard mixtures.

Figure 5-3 shows the variation in MS creatinine compared to some other metabolites (phenylalanine, carnitine, threonine, glutamine and stachydrine) which do not change very much over time for the three subjects; whereas, in Figure 5-4 variations in metabolites of the purine pathway (hypoxanthine, xanthosine, inosine and guanine) are shown in comparison to MS creatinine. As exhibited in Table 5-1 there are large variations across variable as well as non-variable metabolites, and Figure 5-3 futher emphasises that creatinine does not in fact follow a similar pattern to other metabolite markers. It is obvious from the similar trend response across the three subjects, that the purines reflect a very strong acute impact of exercise; with peak levels observed at the first post-exercise sample (PT1). They also exhibit fluctuations over a smaller range on the following day. These metabolites are all in the pathway for ATP catabolism. The levels of adenosine (RSD in Table 5-1) were not affected to the same extent by exercise as the other purines and thus the breakdown of ATP appears not to proceed via this branch of the purine metabolism pathway. The effects of exercise and purine catabolism have been extensively described in the literature (Bianchi et al., 1999; Sahlin et al., 1999; Hellsten et al., 2001; Stathis et al., 2006; Zieliński et al., 2009, 2011, 2013a; Dudzinska et al., 2010a; Zielinski and Kusy, 2012) and in particular, hypoxanthine has been the most studied of the purines.

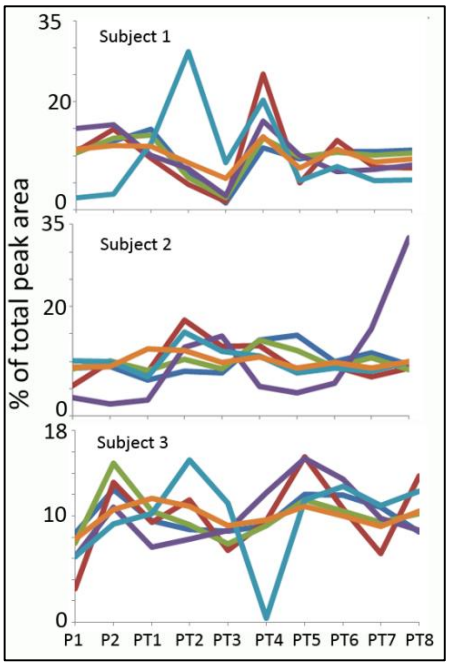


Figure 5-3. Comparison of the exercise response of area percentage for MS creatinine (orange) with phenylalanine (red), carnitine (purple), threonine (green), glutamine (dark blue) and the dietary xenobiotic stachydrine (light blue).

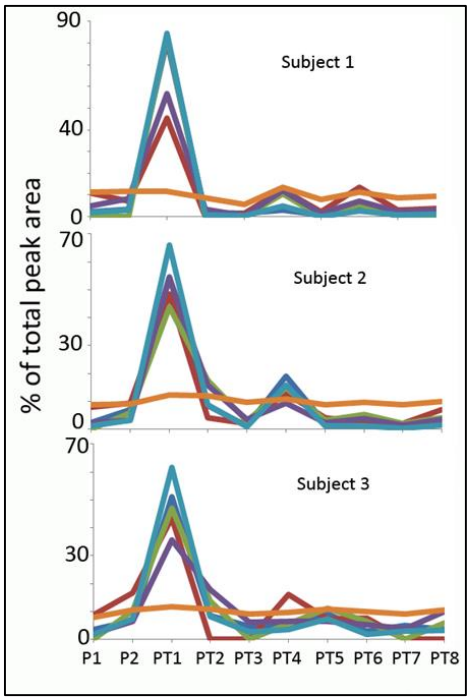


Figure 5-4. Comparison of exercise response of the area percentage for MS creatinine (orange) with the levels of some metabolites from the purine pathway, hypoxanthine (light blue), xanthosine (green), inosine (red) and guanine (purple).

The possible permutations for comparison of the ten sampling points are large, so in order to investigate the impact of exercise on the wider metabolome, the first post-exercise sample was taken as best reflecting the impact of exercise according to the previously established effect on purine metabolism. Hence, statistical analysis was carried out with the first post-exercise sample as the reference point in order to observe the metabolites which were most immediately impacted by exercise (Table 5-2). This would additionally provide a possible time series analysis in order to determine how long it took for the effect of the exercise intervention to subside. This adds statistical power, despite the small sample size, since nine points for each person can be referenced to the first post-exercise sample (PT1). Table 5-2 summarizes the metabolites which were most significantly changed between the P1 and PT1 points. Having established the most significant changes between these two points, subsequent differences between PT1 and some of the other post-exercise points (PT2, 4, 5, and 7) were added to the table. Some of the metabolites affected by exercise took over 24-h to return to the level of the first pre-point. Comparison with the second pre-point (P2) did not produce as distinct differences for certain metabolites as can be seen in the comparison between PT1 and P1. This is probably due to the fact that the physical activity of the subjects (mainly in the morning travel methods of the subjects) prior to the exercise session was not strictly controlled. However, in many cases, there were still significant differences between P2 and PT1.

Table 5-2. Normalised urinary metabolites that are significantly changed by exercise for three subjects. *p*-Value and ratio change for comparisons PT1 (first post-exercise sample) *vs.* P1 (Pre 1), P2 (Pre 2), PT4 (Post 4), PT5 (Post 6), PT5 (Post 7) and PT7 (Post 9). The majority of the metabolites match metabolites in the human metabolome to within 2 ppm and thus are characterised to Metabolomics Standard Initiative (MSI) level 2 where alternative metabolites could be isomers of the identity assigned.

	Mass	RT (min)	PT1/P1		PT1/P2		PT1/PT2		PT1/PT4		PT1/PT5		PT1/PT7	
			Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value
Purine metabolism														
N2-N2-Dimethylguanosine	311.123	9.0	2.59	0.0200	1.57	0.11	1.97	0.12	1.18	0.61	2.68	0.070	4.75	0.0043
Xanthosine *	284.075	12.7	16.18	0.0191	8.24	0.019	3.98	0.014	5.25	0.022	13.7	0.017	18.6	0.019
Inosine *	268.081	11.2	581	0.045	10.8	0.048	5.55	0.049	5.64	0.0591	12.14	0.045	88.8	0.046
Deoxyinosine	252.086	9.0	43.3	0.010	15.6	0.0091	11.9	0.0060	8.87	0.0037	25.13	0.0062	53.4	0.0093
Guanine *	151.049	12.7	5.01	<0.001	4.17	0.0017	33.7	<0.001	3.30	<0.001	10.17	<0.001	27.7	<0.001
Hypoxanthine *	136.038	10.5	22.8	0.025	11.0	0.026	9.86	0.023	6.96	0.016	15.5	0.020	27.3	0.023
Kynurenine pathway														
** 3-Hydroxytryptophan	220.085	10.3	2.69	0.012	1.45	0.12	1.52	0.27	0.93	0.83	2.14	0.095	3.66	0.0095
[†] Xanthurenic acid isomer	205.037	11.3	4.55	0.0023	1.93	0.0072	1.95	0.085	1.76	0.15	2.14	0.044	3.91	0.0001
Kynurenate	189.043	6.6	3.36	0.085	2.71	0.13	1.57	0.42	1.59	0.31	2.55	0.13	3.87	0.092
Hydroxytryptophol	177.079	7.5	3.74	0.0048	0.98	0.97	1.86	0.14	2.22	0.016	2.43	0.026	4.01	0.0040
N1-Methyl-2-pyridone-5-carboxamide	152.059	7.8	2.27	0.042	1.56	0.40	1.28	0.58	0.86	0.68	1.44	0.44	3.12	0.0012
Glycolysis														
Pyruvate *	88.016	8.3	6.79	0.026	5.93	0.02	2.11	0.12	2.81	0.041	9.08	0.024	6.50	0.031
Methyl oxalate	118.027	8.5	3.09	0.011	1.68	0.31	0.95	0.92	1.14	0.82	1.96	0.13	3.12	0.0026
Vitamins														
Riboflavin *	376.138	8.9	3.01	0.047	1.19	0.59	2.63	0.055	0.75	0.50	2.92	0.043	8.94	<0.001
Pantothenate *	219.111	8.9	5.09	0.031	3.18	0.048	1.96	0.13	2.19	0.071	3.44	0.034	4.37	0.042
Neurotransmitter metabolism														
L-Metanephrine *	197.105	18.6	4.56	0.013	2.00	0.050	1.88	0.13	1.45	0.42	2.90	0.035	5.21	0.026

	Mass	RT (min)	PT1/P1		PT1/P2		PT1/PT2		PT1/PT4		PT1/PT5		PT1/PT7	
			Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value
Microbial metabolism														
Indole-3-acetyl-glutamine	303.122	8.2	4.86	0.010	1.61	0.26	3.36	0.024	1.38	0.28	3.64	0.021	8.02	0.011
5-Hydroxyindolepyruvate	219.053	5.3	3.99	0.0045	1.90	0.036	1.92	0.13	1.30	0.46	2.54	0.082	3.35	0.0067
Indoxylsulfate	213.010	7.4	3.07	0.050	2.05	0.19	2.23	0.1155	1.48	0.4326	3.62	0.044	11.57	0.040
Hydroxyferulate	210.053	6.8	3.81	0.042	2.83	0.084	1.36	0.5378	1.55	0.27	6.27	0.040	5.87	0.044
Cresol sulphate	187.008	4.5	2.97	0.037	1.37	0.34	2.14	0.13	0.96	0.91	3.48	0.029	4.05	0.026
Phenol sulphate	173.999	5.0	3.32	0.0042	2.02	0.17	1.84	0.15	1.01	0.99	2.13	0.075	3.83	0.0034
Urocanate	138.043	7.5	2.81	0.054	2.03	0.036	1.73	0.16	2.02	0.049	2.97	0.027	5.50	0.014
Amino acids														
Tryptophan *	204.090	12.0	2.02	0.044	0.90	0.62	1.24	0.58	0.77	0.60	1.49	0.32	2.36	<0.001
O-Acetyl-L-homoserine	161.069	8.6	3.76	0.019	2.91	0.047	1.54	0.42	1.75	0.1177	3.06	0.023	4.23	0.019
Histidine *	155.069	15.0	0.41	0.047	0.39	0.073	0.67	0.44	0.62	0.30	0.29	<0.001	0.34	0.019
D-Methionine *	149.051	7.6	2.40	0.017	1.32	0.16	1.51	0.27	1.11	0.70	1.78	0.21	1.98	0.015
L-Proline *	115.063	13.2	3.88	0.018	1.85	0.091	2.18	0.058	1.74	0.12	2.38	0.047	3.53	0.035
Amino acid metabolism														
N-Acetylvanilalanine	253.095	8.0	7.48	0.0020	3.19	0.035	2.28	0.15	2.52	0.085	4.18	0.018	7.21	< 0.001
N-(Carboxyethyl) arginine	246.133	14.6	5.16	0.027	3.43	0.039	2.98	0.031	3.10	0.027	4.27	0.026	4.13	0.032
N-Acetyl-D-tryptophan	246.101	6.6	3.60	0.051	3.28	0.047	1.29	0.68	1.45	0.25	2.47	0.087	5.42	0.055
N-acetylmethionine	191.062	6.1	4.85	0.039	3.26	0.052	1.85	0.22	1.68	0.19	2.9	0.070	6.07	0.046
Amino acid oxidation														
Indole pyruvate	203.059	6.9	2.36	0.030	1.59	0.40	1.14	0.81	1.44	0.46	3.07	0.025	3.39	0.014
Acetamidooxohexanoate	187.084	8.4	2.71	0.029	1.84	0.24	1.37	0.38	1.44	0.31	2.28	0.031	4.42	0.0075
Hydroxyphenylpyruvate	180.042	7.9	4.43	0.0030	2.63	0.077	1.54	0.41	1.80	0.12	4.66	0.0032	4.94	0.0051
Oxoarginine	173.080	14.7	1.99	0.04	1.78	0.089	1.96	0.052	1.63	0.078	2.88	0.018	2.18	0.045
Guanidovaleramide	158.117	24.7	6.74	0.0015	2.33	0.032	1.61	0.37	1.33	0.65	3.42	0.080	9.27	0.0049

	Mass	RT (min)	PT1/P1		PT1/P2		PT1/PT2		PT1/PT4		PT1/PT5		PT1/PT7	
			Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value	Ratio	*p*-Value
Acetohydroxybutanoate	146.058	7.0	5.67	0.0016	3.51	0.0310	1.99	0.14	1.49	0.31	3.01	0.012	6.47	<0.001
Acetamidobutanoate	145.074	7.1	3.17	0.0095	2.24	0.14	1.69	0.28	1.80	0.13	2.82	0.029	6.39	0.0069
Dihydroxymethylbutanoate	134.058	9.2	8.00	0.030	3.23	0.053	1.74	0.32	0.79	0.74	1.31	0.58	2.54	0.10
Methyloxopentanoic acid	130.063	5.2	4.21	0.026	2.65	0.068	1.51	0.45	1.18	0.66	2.32	0.084	3.68	0.030
Dioxopentanoate	130.027	8.8	2.31	0.046	1.56	0.39	1.10	0.85	0.94	0.84	1.47	0.31	2.18	0.035
Hydroxypentanoate	118.063	7.1	2.72	0.018	1.72	0.28	0.94	0.92	0.71	0.58	1.42	0.48	2.99	0.0035
Methyloxobutanoic acid	116.047	6.9	3.12	0.013	1.95	0.19	1.25	0.70	0.85	0.78	1.46	0.48	3.60	0.0020
Hydroxybutanoic acid	104.047	8.2	3.24	0.010	2.09	0.15	1.31	0.60	0.86	0.75	1.51	0.40	2.96	0.011
Carnitine metabolism														
Dodecenoylcarnitine	341.256	5.5	4.59	0.0025	3.00	0.030	2.67	0.045	1.52	0.13	3.55	0.051	5.19	< 0.001
undecanoylcarnitine	329.256	5.5	3.08	0.026	1.57	0.20	1.48	0.35	1.32	0.40	2.07	0.17	3.83	0.028
Decanoylcarnitine	315.241	5.8	3.07	0.045	2.00	0.065	1.71	0.24	1.18	0.66	2.40	0.1425	5.05	<0.001
L-Octanoylcarnitine	287.209	6.4	2.15	0.016	1.41	0.19	1.52	0.31	0.71	0.51	1.46	0.47	3.18	0.004
Methylglutarylcarnitine	261.121	5.6	1.92	0.078	2.08	0.18	1.24	0.58	0.60	0.23	2.10	0.023	2.06	0.013
Hexanoylcarnitine	259.178	7.7	2.26	0.041	1.17	0.55	1.15	0.77	0.85	0.71	1.55	0.39	3.37	0.027
Valerylcarnitine	245.162	10.7	2.33	0.0186	1.74	0.065	1.36	0.42	0.98	0.96	2.52	0.0082	2.91	0.0075
Dehydroxycarnitine	145.110	15.8	2.76	0.0095	1.89	0.023	1.65	0.20	1.52	0.15	2.69	0.012	3.00	0.0082
Steroid metabolism														
Urocortisol glucuronide	542.273	7.4	6.48	0.0036	2.68	0.062	1.84	0.16	2.16	0.063	2.60	0.045	5.84	0.011
Dihydrocortisone glucuronide	540.257	5.5	5.47	0.029	2.59	0.082	3.70	0.028	2.04	0.075	3.65	0.032	8.43	0.033
Hydrocortisone sulphate	442.166	4.3	8.83	0.060	3.13	0.084	5.16	0.060	2.74	0.092	5.40	0.051	25.26	0.055
Hydroxyandrosterone glucuronide	482.252	5.5	4.27	0.0064	2.21	0.14	2.24	0.068	1.79	0.091	2.90	0.018	5.08	0.017
Androstane diol glucuronide	468.272	5.6	2.77	0.0082	2.11	0.17	1.71	0.21	1.24	0.51	2.38	0.065	4.45	0.0029
Androsterone glucuronide	466.257	4.8	3.59	0.0039	1.99	0.19	1.84	0.19	1.40	0.32	2.43	0.076	5.00	0.0069
Oxoandrostane glucuronide	480.236	5.3	4.13	0.046	2.17	0.15	2.03	0.13	1.47	0.32	2.83	0.061	9.22	0.051

* MSI level 1: finding matches retention time of the authentic standard; ** Retention time earlier than 5-hydroxytryptophan standard; † Retention time later than xanthurenic acid standard.

5.4. Discussion

5.4.1. Tryptophan Metabolism

In addition to the purine pathway a number of other metabolite pathways were significantly affected by exercise. Lewis et al. (2010) observed effects on the kyurenine pathway in response to exercise and we observe the same type of effect in the current study with a weak effect on kynurenate and a clearer effect on 3-hydroxy tryptophan and hydroxyindole pyruvate, hydroxytryptophol as well as pyridone carboxamide, which are also present in this pathway. Initially, we thought that xanthurenate (3-hydroxykynurenate) was also affected by exercise, but the affected compound shown in Table 5-2 appears to be an isomer of xanthurenate. Effects on tryptophan metabolism following exercise were also observed by Lustgarten et al. (2013). There is an early report that kynurenate can be converted non-enzymatically into 6-hydroxykynurenate, and this may occur in urine when levels of kynurenate are high (Takahashi, 1968). We have previously observed in the completely different metabolic system of *Drosophila* that inhibition of the purine metabolism pathway with allopurinol caused a fall in metabolism within the kynurenine pathway (Bratty et al., 2012).

5.4.2. Glycolysis

Pyruvate is strongly elevated by exercise, and this would be expected as a result of increased reliance on glycolysis as the source of energy with the decreased entry of pyruvate into the Krebs cycle. Lewis et al. (2010) observed increased levels of pantothenate in plasma taken from marathon runners, and we also observe a large increase. It was proposed that elevated pantothenate resulted from an increased demand for CoA biosynthesis (Lewis et al., 2010), but in the current case, the pantothenic acid is excreted, which might reflect a decreased demand for CoA. This could also reflect a switch to glycolysis in place of fat metabolism. It has been observed that fat metabolism decreases with an increasing exercise intensity, and there is an increase in reliance on glycogen to supply energy (Romijn et al., 1993). Fatty acid levels in plasma have been found to increase after exercise, and in the current case,

possibly, this is reflected by the rapid fall in urinary panthothenic acid levels post-exercise. Furthermore, the urinary levels of the adrenaline metabolite, metanephrine, are elevated and do not decline to pre-exercise levels until nearly 24-h later.

5.4.3. Microbiome Metabolites

An unexpected and wide ranging effect of exercise is on the levels of microbial metabolites in urine. Five metabolites clearly associated with the gut microflora are moderately to highly elevated by exercise, and include metabolites of tryptophan, such as the uremic toxin, indoxyl sulphate, indole pyruvate and hydroxyindole pyruvate (Wu et al., 2011), the tyrosine metabolites cresol sulphate and phenol sulphate (Nicholson et al., 2012) as well as the histamine metabolite urocanic acid. Elevated levels of a number of other amino acid oxidation products are observed and these may also result from microbial activity (Davila et al., 2013b). This could have important implications for physiological function during exercise when one considers that cresol sulphate and indoxyl sulphate are potent uremic toxins. Again, this effect has been previously observed by Lustgarten et al., highlighting increases in a range of products of gut microflora, including phenol sulfate, p-cresol sulphate, urocanic acid and 3-indoxyl sulphate in physically-impaired adults following an exercise programme (Lustgarten et al., 2014).

5.4.4. Amino Acid Oxidation

As shown in Table 5-2, there are many oxidation products of amino acids produced in response to exercise that is supported in previous studies. Lustgarten et al. (2013) observed increases in α-hydroxyisocaproate, indolelactate hydroxyisovalerate and 2-hydroxy-3-methylvalerate in their study of exercise in physically-impaired adults (Lustgarten et al., 2013). They linked increases in these metabolites with PPAR-α activation. In our study, we have observed the keto acids to a greater extent than the corresponding hydroxyl acids which is more in line with Pechlivanis et al., who observed increased oxidation of branch chain fatty acids in the urine of moderately

trained males post-exercise (three sets of 80-m maximal runs separated by either 10 s or 1 min (Pechlivanis et al., 2010a)).

5.4.5. Carnitines

Another group of metabolites which are affected by exercise are fatty acid carnitine conjugates. In particular we observed increases in C12:0, C10:0 and C8:0 carnitines, which have been reported previously (Lehmann et al., 2010b). Romijn et al. showed that glucose inhibited the metabolism of palmitic acid during exercise (Romijn et al., 2000). Glycolysis is increased during exercise since two molecules of ATP are generated during the conversion of glucose to pyruvate without a requirement for oxygen. Pyruvate then enters mitochondria and is converted to acetyl CoA, which produces one molecule of NADH, but this does not require investment of ATP. In contrast, coupling of a fatty acid to CoA requires one molecule of ATP and the FADH and NADH produced during a fatty acid oxidation step. This requires oxygen in order for them to be converted to ATP in the terminal respiratory chain. Thus, it would seem logical that further oxidation of pyruvate via the Krebs cycle might take precedence over fatty acid oxidation when ATP is at a premium. In order to enter mitochondria. fatty acids have to be converted into acyl- carnitines, where they are then conjugated to CoA and undergo oxidation. Carnitine conjugation is also used to transport fatty acids out of mitochondria in order to ensure that sufficient levels of free CoA are maintained; this allows the Krebs cycle to function (Zammit et al., 2009). CoA is released once acetyl CoA enters the Krebs cycle, but is required once more in the formation of succinyl CoA. Thus, in order to maintain free CoA, fatty acids conjugated to CoA may be converted to acyl- carnitines and be removed from mitochondria and, ultimately, excreted.

Measurement of urinary acyl- carnitine levels is used to diagnose in born errors of fatty acid metabolism, which result from a defect in one of the beta oxidation steps during fatty acid metabolism in the mitochondria. In such conditions, in order to preserve free levels of CoA, the partly metabolised fatty acid is removed and excreted as its carnitine conjugate. The elevation in certain acyl- carnitines may reflect something similar

where fatty acids are removed to ensure the functioning of glycolysis followed by conversion of pyruvate to acetyl CoA and the oxidation of acetate in the Krebs cycle.

5.4.6. Steroid Metabolism

The final major group of metabolites varying between the first post-exercise sample and the other samples in the series are urinary steroid metabolites. Urocortisol glucuronide, which is the major metabolite of hydrocortisone, was greatly elevated in the first post-exercise sample. Cortisol concentration is well-known to vary in the blood and its metabolites urocortisone- 3-glucuronide and urocortisol-3-glucuronide are downstream metabolites of cortisone and cortisol. The peak times for cortisol, cortisone, and urocortisone-3-glucronide concentration were found in a study of plasma levels to be in the afternoon (Kasukawa et al., 2012). This fits with our current observations in urine. However, from the data in Table 5-2 it can be seen that on the non-exercise day the peak level was in the morning- only 50% of the peak level in the first post-exercise sample and the afternoon samples were still lower. Thus, it would appear that exercise does influence the level of hydrocortisone metabolites. This is perhaps not surprising, since hydrocortisone is involved in many physiological functions relating to energy consumption. For instance, it has been found that corticosteroids can directly promote nitric oxide production (Hafezi-Moghadam et al., 2002). There have been previous reports of cortisol metabolism being changed by exercise (Tremblay et al., 2004; Gatti et al., 2005). In addition to hydroxycortisone metabolism, testosterone metabolism was also affected by exercise, and three androgen metabolites were elevated in the first post-exercise sample. There are numerous reports that testosterone is elevated in males following exercise (Dovio et al., 2010).

5.5. Conclusions

There have been longitudinal studies looking at collection of individual urine samples over time in normal subjects following exercise. By collecting 24-h pooled urine samples a lot of interesting information could be lost. The metabolic changes we observed in the current pilot study have been observed across several papers, although they have not been reported in one single study (Bianchi et al., 1999; Sahlin et al., 1999; Hellsten et al., 2001; Tremblay et al., 2004; Gatti et al., 2005; Stathis et al., 2006; Zieliński et al., 2009, 2011, 2013a; Dovio et al., 2010; Dudzinska et al., 2010a; Lehmann et al., 2010b; Lewis et al., 2010; Pechlivanis et al., 2010b; Zielinski and Kusy, 2012; Lustgarten et al., 2013, 2014). It is vital, given the profound effects of exercise that future metabolomics-based investigations also take into consideration the impact exercise has on the metabolome. For instance comparing a relatively sedentary patient cohort with a more active control group would highlight the metabolite changes due to exercise rather than changes due to disease. Another pertinent example is the carnitines, which are widely reported as disease markers (Ganti et al., 2012; Jin et al., 2014), however given their response to exercise, differences could easily result from two groups which are not adequately matched for physical activity. Similarly, purines have been proposed as markers for various types of cancer (Struck et al., 2013), and it is obvious that normalizing against creatinine would not compensate for the large fluctuations in this pathway due to physical activity. From the current study, it is evident that creatinine may fluctuate over time with a similar pattern to numerous metabolites; however, it does not necessarily do so in the same degree. We therefore recommend that the most robust technique for normalization would be to calculate subject-specific area percentages. This technique does not seem to remove any of the abundant features across the subjects, but merely acts to re-stabilize an already variant data set, the latter considered as a norm in metabolomics investigations. In addition, it does not introduce large numbers of significant metabolites, but focuses the metabolites affected by exercise into distinct pathways, which have all been described before in various papers, ultimately adding increased weight to the observations.

The data obtained from this simple study is extensive, and it would be possible to carry out further analysis to uncover further insights into the human metabolic fluctuations. However, this will be better done on a larger sample size with better control over the dose of exercise administered. The purpose of the current study was to demonstrate the underappreciated impact of exercise when carrying out metabolomics studies and that continuous collection of all samples from individuals enables us to gain some confidence in normalizing each metabolite to its total output. The current study, in fact, arose out of the difficulties we experienced when trying to collaborate in a study investigating the effects of recombinant human erythropoietin in the spot urine and plasma samples of endurance trained males taken over 10 weeks (Pitsiladis et al., 2014). Although we expected to see an impact on the metabolome, the metabolite data was thoroughly stochastic. There was no particular information in the literature we could turn to on this, since few studies have been conducted over such a long period. Thus, we hypothesized that, since the individuals concerned were pursuing individual training regimens, the large fluctuations in the metabolites we were observing must be due to exercise masking any other effects. Therefore, the current study supports this view and also could explain why in metabolomics studies of humans it is often difficult to find differences in cohort comparisons since the physical activity level for an individual is not something that is often normalized. Potentially, it might be possible to normalize for this using a metabolite such as hypoxanthine or a combination of the purine markers, which is greatly affected by exercise but this remains to be studied. The effects of exercise on the metabolome have been recently reviewed (Daskalaki et al., 2015b).

Chapter 6. General Summary

The main findings of this Ph.D. thesis are as follows:

1. Subject- specific normalization to total metabolic output, expressed as a percentage, over a period is a practical technique to observe metabolic patterns in urine and plasma, without comprising data integrity. It seems to be a superior method to commonly used urinary creatinine normalization (chapter 3, 4 and 5).

2. Specific gravity, osmolality, MSTUS, as well as creatinine did not improve overall distribution of urine data set. In fact, year seemed to separate experimental phases following application of PLS-DA modelling, in comparison to phase or day order as a factor (chapter 3).

3. Creatinine, and MSTUS did not improve the distribution in the plasma data set either, and once again year seemed to influence separation more so than experimental phase or day order (chapter 3).

4. Normalizing to selected purine metabolites in plasma and urine requires further investigation, but ultimately a better controlled study design would alleviate the need to apply this technique (chapter 3).

5. Urinary and plasma data sets exhibited unique inter-subject variability and clustering in the baseline distribution pattern (in both bio-fluids) and in the wash-out phase (urine) (chapter 3).

6. ROC curve analysis can greatly decrease the volume of significant metabolites, thereby decreasing the chances of false positives, yet at the same time amplify the importance of specific metabolites that could warrant further investigation (chapter 3 and 4).

7. Urinary orotate, intermediate of pyrimidine nucleotide synthesis, could be a potential marker of EPO abuse that requires further investigation (chapter 3). ROC curve analysis (AUC > 0.9) indicated promising marker at EPO2 time-point (after 2 weeks of r-HuEPO injections) as well as at EPO3 (AUC > 0.8). EPO2 *vs.* BASELINE has cut-off at 13.38 % with sensitivity at 88.9 % and specificity at 87.5 %. EPO3 *vs.*

BASELINE has cut-off at 13.37 % with sensitivity at 88.9 % and specificity at 72.2 %.

8. The 14 selected plasma metabolites as a grouped predictive model for POST1 *vs.* PRE, exhibited an AUC of 1 (95% CI 1-1). Average accuracy based on 100 cross validations is 0.99 (AUC). These markers could potentially be traced to the following candidate genes from the WADA gene-expression study: ADIPOR1 (linoelaidyl carnitine and elaidic carnitine), ALAS2 (glycine and potentially histidine metabolite ergothioneine) as well as DPYSL5 (thymine and potentially 2-oxoglutarate). Other markers include: NO related metabolites L-arginine as well as homoarginine, liver generated by-product deoxycholic acid 3-glucuronide, glycosaminoglycan biosynthesis metabolites D-glucuronate as well as D-glucosamine, neurotransmitter 4-aminobutanoate, bacterial peptitoglycan muramic acid, retinoid responsive gene regulation metabolite [PR] Tretinoin/all-trans retinoic acid (chapter 3).

9. The RBC data sets from whole blood and centrifuged blood residue require further investigation (chapter 4). On one hand the RBC profile from whole blood exhibited some interesting findings with regard to glutathione generation, yet in a small subject group. Whereas, the RBC data obtained from the blood residue following centrifugation showed a more cohesive metabolite pattern with regard to fatty acid transport.

10. Serialized urine samples across a short time-period (10 time-points across a 37-h period) allows for a superior method to observe metabolic patterns and natural diurnal effects (chapter 5).

11. Exercise, has a profound effect on the human metabolome. The most evident are those exhibited in the purine metabolism; mainly on hypoxanthine. This should be considered when designing metabolomics-based perturbations, as it acts as a strong confounding variable. We were able to identify metabolites from a variety of pathways (purine pathway, tryptophan, carnitine, cortisol and androgen metabolism, as well as amino acid oxidation and metabolites from the gastrointestinal microbiome) via an untargeted LC-MS approach, presenting good selectivity and coverage that supported

several papers published in the past 15 years, of varying methodological designs (chapter 5).

6.1. Limitations of the WADA Funded Study

One of the main areas that required improvement was in the study design, in order to distinguish key metabolites of interest affected by EPO from those produced by other external stimuli. The present study was not blinded, did not include a control group and the subjects, albeit involved in endurance activities, were not all runners (Durussel et al., 2013). The design was based around the notion that each individual would act as their own control, yet it would have been more informative to have compared the findings to those from a placebo treated cohort, with ideally a crossover design. Diet was not strictly controlled over the ten weeks however this method, arguably, could allow for better anthropological validity. In the field, prospective athletes undergoing doping control will rarely be in a fasted condition or in a tightly controlled environment, hence it would be advisable to create tests which are robust in response to a variety of uncontrolled stimuli. Having said this, there is evidence to suggest that nitrate supplementation in animal models (Wistar rats), under normoxic and hypoxic conditions, suppresses hepatic erythropoietin expression through nitric oxide production (Ashmore et al., 2014), which in turn lowers circulating haemoglobin. In fact, the up-regulation of HIF-1α is not only regulated by oxygen availability but also by nitric oxide as well (Levett et al., 2011). Higher nitrate/ nitrite levels have been shown to be exhibited by high altitude residing Tibetans which could be attributed to a metabolic adaption to hypoxia (Levett et al., 2011). In combination, the points raised above have implications towards the gene-expression component of the WADA study and the relative aspects of EPO regulation that have yet to be fully realized.

Subjects visited the laboratory almost every second day for 10 weeks and were asked a series of questions regarding supplement, caffeine and medication use prior to the general 20 sample collection time-points. They were also informed to abstain from alcohol for the 24 hours prior to a sample collection visit. Subjects were told not to eat or drink anything for at least 1 hour prior to each blood collection time point (Scalbert

et al., 2009). Yet exercise was not controlled prior to a collection and given the findings in the pilot exercise study (chapter 5) there is strong evidence to suggest that this could obscure any effects on metabolite matrix, possibly enough to obscure any signature from the drug itself (Christensen et al., 2013).

The East-African cohort was not analyzed therefore inferences to ethnic variances to r-HuEPO administration cannot be made.

6.2. Future Work

There are a plethora of approaches that can be taken to improve the methodology used for finding markers of r-HuEPO abuse. Recommendations would include investigation, through a continuous time-frame similar to the pilot exercise study, the effect of a single bolus administration of r-HuEPO. From this it would be possible to extend to one week of continuous injections to 2 weeks. The study should follow a double-blinded cross-over design to ensure that a placebo group is included for adequate control. Exercise is another aspect which should be considered. First initial investigations could be carried out on normal sedentary individuals without exercise and then extended to training groups. As such, when sampling from training groups ensure that at least 24 hours have passed post-exercise until collection time-point to avoid metabolome shifts.

Other areas that were not investigated through this thesis that could be potential areas for investigation include:

1. Preparation and analysis of the East-African cohort blood and urine samples. These have already been collected and performance, haematological and demographics of these subjects is being prepared for submission.

2. Combine Caucasian gene-expression and metabolomics profiles for an integrated OMICs approach to detecting r-HuEPO. This may prove to be difficult, as it is naïve to assume that this process is linear in any way and that the up-regulation of a particular

gene would result in specific metabolic changes in complex and hierarchical systems such as human beings.

3. Compare East-African and Caucasian cohorts. This might highlight specific marker differences between sea level and altitude in combination with changes due to r-HuEPO administration. However, even if the data is too stochastic, given the already discussed methodological limitations, the two weeks of baseline measurements may still reveal ethnically driven changes in metabolite profiles which would still be useful for anti-doping authorities and especially for the ABP. This might help reduce the number of false positives in altitude residing ethnic groups.

4. Investigate the responses of the purine metabolites to exercise and hypoxia. There is strong evidence to support the relationship between the effects of exercise and the adenine nucleotides, however recent metabolomics- based research into normobaric hypoxia in the urine of healthy males suggest another possible association (Lou et al., 2014). If exercise and exposure to hypoxia result in similar metabolic responses in the purine pathway, then could this provide another route for determining the overall fitness status or well-being of a particular individual? What implications are there for doping control authorities? This is another avenue that warrants further investigation.

5. Investigate further the relationship between dietary nitrate, which can be converted to nitrite by the microbiome, and EPO regulation. The effects of nitric oxide, a prominent vasodilator with cardio-protective value, could provide valuable insight to anti-doping control authorities as well as patient groups. Therefore it would be of interest to investigate the cumulative as well as the acute effects of the co-administration of nitrate and EPO in human subjects.

6. There is variability across the response of the subjects in the baseline phase, indicative of the possibility of metabotyping. Irrespective of this, there is a great deal of information that can be obtained whilst investigating the 'normal' fluctuations of metabolites across one day, weeks and so on. This could, potentially, benefit anti-doping authorities in their drive to promote and incorporate OMICs technologies to routine doping control. It is equally important to start from the basics, and identify the

state of a particular metabolite or groups of metabolites at a particular time of day and how this could affect the profile of that selected athlete over time.

References

Aachmann-Andersen, N. J.; Just Christensen, S.; Lisbjerg, K.; Oturai, P.; Meinild-Lundby, A.-K.; Holstein-Rathlou, N.-H.; Lundby, C.; Vidiendal Olsen, N. Recombinant Erythropoietin in Humans Has a Prolonged Effect on Circulating Erythropoietin Isoform Distribution. *PLoS One* **2014**, *9*, e110903.

Adamski, J. Genome-Wide Association Studies with Metabolomics. *Genome Med.* **2012**, *4*, 34.

Aguiló, A.; Tauler, P.; Fuentespina, E.; Tur, J. a; Córdova, A.; Pons, A. Antioxidant Response to Oxidative Stress Induced by Exhaustive Exercise. *Physiol. Behav.* **2005**, *84*, 1–7.

Amelio, I.; Cutruzzolá, F.; Antonov, A.; Agostini, M.; Melino, G. Serine and Glycine Metabolism in Cancer. *Trends Biochem. Sci.* **2014**, *39*, 191–198.

Ang, J. E.; Revell, V.; Mann, A.; Mäntele, S.; Otway, D. T.; Johnston, J. D.; Thumser, A. E.; Skene, D. J.; Raynaud, F. Identification of Human Plasma Metabolites Exhibiting Time-of-Day Variation Using an Untargeted Liquid Chromatography-Mass Spectrometry Metabolomic Approach. *Chronobiol. Int.* **2012**, *29*, 868–881.

Appolonova, S. a; Dikunets, M. a; Rodchenkov, G. M. Possible Indirect Detection of rHuEPO Administration in Human Urine by High-Performance Liquid Chromatography Tandem Mass Spectrometry. *Eur. J. Mass Spectrom. (Chichester, Eng).* **2008**, *14*, 201–209.

Appolonova, S. A.; Dikunets, M. A.; Rodchenkov, G. M. HPLC-MS/MS Investigation of Biochemical Markers for the Disclosure of Erythropoietin Abuse in Sports. *Russ. J. Phys. Chem. A* **2009**, *83*, 520–529.

Ashenden, M. Contemporary Issues in the Fight against Blood Doping in Sport. *Haematologica* **2004**, *89*, 901–903.

Ashenden, M.; Gough, C. E.; Garnham, A.; Gore, C. J.; Sharpe, K. Current Markers of the Athlete Blood Passport Do Not Flag Microdose EPO Doping. *Eur. J. Appl. Physiol.* **2011**, *111*, 2307–2314.

Ashmore, T.; Fernandez, B. O.; Evans, C. E.; Huang, Y.; Branco-Price, C.; Griffin, J. L.; Johnson, R. S.; Feelisch, M.; Murray, a. J. Suppression of Erythropoiesis by Dietary Nitrate. *FASEB J.* **2014**, 1–11.

Atamaniuk, J.; Vidotto, C.; Kinzlbauer, M.; Bachl, N.; Tiran, B.; Tschan, H. Cell-Free Plasma DNA and Purine Nucleotide Degradation Markers Following Weightlifting Exercise. *Eur. J. Appl. Physiol.* **2010**, *110*, 695–701.

Audran, M.; Gareau, R.; Matecki, S.; Durand, F.; Chenard, C.; Sicart, M. T.; Marion, B.; Bressolle, F. Effects of Erythropoietin Administration in Training Athletes and Possible Indirect Detection in Doping Control. *Med. Sci. Sports Exerc.* **1999**, *31*, 639–645.

Azzazy, H. M. E. Gene Doping. **2010**, 485–512.

Azzazy, H. M. E.; Mansour, M. M. H. Rogue Athletes and Recombinant DNA Technology: Challenges for Doping Control. *Analyst* **2007**, *132*, 951–957.

Bal, K.; Larsson, L. New and Simple Procedure for the Determination of Muramic Acid in Chemically Complex Environments by Gas Chromatography-Ion Trap Tandem Mass Spectrometry. *J. Chromatogr. B. Biomed. Sci. Appl.* **2000**, *738*, 57–65.

Baranowski, M.; Charmas, M.; Długołęcka, B.; Górski, J. Exercise Increases Plasma Levels of Sphingoid Base-1 Phosphates in Humans. *Acta Physiol. (Oxf).* **2011**, *203*, 373–380.

Baranowski, M.; Górski, J.; Klapcinska, B.; Waskiewicz, Z.; Sadowska-Krepa, E. Ultramarathon Run Markedly Reduces Plasma Sphingosine-1-Phosphate Concentration. *Int. J. Sport Nutr. Exerc. Metab.* **2014**, *24*, 148–156.

Barasa, B.; Slijper, M. Challenges for Red Blood Cell Biomarker Discovery through Proteomics. *Biochim. Biophys. Acta* **2014**, *1844*, 1003–1010.

Berg, J. M.; Tymoczko, J. L.; Stryer, L. The Citric Acid Cycle, 2002.

Berglund, B.; Ekblom, B. Effect of Recombinant Human Erythropoietin Treatment on Blood Pressure and Some Haematological Parameters in Healthy Men. *J. Intern. Med.* **1991**, *229*, 125–130.

Bergman, D. The Endocrinology of Exercise. *Intern. Emerg. Med.* **2013**, *8 Suppl 1*, S17–S21.

Bianchi, G. P.; Grossi, G.; Bargossi, a M.; Fiorella, P. L.; Marchesini, G. Can Oxypurines Plasma Levels Classify the Type of Physical Exercise? *J. Sports Med. Phys. Fitness* **1999**, *39*, 123–127.

Birkeland, K. I.; Stray-Gundersen, J.; Hemmersbach, P.; Hallen, J.; Haug, E.; Bahr, R. Effect of rhEPO Administration on Serum Levels of sTfR and Cycling Performance. *Med. Sci. Sports Exerc.* **2000**, *32*, 1238–1243.

Bosman, G. J. C. G. M. Survival of Red Blood Cells after Transfusion: Processes and Consequences. *Front. Physiol.* **2013**, *4*, 376.

Bouatra, S.; Aziat, F.; Mandal, R.; Guo, A. C.; Wilson, M. R.; Knox, C.; Bjorndahl, T. C.; Krishnamurthy, R.; Saleem, F.; Liu, P.; et al. The Human Urine Metabolome. *PLoS One* **2013**, *8*, e73076.

Bouchard-Mercier, A.; Rudkowska, I.; Lemieux, S.; Couture, P.; Vohl, M.-C. The Metabolic Signature Associated with the Western Dietary Pattern: A Cross-Sectional Study. *Nutr. J.* **2013**, *12*, 158.

Bratty, M. Al; Chintapalli, V. R.; Dow, J. A. T.; Zhang, T.; Watson, D. G. Metabolomic Profiling Reveals That Drosophila Melanogaster Larvae with the Y Mutation Have Altered Lysine Metabolism. *FEBS Open Bio* **2012**, *2*, 217–221.

Brodsky, L.; Moussaieff, A.; Shahaf, N.; Aharoni, A.; Rogachev, I. Evaluation of Peak Picking Quality in LC-MS Metabolomics Data. *Anal. Chem.* **2010**, *82*, 9177–9187.

Brosnan, M. E.; Brosnan, J. T. Orotic Acid Excretion and Arginine Metabolism. *J. Nutr.* **2007**, *137*, 1656S – 1661S.

Burke, M. F.; Dunbar, R. L.; Rader, D. J. Could Exercise Metabolomics Pave the Way for Gymnomimetics? *Sci. Transl. Med.* **2010**, *2*, 41ps35.

Bye, A.; Vettukattil, R.; Aspenes, S. T.; Giskeødegård, G. F.; Gribbestad, I. S.; Wisløff, U.; Bathen, T. F. Serum Levels of Choline-Containing Compounds Are Associated with Aerobic Fitness Level: The HUNT-Study. *PLoS One* **2012**, *7*, e42330.

Cachero, T. G.; Morielli, A. D.; Peralta, E. G. The Small GTP-Binding Protein RhoA Regulates a Delayed Rectifier Potassium Channel. *Cell* **1998**, *93*, 1077–1085.

Campbell, C.; Grapov, D.; Fiehn, O.; Chandler, C. J.; Burnett, D. J.; Souza, E. C.; Casazza, G. A.; Gustafson, M. B.; Keim, N. L.; Newman, J. W.; et al. Improved Metabolic Health Alters Host Metabolism in Parallel with Changes in Systemic Xeno-Metabolites of Gut Origin. *PLoS One* **2014**, *9*, e84260.

Chang, T. W.; Goldberg, A. L. The Origin of Alanine Produced in Skeletal Muscle. *J. Biol. Chem.* **1978**, *253*, 3677–3684.

Chevion, S.; Moran, D. S.; Heled, Y.; Shani, Y.; Regev, G.; Abbou, B.; Berenshtein, E.; Stadtman, E. R.; Epstein, Y. Plasma Antioxidant Status and Cell Injury after Severe Physical Exercise. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 5119–5123.

Chorell, E.; Moritz, T.; Branth, S.; Antti, H.; Svensson, M. B. Predictive Metabolomics Evaluation of Nutrition-Modulated Metabolic Stress Responses in Human Blood Serum During the Early Recovery Phase of Strenuous Physical Exercise Research Articles. *J. Proteome Res.* **2009**, *8*, 2966–2977.

Chorell, E.; Svensson, M. B.; Moritz, T.; Antti, H. Physical Fitness Level Is Reflected by Alterations in the Human Plasma Metabolome. *Mol. Biosyst.* **2012**, *8*, 1187–1196.

Christensen, B.; Sackmann-Sala, L.; Cruz-Topete, D.; Jørgensen, J. O. L.; Jessen, N.; Lundby, C.; Kopchick, J. J. Novel Serum Biomarkers for Erythropoietin Use in Humans: A Proteomic Approach. *J. Appl. Physiol.* **2011**, *110*, 149–156.

Christensen, B.; Vendelbo, M. H.; Krusenstjerna-Hafstrøm, T.; Madsen, M.; Pedersen, S. B.; Jessen, N.; Møller, N.; Jørgensen, J. O. L. Erythropoietin Administration Acutely Stimulates Resting Energy Expenditure in Healthy Young Men. *J. Appl. Physiol.* **2012**, *112*, 1114–1121.

Christensen, B.; Nellemann, B.; Larsen, M. S.; Thams, L.; Sieljacks, P.; Vestergaard, P. F.; Bibby, B. M.; Vissing, K.; Stødkilde-Jørgensen, H.; Pedersen, S. B.; et al. Whole Body Metabolic Effects of Prolonged Endurance Training in Combination with Erythropoietin Treatment in Humans: A Randomized Placebo Controlled Trial. *Am. J. Physiol. Endocrinol. Metab.* **2013**, *305*, E879–E889.

Clouse, A.; Deo, S.; Rampersaud, E.; Farmer, J.; Goldschmidt-Clermont, P. J.; Daunert, S. Defining a Molecular Portrait of Physical Fitness. *Anal. Bioanal. Chem.* **2013**, *405*, 21–26.

Connes, P.; Perrey, S.; Varray, A.; Préfaut, C.; Caillaud, C. Faster Oxygen Uptake Kinetics at the Onset of Submaximal Cycling Exercise Following 4 Weeks Recombinant Human Erythropoietin (r-HuEPO) Treatment. *Pflugers Arch.* **2003**, *447*, 231–238.

Connes, P.; Caillaud, C.; Mercier, J.; Bouix, D.; Casties, J. F. Injections of Recombinant Human Erythropoietin Increases Lactate Influx into Erythrocytes. *J. Appl. Physiol.* **2004**, *97*, 326–332.

Cortese-Krott, M. M.; Kelm, M. Endothelial Nitric Oxide Synthase in Red Blood Cells: Key to a New Erythrocrine Function? *Redox Biol.* **2014**, *2*, 251–258.

Creek, D. J.; Jankevics, A.; Breitling, R.; Watson, D. G.; Barrett, M. P.; Burgess, K. E. V. Toward Global Metabolomics Analysis with Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry: Improved Metabolite Identification by Retention Time Prediction. *Anal. Chem.* **2011**, *83*, 8703–8710.

Creek, D. J.; Jankevics, A.; Burgess, K. E. V; Breitling, R.; Barrett, M. P. IDEOM: An Excel Interface for Analysis of LC-MS-Based Metabolomics Data. *Bioinformatics* **2012**, *28*, 1048–1049.

Cubbon, S.; Antonio, C.; Wilson, J.; Thomas-Oates, J. Metabolomic Applications of HILIC-LC-MS. *Mass Spectrom. Rev.* **2010**, *29*, 671–684.

Dansie, L. E.; Reeves, S.; Miller, K.; Zano, S. P.; Frank, M.; Pate, C.; Wang, J.; Jackowski, S. Physiological Roles of the Pantothenate Kinases. *Biochem. Soc. Trans.* **2014**, *42*, 1033–1036.

Daskalaki, E.; Blackburn, G.; Kalna, G.; Zhang, T.; Anthony, N.; Watson, D. A Study of the Effects of Exercise on the Urinary Metabolome Using Normalisation to Individual Metabolic Output. *Metabolites* **2015a**, *5*, 119–139.

Daskalaki, E.; Easton, C.; Watson, D. G. The Application of Metabolomic Profiling to the Effects of Physical Activity. *Curr. Metabolomics* **2015b**, *2*, 233–263.

Davila, A.-M.; Blachier, F.; Gotteland, M.; Andriamihaja, M.; Benetti, P.-H.; Sanz, Y.; Tomé, D. Intestinal Luminal Nitrogen Metabolism: Role of the Gut Microbiota and Consequences for the Host. *Pharmacol. Res.* **2013a**, *68*, 95–107.

Davila, A.-M.; Blachier, F.; Gotteland, M.; Andriamihaja, M.; Benetti, P.-H.; Sanz, Y.; Tomé, D. Intestinal Luminal Nitrogen Metabolism: Role of the Gut Microbiota and Consequences for the Host. *Pharmacol. Res.* **2013b**, *68*, 95–107.

Debeljak, N.; Solár, P.; Sytkowski, A. J. Erythropoietin and Cancer: The Unintended Consequences of Anemia Correction. *Front. Immunol.* **2014**, *5*, 563.

Degoutte, F.; Jouanel, P.; Filaire, E. Energy Demands during a Judo Match and Recovery. *Br. J. Sports Med.* **2003**, *37*, 245–249.

Dehnes, Y.; Myrvold, L.; Ström, H.; Ericsson, M.; Hemmersbach, P. MAIIA EPO SeLect - a Rapid Screening Kit for the Detection of Recombinant EPO Analogues in Doping Control: Inter-Laboratory Prevalidation and Normative Study of Athlete Urine and Plasma Samples. *Drug Test. Anal.* **2014**, *6*, 1144–1150.

Dovio, A.; Roveda, E.; Sciolla, C.; Montaruli, A.; Raffaelli, A.; Saba, A.; Calogiuri, G.; De Francia, S.; Borrione, P.; Salvadori, P.; et al. Intense Physical Exercise Increases Systemic 11beta-Hydroxysteroid Dehydrogenase Type 1 Activity in Healthy Adult Subjects. *Eur. J. Appl. Physiol.* **2010**, *108*, 681–687.

Dudzinska, W.; Lubkowska, A.; Dolegowska, B.; Safranow, K.; Jakubowska, K. Adenine, Guanine and Pyridine Nucleotides in Blood during Physical Exercise and Restitution in Healthy Subjects. *Eur. J. Appl. Physiol.* **2010a**, *110*, 1155–1162.

Dudzinska, W.; Lubkowska, A.; Dolegowska, B.; Safranow, K. Blood Uridine Concentration May Be an Indicator of the Degradation of Pyrimidine Nucleotides during Physical Exercise with Increasing Intensity. *J. Physiol. Biochem.* **2010b**, *66*, 189–196.

Dudzinska, W.; Lubkowska, a; Jakubowska, K.; Suska, M.; Skotnicka, E. Insulin Resistance Induced by Maximal Exercise Correlates with a Post-Exercise Increase in Uridine Concentration in the Blood of Healthy Young Men. *Physiol. Res.* **2013**, *62*, 163–170.

Dumas, M.-E.; Kinross, J.; Nicholson, J. K. Metabolic Phenotyping and Systems Biology Approaches to Understanding Metabolic Syndrome and Fatty Liver Disease. *Gastroenterology* **2014**, *146*, 46–62.

Dunn, W. B.; Ellis, D. I. Metabolomics: Current Analytical Platforms and Methodologies. *TrAC Trends Anal. Chem.* **2005**, *24*, 285–294.

Dunn, W. B.; Broadhurst, D.; Begley, P.; Zelena, E.; Francis-McIntyre, S.; Anderson, N.; Brown, M.; Knowles, J. D.; Halsall, A.; Haselden, J. N.; 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*, 1060–1083.

Durussel, J.; Daskalaki, E.; Anderson, M.; Chatterji, T.; Wondimu, D. H.; Padmanabhan, N.; Patel, R. K.; McClure, J. D.; Pitsiladis, Y. P. Haemoglobin Mass and Running Time Trial Performance after Recombinant Human Erythropoietin Administration in Trained Men. *PLoS One* **2013**, *8*.

Ellis, D. I.; Dunn, W. B.; Griffin, J. L.; Allwood, J. W.; Goodacre, R. Metabolic Fingerprinting as a Diagnostic Tool. *Pharmacogenomics* **2007**, *8*, 1243–1266.

Emhoff, C.-A. W.; Messonnier, L. A.; Horning, M. A.; Fattor, J. A.; Carlson, T. J.; Brooks, G. A. Gluconeogenesis and Hepatic Glycogenolysis during Exercise at the Lactate Threshold. *J. Appl. Physiol.* **2013**, *114*, 297–306.

Enea, C.; Seguin, F.; Petitpas-Mulliez, J.; Boildieu, N.; Boisseau, N.; Delpech, N.; Diaz, V.; Eugène, M.; Dugué, B. (1)H NMR-Based Metabolomics Approach for Exploring Urinary Metabolome Modifications after Acute and Chronic Physical Exercise. *Anal. Bioanal. Chem.* **2010**, *396*, 1167–1176.

Fanelli, C.; Calderone, S.; Epifano, L.; De Vincenzo, A.; Modarelli, F.; Pampanelli, S.; Perriello, G.; De Feo, P.; Brunetti, P.; Gerich, J. E. Demonstration of a Critical Role for Free Fatty Acids in Mediating Counterregulatory Stimulation of Gluconeogenesis and Suppression of Glucose Utilization in Humans. *J. Clin. Invest.* **1993**, *92*, 1617–1622.

Fiedler, L. The DDAH/ADMA Pathway Is a Critical Regulator of NO Signalling in Vascular Homeostasis. *Cell Adh. Migr.* **2008**, *2*, 149–150.

Fiehn, O.; Robertson, D.; Griffin, J.; van der Werf, M.; Nikolau, B.; Morrison, N.; Sumner, L. W.; Goodacre, R.; Hardy, N. W.; Taylor, C.; et al. The Metabolomics Standards Initiative (MSI). *Metabolomics* **2007**, *3*, 175–178.

Filippakopoulos, P.; Knapp, S. Targeting Bromodomains: Epigenetic Readers of Lysine Acetylation. *Nat. Rev. Drug Discov.* **2014**, *13*, 337–356.

Forrester, T. The Identification and Assay of Acetylcholine and Adenosine Triphorphate Released from Active Skeletal Muscle, University of Glasgow, 1967.

Forrester, T.; Lind, A. R. Identification of Adenosine Triphosphate in Human Plasma and the Concentration in the Venous Effluent of Forearm Muscles Before, during and after Sustained Contractions. *J. Physiol.* **1969**, *204*, 347–364.

Franco, R. S. Measurement of Red Cell Lifespan and Aging. *Transfus. Med. hemotherapy Off. Organ der Dtsch. Gesellschaft fur̈ Transfusionsmedizin und Immunhamatologie* **2012**, *39*, 302–307.

Franco, R. S.; Puchulu-Campanella, M. E.; Barber, L. a; Palascak, M. B.; Joiner, C. H.; Low, P. S.; Cohen, R. M. Changes in the Properties of Normal Human Red Blood Cells during in Vivo Aging. *Am. J. Hematol.* **2013**, *88*, 44–51.

Gangemi, S.; Luciotti, G.; D'Urbano, E.; Mallamace, A.; Santoro, D.; Bellinghieri, G.; Davi, G.; Romano, M. Physical Exercise Increases Urinary Excretion of Lipoxin A4 and Related Compounds. *J. Appl. Physiol.* **2003**, *94*, 2237–2240.

Ganti, S.; Taylor, S. L.; Kim, K.; Hoppel, C. L.; Guo, L.; Yang, J.; Evans, C.; Weiss, R. H. Urinary Acylcarnitines Are Altered in Human Kidney Cancer. *Int. J. Cancer* **2012**, *130*, 2791–2800.

Gatti, R.; Cappellin, E.; Zecchin, B.; Antonelli, G.; Spinella, P.; Mantero, F.; De Palo, E. F. Urinary High Performance Reverse Phase Chromatography Cortisol and Cortisone Analyses before and at the End of a Race in Elite Cyclists. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2005**, *824*, 51–56.

Gaudard, A.; Varlet-marie, E.; Bressolle, F.; Audran, M. Drugs for Increasing Oxygen Transport and Their Potential Use in Doping, A Review. *Sport. Med.* **2003**, *33*, 187–212.

Gavaghan, C. L.; Wilson, I. D.; Nicholson, J. K. Physiological Variation in Metabolic Phenotyping and Functional Genomic Studies: Use of Orthogonal Signal Correction and PLS-DA. *FEBS Lett.* **2002**, *530*, 191–196.

Ge, R.-L.; Witkowski, S.; Zhang, Y.; Alfrey, C.; Sivieri, M.; Karlsen, T.; Resaland, G. K.; Harber, M.; Stray-Gundersen, J.; Levine, B. D. Determinants of Erythropoietin Release in Response to Short-Term Hypobaric Hypoxia. *J. Appl. Physiol.* **2002**, *92*, 2361–2367.

Gerber, T.; Borg, M. L.; Hayes, A.; Stathis, C. G. High-Intensity Intermittent Cycling Increases Purine Loss Compared with Workload-Matched Continuous Moderate Intensity Cycling. *Eur. J. Appl. Physiol.* **2014**, *114*, 1513–1520.

Gieger, C.; Geistlinger, L.; Altmaier, E.; Hrabé de Angelis, M.; Kronenberg, F.; Meitinger, T.; Mewes, H.-W.; Wichmann, H.-E.; Weinberger, K. M.; Adamski, J.; et al. Genetics Meets Metabolomics: A Genome-Wide Association Study of Metabolite Profiles in Human Serum. *PLoS Genet.* **2008**, *4*, e1000282.

Gika, H. G.; Theodoridis, G. a.; Plumb, R. S.; Wilson, I. D. Current Practice of Liquid Chromatography-Mass Spectrometry in Metabolomics and Metabonomics. *J. Pharm. Biomed. Anal.* **2014**, *87*, 12–25.

Giraldo, E.; Garcia, J. J.; Hinchado, M. D.; Ortega, E. Exercise Intensity-Dependent Changes in the Inflammatory Response in Sedentary Women: Role of Neuroendocrine Parameters in the Neutrophil Phagocytic Process and the pro-/anti-Inflammatory Cytokine Balance. *Neuroimmunomodulation* **2009**, *16*, 237–244.

Girasole, M.; Dinarelli, S.; Boumis, G. Structural, Morphological and Nanomechanical Characterisation of Intermediate States in the Ageing of Erythrocytes. *J. Mol. Recognit.* **2012**, *25*, 285–291.

Goodyear, L. J. The Exercise Pill--Too Good to Be True? *N. Engl. J. Med.* **2008**, *359*, 1842–1844.

Gooley, J. J. Applications of Circadian Metabolomics. *Curr. Metabolomics* **2014**, *2*, 2–14.

Green, H. J.; Bombardier, E.; Duhamel, T. A.; Stewart, R. D.; Tupling, A. R.; Ouyang, J. Metabolic, Enzymatic, and Transporter Responses in Human Muscle during Three Consecutive Days of Exercise and Recovery. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2008**, *295*, R1238–R1250.

Green, H. J.; Bombardier, E.; Burnett, M. E.; Smith, I. C.; Tupling, S. M.; Ranney, D. A. Time-Dependent Effects of Short-Term Training on Muscle Metabolism during the Early Phase of Exercise. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2009**, *297*, R1383–R1391.

Green, H. J.; Burnett, M.; Jacobs, I.; Ranney, D.; Smith, I.; Tupling, S. Adaptations in Muscle Metabolic Regulation Require Only a Small Dose of Aerobic-Based Exercise. *Eur. J. Appl. Physiol.* **2013a**, *113*, 313–324.

Green, H. J.; Burnett, M.; Carter, S.; Jacobs, I.; Ranney, D.; Smith, I.; Tupling, S. Role of Exercise Duration on Metabolic Adaptations in Working Muscle to Short-Term Moderate-to-Heavy Aerobic-Based Cycle Training. *Eur. J. Appl. Physiol.* **2013b**, *113*, 1965–1978.

Green, H.; Grant, S.; Bombardier, E.; Ranney, D. Initial Aerobic Power Does Not Alter Muscle Metabolic Adaptations to Short-Term Training. *Am. J. Physiol.* **1999**, *277*, E39–E48.

Griffin, J. L.; Bonney, S. a; Mann, C.; Hebbachi, A. M.; Gibbons, G. F.; Nicholson, J. K.; Shoulders, C. C.; Scott, J. An Integrated Reverse Functional Genomic and Metabolic Approach to Understanding Orotic Acid-Induced Fatty Liver. *Physiol. Genomics* **2004**, *17*, 140–149.

Gründemann, D.; Harlfinger, S.; Golz, S.; Geerts, A.; Lazar, A.; Berkels, R.; Jung, N.; Rubbert, A.; Schömig, E. Discovery of the Ergothioneine Transporter. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5256–5261.

Haase, V. H. Regulation of Erythropoiesis by Hypoxia-Inducible Factors. *Blood Rev.* **2013**, *27*, 41–53.

Hafezi-Moghadam, A.; Simoncini, T.; Yang, Z.; Limbourg, F. P.; Plumier, J.-C.; Rebsamen, M. C.; Hsieh, C.-M.; Chui, D.-S.; Thomas, K. L.; Prorock, A. J.; et al. Acute Cardiovascular Protective Effects of Corticosteroids Are Mediated by Non-Transcriptional Activation of Endothelial Nitric Oxide Synthase. *Nat. Med.* **2002**, *8*, 473–479.

Hailey, N. A False Start in the Race Against Doping in Sport : Concerns with Cycling's Biological Passport. *Duke Law J.* **2010**, *61*, 393–432.

Hellsten, Y.; Svensson, M.; Sjödin, B.; Smith, S.; Christensen, A.; Richter, E. .; Bangsbo, J. Allantoin Formation and Urate and Glutathione Exchange in Human Muscle during Submaximal Exercise. *Free Radic. Biol. Med.* **2001**, *31*, 1313–1322.

Heyman, E.; Gamelin, F.-X.; Goekint, M.; Piscitelli, F.; Roelands, B.; Leclair, E.; Di Marzo, V.; Meeusen, R. Intense Exercise Increases Circulating Endocannabinoid and BDNF Levels in Humans--Possible Implications for Reward and Depression. *Psychoneuroendocrinology* **2012**, *37*, 844–851.

Holecek, M. Relation between Glutamine, Branched-Chain Amino Acids, and Protein Metabolism. *Nutrition* **2002**, *18*, 130–133.

Hsu, J.; Johansen, K. L.; Hsu, C.-Y.; Kaysen, G. A.; Chertow, G. M. Higher Serum Creatinine Concentrations in Black Patients with Chronic Kidney Disease: Beyond Nutritional Status and Body Composition. *Clin. J. Am. Soc. Nephrol.* **2008**, *3*, 992–997.

Hunter, P. Reading the Metabolic Fine Print. The Application of Metabolomics to Diagnostics, Drug Research and Nutrition Might Be Integral to Improved Health and Personalized Medicine. *EMBO Rep.* **2009**, *10*, 20–23.

Imai, H.; Hayashi, T.; Negawa, T.; Nakamura, K.; Tomida, M.; Koda, K.; Tajima, T.; Koda, Y.; Suda, K.; Era, S. Strenuous Exercise-Induced Change in Redox State of Human Serum Albumin during Intensive Kendo Training. *Jpn. J. Physiol.* **2002**, *52*, 135–140.

Jelkmann, W. Erythropoietin after a Century of Research: Younger than Ever. *Eur. J. Haematol.* **2007**, *78*, 183–205.

Jelkmann, W. Erythropoietin: Back to Basics. *Blood* **2010**, *115*, 4151–4152.

Jin, X.; Yun, S. J.; Jeong, P.; Kim, I. Y.; Kim, W.-J.; Park, S. Diagnosis of Bladder Cancer and Prediction of Survival by Urinary Metabolomics. *Oncotarget* **2014**, *5*, 1635–1645.

Juel, C.; Thomsen, J. J.; Rentsch, R. L.; Lundby, C. Effects of Prolonged Recombinant Human Erythropoietin Administration on Muscle Membrane Transport Systems and Metabolic Marker Enzymes. *Eur. J. Appl. Physiol.* **2007**, *102*, 41–44.

Kanďár, R.; Stramová, X.; Drábková, P.; Křenková, J. A Monitoring of Allantoin, Uric Acid, and Malondialdehyde Levels in Plasma and Erythrocytes after a 10-Minute Running Activity. *Physiol. Res.* **2014**.

Karatzaferi, C.; Haan, A. De; Mechelen, W. Van; Sargeant, A. J. Metabolic Changes in Single Human Muscle Fibres during Brief Maximal Exercise. *Exp. Physiol.* **2014**, *86*, 411–415.

Kasukawa, T.; Sugimoto, M.; Hida, A.; Minami, Y.; Mori, M.; Honma, S.; Honma, K.; Mishima, K.; Soga, T.; Ueda, H. R. Human Blood Metabolite Timetable Indicates Internal Body Time. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 15036–15041.

Katajamaa, M.; Oresic, M. Processing Methods for Differential Analysis of LC/MS Profile Data. *BMC Bioinformatics* **2005**, *6*, 179.

Katajamaa, M.; Miettinen, J.; Oresic, M. MZmine: Toolbox for Processing and Visualization of Mass Spectrometry Based Molecular Profile Data. *Bioinformatics* **2006**, *22*, 634–636.

Katz, O.; Stuible, M.; Golishevski, N.; Lifshitz, L.; Tremblay, M. L.; Gassmann, M.; Mittelman, M.; Neumann, D. Erythropoietin Treatment Leads to Reduced Blood Glucose Levels and Body Mass: Insights from Murine Models. *J. Endocrinol.* **2010**, *205*, 87–95.

Kirkwood, J. S.; Lebold, K. M.; Miranda, C. L.; Wright, C. L.; Miller, G. W.; Tanguay, R. L.; Barton, C. L.; Traber, M. G.; Stevens, J. F. Vitamin C Deficiency Activates the Purine Nucleotide Cycle in Zebrafish. *J. Biol. Chem.* **2012**, *287*, 3833–3841.

De Kloet, E. R.; Karst, H.; Joëls, M. Corticosteroid Hormones in the Central Stress Response: Quick-and-Slow. *Front. Neuroendocrinol.* **2008**, *29*, 268–272.

Koek, M. M.; Jellema, R. H.; van der Greef, J.; Tas, A. C.; Hankemeier, T. Quantitative Metabolomics Based on Gas Chromatography Mass Spectrometry: Status and Perspectives. *Metabolomics* **2011**, *7*, 307–328.

Koopman, R.; Wagenmakers, A. J. M.; Manders, R. J. F.; Zorenc, A. H. G.; Senden, J. M. G.; Gorselink, M.; Keizer, H. A.; van Loon, L. J. C. Combined Ingestion of Protein and Free Leucine with Carbohydrate Increases Postexercise Muscle Protein Synthesis in Vivo in Male Subjects. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E645–E653.

Krug, S.; Kastenmüller, G.; Stückler, F.; Rist, M. J.; Skurk, T.; Sailer, M.; Raffler, J.; Römisch-Margl, W.; Adamski, J.; Prehn, C.; et al. The Dynamic Range of the Human Metabolome Revealed by Challenges. *FASEB J.* **2012**, *26*, 2607–2619.

Krzyzanski, W.; Brier, M. E.; Creed, T. M.; Gaweda, A. E. Reticulocyte-Based Estimation of Red Blood Cell Lifespan. *Exp. Hematol.* **2013**, *41*, 817–822.

Kuehnbaum, N. L.; Britz-McKibbin, P. New Advances in Separation Science for Metabolomics: Resolving Chemical Diversity in a Post-Genomic Era. *Chem. Rev.* **2013**, *113*, 2437–2468.

Van Kuilenburg, A. B. P. Analysis of Pyrimidine Synthesis "de Novo" Intermediates in Urine and Dried Urine Filter- Paper Strips with HPLC-Electrospray Tandem Mass Spectrometry. *Clin. Chem.* **2004**, *50*, 2117–2124.

Kujala, U. M.; Mäkinen, V.-P.; Heinonen, I.; Soininen, P.; Kangas, A. J.; Leskinen, T. H.; Rahkila, P.; Würtz, P.; Kovanen, V.; Cheng, S.; et al. Long-Term Leisure-Time Physical Activity and Serum Metabolome. *Circulation* **2013**, *127*, 340–348.

Ladep, N. G.; Dona, A. C.; Lewis, M. R.; Crossey, M. M. E.; Lemoine, M.; Okeke, E.; Shimakawa, Y.; Duguru, M.; Njai, H. F.; Fye, H. K. S.; et al. Discovery and Validation of Urinary Metabotypes for the Diagnosis of Hepatocellular Carcinoma in West Africans. *Hepatology* **2014**, *60*, 1291–1301.

Lee, J.; Park, J.; Lim, M.; Seong, S. J.; Seo, J. J.; Park, S. M.; Lee, H. W.; Yoon, Y.-R. Quantile Normalization Approach for Liquid Chromatography-Mass Spectrometry-Based Metabolomic Data from Healthy Human Volunteers. *Anal. Sci.* **2012**, *28*, 801–805.

Lehmann, R.; Zhao, X.; Weigert, C.; Simon, P.; Fehrenbach, E.; Fritsche, J.; Machann, J.; Schick, F.; Wang, J.; Hoene, M.; et al. Medium Chain Acylcarnitines Dominate the Metabolite Pattern in Humans under Moderate Intensity Exercise and Support Lipid Oxidation. *PLoS One* **2010a**, *5*, e11519.

Lehmann, R.; Zhao, X.; Weigert, C.; Simon, P.; Fehrenbach, E.; Fritsche, J.; Machann, J.; Schick, F.; Wang, J.; Hoene, M.; et al. Medium Chain Acylcarnitines Dominate the Metabolite Pattern in Humans under Moderate Intensity Exercise and Support Lipid Oxidation. *PLoS One* **2010b**, *5*, e11519.

Levett, D. Z.; Fernandez, B. O.; Riley, H. L.; Martin, D. S.; Mitchell, K.; Leckstrom, C. A.; Ince, C.; Whipp, B. J.; Mythen, M. G.; Montgomery, H. E.; et al. The Role of Nitrogen Oxides in Human Adaptation to Hypoxia. *Sci. Rep.* **2011**, *1*, 109.

Lewis, G. D.; Farrell, L.; Wood, M. J.; Martinovic, M.; Arany, Z.; Rowe, G. C.; Souza, A.; Cheng, S.; McCabe, E. L.; Yang, E.; et al. Metabolic Signatures of Exercise in Human Plasma. *Sci. Transl. Med.* **2010**, *2*, 33ra37.

Lou, B.-S.; Wu, P.-S.; Liu, Y.; Wang, J.-S. Effects of Acute Systematic Hypoxia on Human Urinary Metabolites Using LC-MS-Based Metabolomics. *High Alt. Med. Biol.* **2014**, *15*, 192–202.

Luiking, Y. C.; Deutz, N. E. P. Biomarkers of Arginine and Lysine Excess. *J. Nutr.* **2007**, *137*, 1662S – 1668.

Lundby, C.; Olsen, N. V. Effects of Recombinant Human Erythropoietin in Normal Humans. *J. Physiol.* **2011**, *589*, 1265–1271.

Lundby, C.; Thomsen, J. J.; Boushel, R.; Koskolou, M.; Warberg, J.; Calbet, J. A. L.; Robach, P. Erythropoietin Treatment Elevates Haemoglobin Concentration by Increasing Red Cell Volume and Depressing Plasma Volume. *J. Physiol.* **2007**, *578*, 309–314.

Lundby, C.; Robach, P.; Boushel, R.; Thomsen, J. J.; Rasmussen, P.; Koskolou, M.; Calbet, J. A. L. Does Recombinant Human Epo Increase Exercise Capacity by Means Other than Augmenting Oxygen Transport? *J. Appl. Physiol.* **2008a**, *105*, 581–587.

Lundby, C.; Achman-Andersen, N. J.; Thomsen, J. J.; Norgaard, A. M.; Robach, P. Testing for Recombinant Human Erythropoietin in Urine: Problems Associated with Current Anti-Doping Testing. *J. Appl. Physiol.* **2008b**, *105*, 417–419.

Lundby, C.; Robach, P.; Saltin, B. The Evolving Science of Detection of "Blood Doping". *Br. J. Pharmacol.* **2012**, *165*, 1306–1315.

Lustgarten, M. S.; Price, L. L.; Logvinenko, T.; Hatzis, C.; Padukone, N.; Reo, N. V; Phillips, E. M.; Kirn, D.; Mills, J.; Fielding, R. A. Identification of Serum Analytes and Metabolites Associated with Aerobic Capacity. *Eur. J. Appl. Physiol.* **2013**, *113*, 1311–1320.

Lustgarten, M. S.; Price, L. L.; Chalé, A.; Fielding, R. A. Metabolites Related to Gut Bacterial Metabolism, Peroxisome Proliferator-Activated Receptor-Alpha Activation, and Insulin Sensitivity Are Associated with Physical Function in Functionally-Limited Older Adults. *Aging Cell* **2014**, *13*, 918–925.

Lutz, H. U.; Bogdanova, A. Mechanisms Tagging Senescent Red Blood Cells for Clearance in Healthy Humans. *Front. Physiol.* **2013**, *4*, 387.

Malcovati, L.; Pascutto, C.; Cazzola, M. Hematologic Passport for Athletes Competing in Endurance Sports: A Feasibility Study. *Haematologica* **2003**, *88*, 570–581.

Mehaffey, J. Doping-Undetectable new blood boosters available says expert http://www.reuters.com/article/2012/03/15/doping-epo-idUSL4E8EF70520120315 (accessed Jan 7, 2015).

Menshikova, E. V; Ritov, V. B.; Fairfull, L.; Ferrell, R. E.; Kelley, D. E.; Goodpaster, B. H. Effects of Exercise on Mitochondrial Content and Function in Aging Human Skeletal Muscle. *J. Gerontol. A. Biol. Sci. Med. Sci.* **2006**, *61*, 534–540.

Michlmayr, A.; Oehler, R. "OMICS": State of the Art in Vitro Techniques Employed in Surgical Research. *Eur. Surg.* **2010**, *42*, 127–133.

Miskowiak, K. W.; Vinberg, M.; Harmer, C. J.; Ehrenreich, H.; Kessing, L. V. Erythropoietin: A Candidate Treatment for Mood Symptoms and Memory Dysfunction in Depression. *Psychopharmacology (Berl).* **2011**, *219*, 687–698.

Miskowiak, K.; Inkster, B.; Selvaraj, S.; Wise, R.; Goodwin, G. M.; Harmer, C. J. Erythropoietin Improves Mood and Modulates the Cognitive and Neural Processing of Emotion 3 Days Post Administration. *Neuropsychopharmacology* **2008**, *33*, 611–618.

Miyake, T.; Kung, C. K.; Goldwasser, E. Purification of Human Erythropoietin. *J. Biol. Chem.* **1977**, *252*, 5558–5564.

Moali, C.; Boucher, J. L.; Sari, M. A.; Stuehr, D. J.; Mansuy, D. Substrate Specificity of NO Synthases: Detailed Comparison of L-Arginine, Homo-L-Arginine, Their N Omega-Hydroxy Derivatives, and N Omega-Hydroxynor-L-Arginine. *Biochemistry* **1998**, *37*, 10453–10460.

Moffett, J. R.; Namboodiri, M. A. Tryptophan and the Immune Response. *Immunol. Cell Biol.* **2003**, *81*, 247–265.

Morris, C.; Grada, C. O.; Ryan, M.; Roche, H. M.; De Vito, G.; Gibney, M. J.; Gibney, E. R.; Brennan, L. The Relationship between Aerobic Fitness Level and Metabolic Profiles in Healthy Adults. *Mol. Nutr. Food Res.* **2013**, *57*, 1246–1254.

Muggeridge, D. J.; Howe, C. C. F.; Spendiff, O.; Pedlar, C.; James, P. E.; Easton, C. A Single Dose of Beetroot Juice Enhances Cycling Performance in Simulated Altitude. *Med. Sci. Sports Exerc.* **2014**, *46*, 143–150.

Mukherjee, K.; Edgett, B. A.; Burrows, H. W.; Castro, C.; Griffin, J. L.; Schwertani, A. G.; Gurd, B. J.; Funk, C. D. Whole Blood Transcriptomics and Urinary Metabolomics to Define Adaptive Biochemical Pathways of High-Intensity Exercise in 50-60 Year Old Masters Athletes. *PLoS One* **2014a**, *9*, e92031.

Mukherjee, K.; Edgett, B. A.; Burrows, H. W.; Castro, C.; Griffin, J. L.; Schwertani, A. G.; Gurd, B. J.; Funk, C. D. Whole Blood Transcriptomics and Urinary Metabolomics to Define Adaptive Biochemical Pathways of High-Intensity Exercise in 50-60 Year Old Masters Athletes. *PLoS One* **2014b**, *9*, e92031.

Nasrallah, S.; Al-Khalidi, U. Nature of Purines Excreted in Urine during Muscular Exercise. *J Appl Physiol* **1964**, *19*, 246–248.

Navarrete, A.; Armitage, E. G.; Musteanu, M.; García, A.; Mastrangelo, A.; Bujak, R.; López-Casas, P. P.; Hidalgo, M.; Barbas, C. Metabolomic Evaluation of Mitomycin C and Rapamycin in a Personalized Treatment of Pancreatic Cancer. *Pharmacol. Res. Perspect.* **2014**, *2*, n/a – n/a.

Neal, C. M.; Hunter, A. M.; Brennan, L.; O'Sullivan, A.; Hamilton, D. L.; De Vito, G.; Galloway, S. D. R. Six Weeks of a Polarized Training-Intensity Distribution Leads to Greater Physiological and Performance Adaptations than a Threshold Model in Trained Cyclists. *J. Appl. Physiol.* **2013**, *114*, 461–471.

Netzer, M.; Weinberger, K. M.; Handler, M.; Seger, M.; Fang, X.; Kugler, K. G.; Graber, A.; Baumgartner, C. Profiling the Human Response to Physical Exercise: A Computational Strategy for the Identification and Kinetic Analysis of Metabolic Biomarkers. *J. Clin. Bioinforma.* **2011a**, *1*, 34.

Netzer, M.; Weinberger, K. M.; Handler, M.; Seger, M.; Fang, X.; Kugler, K. G.; Graber, A.; Baumgartner, C. Profiling the Human Response to Physical Exercise: A Computational Strategy for the Identification and Kinetic Analysis of Metabolic Biomarkers. *J. Clin. Bioinforma.* **2011b**, *1*, 34.

Nicholson, J. K.; Lindon, J. C. Systems Biology: Metabonomics. *Nature* **2008**, *455*, 1054–1056.

Nicholson, J. K.; Holmes, E.; Elliott, P. The Metabolome-Wide Association Study: A New Look at Human Disease Risk Factors. *J. Proteome Res.* **2008**, *7*, 3637–3638.

Nicholson, J. K.; Holmes, E.; Kinross, J.; Burcelin, R.; Gibson, G.; Jia, W.; Pettersson, S. Host-Gut Microbiota Metabolic Interactions. *Science* **2012**, *336*, 1262–1267.

Nieman, D. C.; Gillitt, N. D.; Henson, D. A.; Sha, W.; Shanely, R. A.; Knab, A. M.; Cialdella-Kam, L.; Jin, F. Bananas as an Energy Source during Exercise: A Metabolomics Approach. *PLoS One* **2012**, *7*, e37479.

Nieman, D. C.; Gillitt, N. D.; Knab, A. M.; Shanely, R. A.; Pappan, K. L.; Jin, F.; Lila, M. A. Influence of a Polyphenol-Enriched Protein Powder on Exercise-Induced Inflammation and Oxidative Stress in Athletes: A Randomized Trial Using a Metabolomics Approach. *PLoS One* **2013a**, *8*, e72215.

Nieman, D. C.; Shanely, R. A.; Gillitt, N. D.; Pappan, K. L.; Lila, M. A. Serum Metabolic Signatures Induced by a Three-Day Intensified Exercise Period Persist after 14 H of Recovery in Runners. *J. Proteome Res.* **2013b**, *12*, 4577–4584.

Nieman, D. C.; Shanely, R. A.; Luo, B.; Meaney, M. P.; Dew, D. A.; Pappan, K. L. Metabolomics Approach to Assessing Plasma 13- and 9-Hydroxy-Octadecadienoic Acid and Linoleic Acid Metabolite Responses to 75-Km Cycling. *AJP Regul. Integr. Comp. Physiol.* **2014**, *307*, R68–R74.

Ninot, G.; Connes, P.; Caillaud, C. Effects of Recombinant Human Erythropoietin Injections on Physical Self in Endurance Athletes. *J. Sports Sci.* **2006**, *24*, 383–391.

Nurjhan, N.; Bucci, A.; Perriello, G.; Stumvoll, M.; Dailey, G.; Bier, D. M.; Toft, I.; Jenssen, T. G.; Gerich, J. E. Glutamine: A Major Gluconeogenic Precursor and Vehicle for Interorgan Carbon Transport in Man. *J. Clin. Invest.* **1995**, *95*, 272–277.

O'Donovan, C. B.; Walsh, M. C.; Nugent, A. P.; McNulty, B.; Walton, J.; Flynn, A.; Gibney, M. J.; Gibney, E. R.; Brennan, L. Use of Metabotyping for the Delivery of Personalised Nutrition. *Mol. Nutr. Food Res.* **2014**.

Oberbach, A.; Blüher, M.; Wirth, H.; Till, H.; Kovacs, P.; Kullnick, Y.; Schlichting, N.; Tomm, J. M.; Rolle-Kampczyk, U.; Murugaiyan, J.; et al. Combined Proteomic and Metabolomic Profiling of Serum Reveals Association of the Complement System with Obesity and Identifies Novel Markers of Body Fat Mass Changes. *J. Proteome Res.* **2011**, *10*, 4769–4788.

Ojiambo, R.; Mohammad, Y.; Fudge, B.; Kingsmore, D.; Parisotto, R.; Magnus, L.; Pitsiladis, Y. Haematological Profiles of Elite East African Athletes over a Nine Year Period. In *13th Annual Congress of the European College of Sport Science (ECSS)*; 2008.

Okano, M.; Sato, M.; Kageyama, S. Identification of the Long-Acting Erythropoiesis-Stimulating Agent Darbepoetin Alfa in Human Urine by Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* **2014**, *406*, 1317–1329.

Okura, T.; Rankinen, T.; Gagnon, J.; Lussier-Cacan, S.; Davignon, J.; Leon, A. S.; Rao, D. C.; Skinner, J. S.; Wilmore, J. H.; Bouchard, C. Effect of Regular Exercise on Homocysteine Concentrations: The HERITAGE Family Study. *Eur. J. Appl. Physiol.* **2006**, *98*, 394–401.

Olsen, N. V.; Aachmann-Andersen, N.-J.; Oturai, P.; Munch-Andersen, T.; Bornø, A.; Hulston, C.; Holstein-Rathlou, N.-H.; Robach, P.; Lundby, C. Erythropoietin down-Regulates Proximal Renal Tubular Reabsorption and Causes a Fall in Glomerular Filtration Rate in Humans. *J. Physiol.* **2011**, *589*, 1273–1281.

Parisotto, R.; Gore, C. J.; Emslie, K. R.; Ashenden, M. J.; Brugnara, C.; Howe, C.; Martin, D. T.; Trout, G. J.; Hahn, A. G. A Novel Method Utilising Markers of Altered Erythropoiesis for the Detection of Recombinant Human Erythropoietin Abuse in Athletes. *Haematologica* **2000**, *85*, 564–572.

Pasikanti, K. K.; Esuvaranathan, K.; Hong, Y.; Ho, P. C.; Mahendran, R.; Raman Nee Mani, L.; Chiong, E.; Chan, E. C. Y. Urinary Metabotyping of Bladder Cancer Using Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry. *J. Proteome Res.* **2013**, *12*, 3865–3873.

Patrick, K. Passport to Clean Competition. *Br. Med. J.* **2012**, *2077*, 2010–2013.

Pavese, P.; Kuentz, F.; Belleville, C.; Rougé, P. E.; Elsener, M. Renal Impairment Induced by Isotretinoin. *Nephrol. Dial. Transplant* **1997**, *12*, 1299.

Peake, J. M.; Tan, S. J.; Markworth, J. F.; Broadbent, J. A.; Skinner, T. L.; Cameron-Smith, D. Metabolic and Hormonal Responses to Isoenergetic High-Intensity Interval Exercise and Continuous Moderate-Intensity Exercise. *Am. J. Physiol. Endocrinol. Metab.* **2014**, *307*, E539–E552.

Pechlivanis, A.; Kostidis, S.; Saraslanidis, P.; Petridou, A.; Tsalis, G.; Mougios, V.; Gika, H. G.; Mikros, E.; Theodoridis, G. A. (1)H NMR-Based Metabonomic Investigation of the Effect of Two Different Exercise Sessions on the Metabolic Fingerprint of Human Urine. *J. Proteome Res.* **2010a**, *9*, 6405–6416.

Pechlivanis, A.; Kostidis, S.; Saraslanidis, P.; Petridou, A.; Tsalis, G.; Mougios, V.; Gika, H. G.; Mikros, E.; Theodoridis, G. A. H NMR-Based Metabonomic Investigation of the Effect of Two Different Exercise Sessions on the Metabolic Fingerprint of Human Urine Research Articles. *J. Proteome Res.* **2010b**, *9*, 6405–6416.

Pechlivanis, A.; Kostidis, S.; Saraslanidis, P.; Petridou, A.; Tsalis, G.; Veselkov, K.; Mikros, E.; Mougios, V.; Theodoridis, G. A. 1H NMR Study on the Short- and Long-Term Impact of Two Training Programs of Sprint Running on the Metabolic Fingerprint of Human Serum. *J. Proteome Res.* **2013**, *12*, 470–480.

Peckett, A. J.; Wright, D. C.; Riddell, M. C. The Effects of Glucocorticoids on Adipose Tissue Lipid Metabolism. *Metabolism.* **2011**, *60*, 1500–1510.

Perkel, J. Metabolomics: Where Seeing Is Believing. *Biotechniques* **2011**, *50*, 285–289.

Pitsiladis, Y. P.; Durussel, J.; Rabin, O. An Integrative "Omics" Solution to the Detection of Recombinant Human Erythropoietin and Blood Doping. *Br. J. Sports Med.* **2014**, *48*, 856–861.

Pluskal, T.; Castillo, S.; Villar-Briones, A.; Oresic, M. MZmine 2: Modular Framework for Processing, Visualizing, and Analyzing Mass Spectrometry-Based Molecular Profile Data. *BMC Bioinformatics* **2010**, *11*, 395.

Pohjanen, E.; Thysell, E.; Jonsson, P.; Eklund, C.; Silfver, A.; Carlsson, I.-B.; Lundgren, K.; Moritz, T.; Svensson, M. B.; Antti, H. A Multivariate Screening Strategy for Investigating Metabolic Effects of Strenuous Physical Exercise in Human Serum. *J. Proteome Res.* **2007**, *6*, 2113–2120.

Pritchard, J.; Després, J.-P.; Gagnon, J.; Tchernof, A.; Nadeau, A.; Tremblay, A.; Bouchard, C. Plasma Adrenal, Gonadal, and Conjugated Steroids Following Long-Term Exercise-Induced Negative Energy Balance in Identical Twins. *Metabolism* **1999**, *48*, 1120–1127.

Ramel, A.; Wagner, K.-H.; Elmadfa, I. Plasma Antioxidants and Lipid Oxidation after Submaximal Resistance Exercise in Men. *Eur. J. Nutr.* **2004**, *43*, 2–6.

Reichel, C. OMICS-Strategies and Methods in the Fight against Doping. *Forensic Sci. Int.* **2011**, *213*, 20–34.

Reichel, C.; Gmeiner, G. Erythropoietin and Analogs. In *Handbook of Experimental Pharmacology*; 2010; pp. 251–294.

Reuter, S. E.; Faull, R. J.; Ranieri, E.; Evans, A. M. Endogenous Plasma Carnitine Pool Composition and Response to Erythropoietin Treatment in Chronic Haemodialysis Patients. *Nephrol. Dial. Transplant* **2009**, *24*, 990–996.

Robach, P.; Recalcati, S.; Girelli, D.; Gelfi, C.; Aachmann-Andersen, N. J.; Thomsen, J. J.; Norgaard, A. M.; Alberghini, A.; Campostrini, N.; Castagna, A.; et al. Alterations of Systemic and Muscle Iron Metabolism in Human Subjects Treated with Low-Dose Recombinant Erythropoietin. *Blood* **2009**, *113*, 6707–6715.

Romijn, J. A.; Coyle, E. F.; Sidossis, L. S.; Gastaldelli, A.; Horowitz, J. F.; Endert, E.; Wolfe, R. R. Regulation of Endogenous Fat and Carbohydrate Metabolism in Relation to Exercise Intensity and Duration. *Am. J. Physiol.* **1993**, *265*, E380–E391.

Romijn, J. A.; Coyle, E. F.; Sidossis, L. S.; Rosenblatt, J.; Wolfe, R. R. Substrate Metabolism during Different Exercise Intensities in Endurance-Trained Women. *J. Appl. Physiol.* **2000**, *88*, 1707–1714.

Røsjø, H.; Opstad, P.-K.; Hoff, J. E.; Godang, K.; Christensen, G.; Stridsberg, M.; Omland, T. Effect of Short- and Long-Term Physical Activities on Circulating Granin Protein Levels. *Regul. Pept.* **2013**, *185*, 14–19.

Russell, G.; Gore, C. J.; Ashenden, M. J.; Parisotto, R.; Hahn, A. G. Effects of Prolonged Low Doses of Recombinant Human Erythropoietin during Submaximal and Maximal Exercise. *Eur. J. Appl. Physiol.* **2002**, *86*, 442–449.

Ryan, D.; Robards, K.; Prenzler, P. D.; Kendall, M. Recent and Potential Developments in the Analysis of Urine: A Review. *Anal. Chim. Acta* **2011a**, *684*, 8–20.

Ryan, D.; Robards, K.; Prenzler, P. D.; Kendall, M. Recent and Potential Developments in the Analysis of Urine: A Review. *Anal. Chim. Acta* **2011b**, *684*, 8–20.

Sahlin, K.; Tonkonogi, M.; Söderlund, K. Plasma Hypoxanthine and Ammonia in Humans during Prolonged Exercise. *Eur. J. Appl. Physiol. Occup. Physiol.* **1999**, *80*, 417–422.

Sakata, S.; Shimizu, S.; Kishi, T.; Hirai, K.; Mori, I.; Ohno, Y.; Ueda, M.; Takaki, M.; Kohzuki, H.; Okamoto, S.; et al. Correlation between Erythropoietin and Lactate in Humans during Altitude Exposure. *Jpn. J. Physiol.* **2000**, *50*, 285–288.

Salek, R. M.; Steinbeck, C.; Viant, M. R.; Goodacre, R.; Dunn, W. B. The Role of Reporting Standards for Metabolite Annotation and Identification in Metabolomic Studies. *Gigascience* **2013**, *2*, 13.

Santone, C.; Dinallo, V.; Paci, M.; D'Ottavio, S.; Barbato, G.; Bernardini, S. Saliva Metabolomics by NMR for the Evaluation of Sport Performance. *J. Pharm. Biomed. Anal.* **2014**, *88*, 441–446.

Sarris, J.; O'Neil, A.; Coulson, C. E.; Schweitzer, I.; Berk, M. Lifestyle Medicine for Depression. *BMC Psychiatry* **2014**, *14*, 107.

Saugy, M.; Lundby, C.; Robinson, N. Monitoring of Biological Markers Indicative of Doping: The Athlete Biological Passport. *Br. J. Sports Med.* **2014**, *48*, 827–832.

Scalbert, A.; Brennan, L.; Fiehn, O.; Hankemeier, T.; Kristal, B. S.; van Ommen, B.; Pujos-Guillot, E.; Verheij, E.; Wishart, D.; Wopereis, S. Mass-Spectrometry-Based Metabolomics: Limitations and Recommendations for Future Progress with Particular Focus on Nutrition Research. *Metabolomics* **2009**, *5*, 435–458.

Scalera, F.; Kielstein, J. T.; Martens-Lobenhoffer, J.; Postel, S. C.; Täger, M.; Bode-Böger, S. M. Erythropoietin Increases Asymmetric Dimethylarginine in Endothelial Cells: Role of Dimethylarginine Dimethylaminohydrolase. *J. Am. Soc. Nephrol.* **2005**, *16*, 892–898.

Scheltema, R. A.; Jankevics, A.; Jansen, R. C.; Swertz, M. A.; Breitling, R. PeakML/mzMatch: A File Format, Java Library, R Library, and Tool-Chain for Mass Spectrometry Data Analysis. *Anal. Chem.* **2011**, *83*, 2786–2793.

Schenk, T.; Stengel, S.; Zelent, A. Unlocking the Potential of Retinoic Acid in Anticancer Therapy. *Br. J. Cancer* **2014**, *111*, 2039–2045.

Sharp, C. P. M.; Pearson, D. R. Amino Acid Supplements and Recovery from High-Intensity Resistance Training. *J. Strength Cond. Res.* **2010**, *24*, 1125–1130.

Sheedy, D. J. R.; Gooley, P. R.; Nahid, A.; Tull, D. L.; McConville, M. J.; Kukuljan, S.; Nowson, C. A.; Daly, R. M.; Ebeling, P. R. 1H-NMR Analysis of the Human Urinary Metabolome in Response to an 18-Month Multi-Component Exercise Program and Calcium-Vitamin-D3 Supplementation in Older Men. **2014**.

Skelton, M. S.; Kremer, D. E.; Smith, E. W.; Gladden, L. B. Lactate Influx into Red Blood Cells of Athletic and Nonathletic Species. *Am. J. Physiol.* **1995**, *268*, R1121–R1128.

Skelton, M. S.; Kremer, D. E.; Smith, E. W.; Gladden, L. B. Lactate Influx into Red Blood Cells from Trained and Untrained Human Subjects. *Med. Sci. Sports Exerc.* **1998**, *30*, 536–542.

Sloutsky, R.; Jimenez, N.; Swamidass, S. J.; Naegle, K. M. Accounting for Noise When Clustering Biological Data. *Brief. Bioinform.* **2013**, *14*, 423–436.

Sottas, P.-E.; Robinson, N.; Giraud, S.; Taroni, F.; Kamber, M.; Mangin, P.; Saugy, M. Statistical Classification of Abnormal Blood Profiles in Athletes. *Int. J. Biostat.* **2006**, *2*, 1–23.

Sottas, P.-E.; Robinson, N.; Saugy, M. The Athlete's Biological Passport and Indirect Markers of Blood Doping. *Handb. Exp. Pharmacol.* **2010**, 305–326.

Sottas, P.-E.; Robinson, N.; Rabin, O.; Saugy, M. The Athlete Biological Passport. *Clin. Chem.* **2011**, *57*, 969–976.

Sparling, P. B.; Giuffrida, A.; Piomelli, D.; Rosskopf, L.; Dietrich, A. Exercise Activates the Endocannabinoid System. *Neuroreport* **2003**, *14*, 2209–2211.

Spirlandeli, A. L.; Deminice, R.; Jordao, A. A.; Preto, R. Plasma Malondialdehyde as Biomarker of Lipid Peroxidation : E Ff Ects of Acute Exercise. *Int. J. Sports Med.* **2014**, *35*, 14–18.

Spriet, L. L.; Watt, M. J. Regulatory Mechanisms in the Interaction between Carbohydrate and Lipid Oxidation during Exercise. *Acta Physiol. Scand.* **2003**, *178*, 443–452.

Stathis, C. G.; Carey, M. F.; Snow, R. J. The Influence of Allopurinol on Urinary Purine Loss after Repeated Sprint Exercise in Man. *Metabolism.* **2005**, *54*, 1269–1275.

Stathis, C. G.; Carey, M. F.; Hayes, A.; Garnham, A. P.; Snow, R. J. Sprint Training Reduces Urinary Purine Loss Following Intense Exercise in Humans. *Appl. Physiol. Nutr. Metab.* **2006**, *31*, 702–708.

Stray-Gundersen, J.; Chapman, R. F.; Levine, B. D. "Living High-Training Low" Altitude Training Improves Sea Level Performance in Male and Female Elite Runners. *J. Appl. Physiol.* **2001**, *91*, 1113–1120.

Struck, W.; Siluk, D.; Yumba-Mpanga, A.; Markuszewski, M.; Kaliszan, R.; Markuszewski, M. J. Liquid Chromatography Tandem Mass Spectrometry Study of Urinary Nucleosides as Potential Cancer Markers. *J. Chromatogr. A* **2013**, *1283*, 122–131.

Subudhi, A. W.; Davis, S. L.; Kipp, R. W.; Wayne Askew, E. Antioxidant Status and Oxidative Stress in Elite Alpine Ski Racers. *Int. J. Sport. Nutr. Exerc. Metab.* **2001**, *11*, 32–41.

Sugimoto, M.; Kawakami, M.; Robert, M.; Soga, T.; Tomita, M. Bioinformatics Tools for Mass Spectroscopy-Based Metabolomic Data Processing and Analysis. *Curr. Bioinform.* **2012**, *7*, 96–108.

Sweedler, J.; James, R. Tech News Metabolomics : Where Seeing Is Believing. *Biotechniques* **2011**, *50*, 285–289.

Takahashi, H. Non-Enzymatic Hydroxylation of Kynurenic Acid to 6-Hydroxykynurenic Acid. *J. Biochem.* **1968**, *63*, 789–791.

Takahashi, R.; Shiono, T.; Tamai, M.; Itoh, K.; Mizugaki, M. [Expression of Modified Nucleoside, 1-Methyladenosine in Intraocular Tumors and the Retinas]. *Nihon. Ganka Gakkai Zasshi* **1993**, *97*, 43–49.

Theodoridis, G. a; Gika, H. G.; Want, E. J.; Wilson, I. D. Liquid Chromatography-Mass Spectrometry Based Global Metabolite Profiling: A Review. *Anal. Chim. Acta* **2012**, *711*, 7–16.

Thevis, M.; Kuuranne, T.; Geyer, H.; Schänzer, W. Annual Banned-Substance Review: Analytical Approaches in Human Sports Drug Testing. *Drug Test. Anal.* **2012**, *4*, 2–16.

Thomsen, J. J.; Rentsch, R. L.; Robach, P.; Calbet, J. a L.; Boushel, R.; Rasmussen, P.; Juel, C.; Lundby, C. Prolonged Administration of Recombinant Human Erythropoietin Increases Submaximal Performance More than Maximal Aerobic Capacity. *Eur. J. Appl. Physiol.* **2007**, *101*, 481–486.

Thysell, E.; Chorell, E.; Svensson, M. B.; Jonsson, P.; Antti, H. Validated and Predictive Processing of Gas Chromatography-Mass Spectrometry Based Metabolomics Data for Large Scale Screening Studies, Diagnostics and Metabolite Pattern Verification. *Metabolites* **2012**, *2*, 796–817.

Tremblay, M. S.; Copeland, J. L.; Van Helder, W. Effect of Training Status and Exercise Mode on Endogenous Steroid Hormones in Men. *J. Appl. Physiol.* **2004**, *96*, 531–539.

Urvalek, A.; Laursen, K. B.; Gudas, L. J. The Roles of Retinoic Acid and Retinoic Acid Receptors in Inducing Epigenetic Changes. *Subcell. Biochem.* **2014**, *70*, 129–149.

Veselkov, K. A.; Vingara, L. K.; Masson, P.; Robinette, S. L.; Want, E.; Li, J. V; Barton, R. H.; Boursier-Neyret, C.; Walther, B.; Ebbels, T. M.; et al. Optimized Preprocessing of Ultra-Performance Liquid Chromatography/mass Spectrometry Urinary Metabolic Profiles for Improved Information Recovery. *Anal. Chem.* **2011**, *83*, 5864–5872.

Vogel, M.; Blobel, M.; Thomas, A.; Walpurgis, K.; Schänzer, W.; Reichel, C.; Thevis, M. Isolation, Enrichment, and Analysis of Erythropoietins in Anti-Doping

Analysis by Receptor-Coated Magnetic Beads and Liquid Chromatography-Mass Spectrometry. *Anal. Chem.* **2014**, *86*, 12014–12021.

Volpe, D. A.; Tobin, G. A.; Tavakkoli, F.; Dowling, T. C.; Light, P. D.; Parker, R. J. Effect of Uremic Serum and Uremic Toxins on Drug Metabolism in Human Microsomes. *Regul. Toxicol. Pharmacol.* **2014**, *68*, 297–303.

Wang, X. Q.; Vaziri, N. D. Erythropoietin Depresses Nitric Oxide Synthase Expression by Human Endothelial Cells. *Hypertension* **1999**, *33*, 894–899.

Warrack, B. M.; Hnatyshyn, S.; Ott, K.-H.; Reily, M. D.; Sanders, M.; Zhang, H.; Drexler, D. M. Normalization Strategies for Metabonomic Analysis of Urine Samples. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2009**, *877*, 547–552.

Watson, D. G. The Potential of Mass Spectrometry for the Global Profiling of Parasite Metabolomes. *Parasitology* **2010**, *137*, 1409–1423.

Weiss, R. H.; Kim, K. Metabolomics in the Study of Kidney Diseases. *Nat. Rev. Nephrol.* **2012**, *8*, 22–33.

Whillier, S.; Garcia, B.; Chapman, B. E.; Kuchel, P. W.; Raftos, J. E. Glutamine and A-Ketoglutarate as Glutamate Sources for Glutathione Synthesis in Human Erythrocytes. *FEBS J.* **2011**, *278*, 3152–3163.

WHO. *UNHEALTHY DIETS & PHYSICAL INACTIVITY*; 2009.

Wilkerson, D. P.; Rittweger, J.; Berger, N. J. A.; Naish, P. F.; Jones, A. M. Influence of Recombinant Human Erythropoietin Treatment on Pulmonary O2 Uptake Kinetics during Exercise in Humans. *J. Physiol.* **2005**, *568*, 639–652.

Woldt, E.; Sebti, Y.; Solt, L. A.; Duhem, C.; Lancel, S.; Eeckhoute, J.; Hesselink, M. K. C.; Paquet, C.; Delhaye, S.; Shin, Y.; et al. Rev-Erb-A Modulates Skeletal Muscle Oxidative Capacity by Regulating Mitochondrial Biogenesis and Autophagy. *Nat. Med.* **2013**, *19*, 1039–1046.

Wu, I.-W.; Hsu, K.-H.; Lee, C.-C.; Sun, C.-Y.; Hsu, H.-J.; Tsai, C.-J.; Tzen, C.-Y.; Wang, Y.-C.; Lin, C.-Y.; Wu, M.-S. P-Cresyl Sulphate and Indoxyl Sulphate Predict Progression of Chronic Kidney Disease. *Nephrol. Dial. Transplant* **2011**, *26*, 938–947.

Xia, J.; Psychogios, N.; Young, N.; Wishart, D. S. MetaboAnalyst: A Web Server for Metabolomic Data Analysis and Interpretation. *Nucleic Acids Res.* **2009**, *37*, W652–W660.

Xia, J.; Mandal, R.; Sinelnikov, I. V; Broadhurst, D.; Wishart, D. S. MetaboAnalyst 2.0--a Comprehensive Server for Metabolomic Data Analysis. *Nucleic Acids Res.* **2012**, *40*, W127–W133.

Xia, J.; Broadhurst, D. I.; Wilson, M.; Wishart, D. S. Translational Biomarker Discovery in Clinical Metabolomics: An Introductory Tutorial. *Metabolomics* **2013**, *9*, 280–299.

Xiao, J. F.; Varghese, R. S.; Zhou, B.; Nezami Ranjbar, M. R.; Zhao, Y.; Tsai, T. H.; Di Poto, C.; Wang, J.; Goerlitz, D.; Luo, Y.; et al. LC-MS Based Serum Metabolomics for Identification of Hepatocellular Carcinoma Biomarkers in Egyptian Cohort. *J. Proteome Res.* **2012**, *11*, 5914–5923.

Yan, B.; A, J.; Wang, G.; Lu, H.; Huang, X.; Liu, Y.; Zha, W.; Hao, H.; Zhang, Y.; Liu, L.; et al. Metabolomic Investigation into Variation of Endogenous Metabolites in Professional Athletes Subject to Strength-Endurance Training. *J. Appl. Physiol.* **2009**, *106*, 531–538.

Yoshizumi, W. M.; Tsourounis, C. Effects of Creatine Supplementation on Renal Function. *J. Herb. Pharmacother.* **2004**, *4*, 1–7.

Zafeiridis, A. The Effects of Dietary Nitrate (Beetroot Juice) Supplementation on Exercise Performance: A Review. *Am. J. Sport. Sci.* **2014**, *2*, 97–110.

Zajac, A.; Poprzecki, S.; Maszczyk, A.; Czuba, M.; Michalczyk, M.; Zydek, G. The Effects of a Ketogenic Diet on Exercise Metabolism and Physical Performance in off-Road Cyclists. *Nutrients* **2014**, *6*, 2493–2508.

Zammit, V. A.; Ramsay, R. R.; Bonomini, M.; Arduini, A. Carnitine, Mitochondrial Function and Therapy. *Adv. Drug Deliv. Rev.* **2009**, *61*, 1353–1362.

Zhang, R.; Watson, D. G.; Wang, L.; Westrop, G. D.; Coombs, G. H.; Zhang, T. Evaluation of Mobile Phase Characteristics on Three Zwitterionic Columns in Hydrophilic Interaction Liquid Chromatography Mode for Liquid Chromatography-High Resolution Mass Spectrometry Based Untargeted Metabolite Profiling of Leishmania Parasites. *J. Chromatogr. A* **2014**, *1362*, 168–179.

Zhang, T.; Watson, D. G. High Performance Liquid Chromatographic Approaches to Mass Spectrometry Based Metabolomics. *Curr. Metabolomics* **2013**, *1*, 58–83.

Zhang, T.; Creek, D. J.; Barrett, M. P.; Blackburn, G.; Watson, D. G. Evaluation of Coupling Reversed Phase, Aqueous Normal Phase, and Hydrophilic Interaction Liquid Chromatography with Orbitrap Mass Spectrometry for Metabolomic Studies of Human Urine. *Anal. Chem.* **2012**, *84*, 1994–2001.

Zhang, T.; Watson, D. G.; Wang, L.; Abbas, M.; Murdoch, L.; Bashford, L.; Ahmad, I.; Lam, N. Y.; Ng, A. C. F.; Leung, H. Y. Application of Holistic Liquid Chromatography-High Resolution Mass Spectrometry Based Urinary Metabolomics for Prostate Cancer Detection and Biomarker Discovery. *PLoS One* **2013**, *8*, e65880.

Zhao, S.; Snow, R. J.; Stathis, C. G.; Febbraio, M. A.; Carey, M. F. Muscle Adenine Nucleotide Metabolism during and in Recovery from Maximal Exercise in Humans. *J Appl Physiol* **2000**, *88*, 1513–1519.

Zielinski, J.; Kusy, K. Training-Induced Adaptation in Purine Metabolism in High-Level Sprinters vs. Triathletes. *J. Appl. Physiol.* **2012**, *112*, 542–551.

Zieliński, J.; Rychlewski, T.; Kusy, K.; Domaszewska, K.; Laurentowska, M. The Effect of Endurance Training on Changes in Purine Metabolism: A Longitudinal Study of Competitive Long-Distance Runners. *Eur. J. Appl. Physiol.* **2009**, *106*, 867–876.

Zieliński, J.; Kusy, K.; Rychlewski, T. Effect of Training Load Structure on Purine Metabolism in Middle-Distance Runners. *Med. Sci. Sports Exerc.* **2011**, *43*, 1798–1807.

Zieliński, J.; Kusy, K.; Słomińska, E. Alterations in Purine Metabolism in Middle-Aged Elite, Amateur, and Recreational Runners across a 1-Year Training Cycle. *Eur. J. Appl. Physiol.* **2013a**, *113*, 763–773.

Zieliński, J.; Krasińska, B.; Kusy, K. Hypoxanthine as a Predictor of Performance in Highly Trained Athletes. *Int. J. Sports Med.* **2013b**, *34*, 1079–1086.

Zorzoli, M.; Pipe, A.; Garnier, P. Y.; Vouillamoz, M.; Dvorak, J. Practical Experience with the Implementation of an Athlete's Biological Profile in Athletics, Cycling, Football and Swimming. *Br. J. Sports Med.* **2014**, *48*, 862–866.

Appendix i. Publications

Publication related to the Caucasian subject demographics, haematological parameters and exercise performance:

1. Durussel J, **Daskalaki E**, Anderson M, Chatterji T, Wondimu DH, et al. (2013) Haemoglobin Mass and Running Time Trial Performance after Recombinant Human Erythropoietin Administration in Trained Men. *PLoS One,* 8(2) e56151. doi:10.1371/journal.pone.0056151

Submitted as a direct result of the Ph.D. thesis:

2. **Evangelia Daskalaki**, Gavin Blackburn, Gabriela Kalna, Tong Zhang, Nahoum Anthony, David Watson (2015) A study of the effects of exercise on the urinary metabolome using normalisation to individual metabolic output. *Metabolites*, 5(1):119-139. doi:10.3390/metabo5010119
3. **Evangelia Daskalaki**, Chris Easton and David G. Watson (2015) The Application of Metabolomic Profiling to the Effects of Physical Activity. *Current Metabolomics*, 2(4):233-263. doi: 10.2174/2213235X03666150211000831

Submitted as an external initiate as a member of the metabolomics community:

4. David Leisenfeld, Nicholas Rattray, Justin Van Der Hooft, **Evangelia Daskalaki**, Ralf Weber, Thomas Payne and Sastia P. Putri (2015) Activity Update from the Early-career Members Network (commissioned and published in *Metabolomics*)

Appendix ii. Oral and Poster Presentations

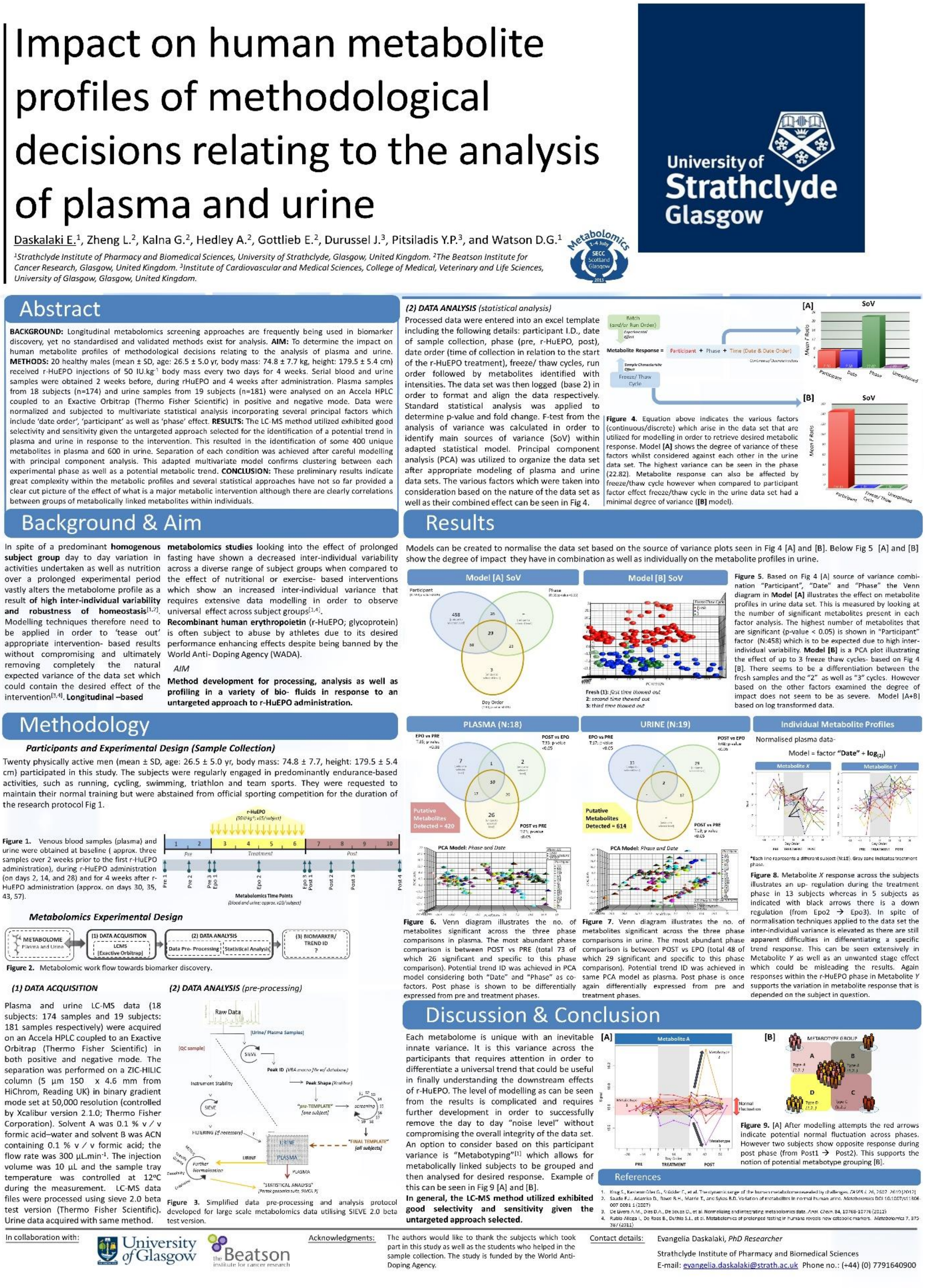
1. **Evangelia Daskalaki**, Gavin Blackburn and David Watson**.** Defining the training effect of prolonged exercise in combination with recombinant human erythropoietin (r-HuEPO) treatment in healthy trained males. Metabolomics Society, Tenth Annual International Conference of the Metabolomics Society and the Official Joint Conference of the Metabolomics Society and Plant Metabolomics Platform; Tsuruoka, Japan, 23-26 June 2014. (Poster, available in appendix iii)

2. **Daskalaki E.**, Zheng L., Kalna G., Hedley A., Gottlieb E., Durussel J., Pitsiladis Y.P., and Watson D. Impact on human metabolite profiles of methodological decisions relating to the analysis of plasma and urine. 9th Annual Conference of the Metabolomics Society, SECC, Glasgow, United Kingdom, 1st – 4th July 2013. (Poster, available in appendix iv)

3. **Daskalaki E.**, Zheng L., Kalna G., Gottlieb E., Durussel J., Chatterji T., Frezza C., Hedley A., Padmanabhan N., Padmanabhan S., Patel R.K., Gmeiner G., Watson D., Pitsiladis Y.P. Effects of Recombinant Human Erythropoietin on Targeted Blood Metabolomic Profiles in Endurance Trained Individuals; Glasgow Polyomics Symposium, University of Glasgow- Glasgow, September 14th 2012.

4. **Evangelia Daskalaki**, Liang Zheng, Tushar Chatterji, Jérôme Durussel, Gabriela Kalna, Christian Frezza, Ann Hedley, Eyal Gottlieb, Neal Padmanabhan, Sandosh Padmanabhan, Rajan K. Patel, Günter Gmeiner, Yannis P. Pitsiladis. Effects of Recombinant Human Erythropoietin on Targeted Blood Metabolomic Profiles in Endurance Trained Individuals. International Convention on Science, Education and Medicine in Sport (ICSEMIS),SECC Glasgow, July 19th – 24th 2012. (Oral Presentation)

Appendix iii. International Metabolomics Conference Poster 2013



Impact on human metabolite profiles of methodological decisions relating to the analysis of plasma and urine

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Metabolomics 1-4 July SECC Scotland Glasgow 2013

University of Strathclyde Glasgow

Abstract

BACKGROUND: Longitudinal metabolomics screening approaches are frequently being used in biomarker discovery, yet no standardised and validated methods exist for analysis. **AIM:** To determine the impact on human metabolite profiles of methodological decisions relating to the analysis of plasma and urine. **METHODS:** 20 healthy males (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7 kg, height: 179.5 ± 5.4 cm) received r-HuEPO injections of 50 IU.kg^{-1} body mass every two days for 4 weeks. Serial blood and urine samples were obtained 2 weeks before, during rHuEPO and 4 weeks after administration. Plasma samples from 18 subjects (n=174) and urine samples from 19 subjects (n=181) were analysed on an Accela HPLC coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in positive and negative mode. Data were normalized and subjected to multivariate statistical analysis incorporating several principal factors which include 'date order', 'participant' as well as 'phase' effect. **RESULTS:** The LC-MS method utilized exhibited good selectivity and sensitivity given the untargeted approach selected for the identification of a potential trend in plasma and urine in response to the intervention. This resulted in the identification of some 400 unique metabolites in plasma and 600 in urine. Separation of each condition was achieved after careful modelling with principal component analysis. This adapted multivariate model confirms clustering between each experimental phase as well as a potential metabolic trend. **CONCLUSION:** These preliminary results indicate great complexity within the metabolic profiles and several statistical approaches have not so far provided a clear cut picture of the effect of what is a major metabolic intervention although there are clearly correlations between groups of metabolically linked metabolites within individuals.

Background & Aim

In spite of a predominant **homogenous subject group** day to day variation in activities undertaken as well as nutrition over a prolonged experimental period vastly alters the metabolome profile as a result **of high inter-individual variability and robustness of homeostasis**[1,2]. Modelling techniques therefore need to be applied in order to 'tease out' appropriate intervention- based results without compromising and ultimately removing completely the natural expected variance of the data set which could contain the desired effect of the intervention[3,4]. **Longitudinal –based metabolomics studies** looking into the effect of prolonged fasting have shown a decreased inter-individual variability across a diverse range of subject groups when compared to the effect of nutritional or exercise- based interventions which show an increased inter-individual variance that requires extensive data modelling in order to observe universal effect across subject groups[1,4].

Recombinant human erythropoietin (r-HuEPO; glycoprotein) is often subject to abuse by athletes due to its desired performance enhancing effects despite being banned by the World Anti- Doping Agency (WADA).

AIM

Method development for processing, analysis as well as profiling in a variety of bio- fluids in response to an untargeted approach to r-HuEPO administration.

Methodology

Participants and Experimental Design (Sample Collection)

Twenty physically active men (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7, height: 179.5 ± 5.4 cm) participated in this study. The subjects were regularly engaged in predominantly endurance-based activities, such as running, cycling, swimming, triathlon and team sports. They were requested to maintain their normal training but were abstained from official sporting competition for the duration of the research protocol Fig 1.

r-HuEPO (50 IU·kg^{-1}; x15/subject)

1 2 | 3 4 5 6 | 7 8 9 10

Pre | Treatment | Post

Pre 1, Pre 2, Pre 3, Epo 1, Epo 2, Epo 3, Post 1, Post 2, Post 3, Post 4

Metabolomics Time Points (blood and urine; approx. x10/subject)

Figure 1. Venous blood samples (plasma) and urine were obtained at baseline (approx. three samples over 2 weeks prior to the first r-HuEPO administration), during r-HuEPO administration (on days 2, 14, and 28) and for 4 weeks after r-HuEPO administration (approx. on days 30, 35, 43, 57).

Metabolomics Experimental Design

METABOLOME Plasma and Urine → (1) DATA ACQUISITION LCMS (Exactive Orbitrap) → (2) DATA ANALYSIS Data Pre- Processing | Statistical Analysis → (3) BIOMARKER/ TREND ID ?

Figure 2. Metabolomic work flow towards biomarker discovery.

(1) DATA ACQUISITION

Plasma and urine LC-MS data (18 subjects: 174 samples and 19 subjects: 181 samples respectively) were acquired on an Accela HPLC coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in both positive and negative mode. The separation was performed on a ZIC-HILIC column (5 µm 150 x 4.6 mm from HiChrom, Reading UK) in binary gradient mode set at 50,000 resolution (controlled by Xcalibur version 2.1.0; Thermo Fisher Corporation). Solvent A was 0.1 % v / v formic acid–water and solvent B was ACN containing 0.1 % v / v formic acid; the flow rate was 300 µL.min^{-1}. The injection volume was 10 µL and the sample tray temperature was controlled at 12ºC during the measurement. LC-MS data files were processed using sieve 2.0 beta test version (Thermo Fisher Scientific). Urine data acquired with same method.

***(2) DATA ANALYSIS** (pre-processing)*

Raw Data; [Urine/ Plasma Samples]; [QC sample]; SIEVE; Peak ID (VBA macro file w/ database); Peak Shape (Xcalibur); Instrument Stability; SIEVE; "pre-TEMPLATE" (one subject); screening; FILTERING (if necessary); "FINAL TEMPLATE" (all subjects); URINE; PLASMA; Further Normalisation; "STATISTICAL ANALYSIS" (Partek genomics suite, SIMCA P)

Figure 3. Simplified data pre-processing and analysis protocol developed for large scale metabolomics data utilising SIEVE 2.0 beta test version.

***(2) DATA ANALYSIS** (statistical analysis)*

Processed data were entered into an excel template including the following details: participant I.D., date of sample collection, phase (pre, r-HuEPO, post), date order (time of collection in relation to the start of the r-HuEPO treatment), freeze/ thaw cycles, run order followed by metabolites identified with intensities. The data set was then logged (base 2) in order to format and align the data respectively. Standard statistical analysis was applied to determine p-value and fold change. F-test from the analysis of variance was calculated in order to identify main sources of variance (SoV) within adapted statistical model. Principal component analysis (PCA) was utilized to organize the data set after appropriate modeling of plasma and urine data sets. The various factors which were taken into consideration based on the nature of the data set as well as their combined effect can be seen in Fig 4.

Batch (and/or Run Order) — Experimental Effect

Metabolite Response = Participant + Phase + Time (Date & Date Order)

Freeze/ Thaw Cycle — Sample Characteristic Effect

[A] SoV: Participant, Date, Phase, Unexplained

[B] SoV: Participant, Freeze/ Thaw Cycle, Unexplained

Figure 4. Equation above indicates the various factors (continuous/discrete) which arise in the data set that are utilized for modelling in order to retrieve desired metabolic response. Model **[A]** shows the degree of variance of these factors whilst considered against each other in the urine data set. The highest variance can be seen in the phase (22.82). Metabolite response can also be affected by freeze/thaw cycle however when compared to participant factor effect freeze/thaw cycle in the urine data set had a minimal degree of variance (**[B]** model).

Results

Models can be created to normalise the data set based on the source of variance plots seen in Fig 4 [A] and [B]. Below Fig 5 [A] and [B] show the degree of impact they have in combination as well as individually on the metabolite profiles in urine.

Model [A] SoV — Participant, Phase, Day Order: 458, 35, 23, 50, 21, 3

Model [B] SoV — Fresh (1): *first time thawed out*; 2: *second time thawed out*; 3: *third time thawed out*

Figure 5. Based on Fig 4 [A] source of variance combination "Participant", "Date" and "Phase" the Venn diagram in **Model [A]** illustrates the effect on metabolite profiles in urine data set. This is measured by looking at the number of significant metabolites present in each factor analysis. The highest number of metabolites that are significant (p-value < 0.05) is shown in "Participant" factor (N:458) which is to be expected due to high inter-individual variability. **Model [B]** is a PCA plot illustrating the effect of up to 3 freeze thaw cycles- based on Fig 4 [B]. There seems to be a differentiation between the fresh samples and the "2" as well as "3" cycles. However based on the other factors examined the degree of impact does not seem to be as severe. Model [A+B] based on log transformed data.

PLASMA (N:18)

EPO vs PRE T:35; p-value <0.05; POST vs EPO T:33; p-value <0.05; POST vs PRE T:73; p-value <0.05

Putative Metabolites Detected = 420

PCA Model: *Phase and Date*

Figure 6. Venn diagram illustrates the no. of metabolites significant across the three phase comparisons in plasma. The most abundant phase comparison is between POST vs PRE (total 73 of which 26 significant and specific to this phase comparison). Potential trend ID was achieved in PCA model considering both "Date" and "Phase" as co-factors. Post phase is shown to be differentially expressed from pre and treatment phases.

URINE (N:19)

EPO vs PRE T:17; p-value <0.05; POST vs EPO T:48; p-value <0.05; POST vs PRE T:19; p-value <0.05

Putative Metabolites Detected = 614

PCA Model: *Phase and Date*

Figure 7. Venn diagram illustrates the no. of metabolites significant across the three phase comparisons in urine. The most abundant phase comparison is between POST vs EPO (total 48 of which 29 significant and specific to this phase comparison). Potential trend ID was achieved in same PCA model as plasma. Post phase is once again differentially expressed from pre and treatment phases.

Individual Metabolite Profiles

Normalised plasma data-

Model = factor **"Date"** + **log(2)**

Metabolite *X*; Metabolite *Y*

*Each line represents a different subject (N:18). Grey zone indicates treatment phase.

Figure 8. Metabolite *X* response across the subjects illustrates an up- regulation during the treatment phase in 13 subjects whereas in 5 subjects as indicated with black arrows there is a down regulation (from Epo2 → Epo3). In spite of normalisation techniques applied to the data set the inter-individual variance is elevated as there are still apparent difficulties in differentiating a specific trend response. This can be seen extensively in Metabolite *Y* as well as an unwanted stage effect which could be misleading the results. Again responses within the r-HuEPO phase in Metabolite *Y* supports the variation in metabolite response that is depended on the subject in question.

Discussion & Conclusion

Each metabolome is unique with an inevitable innate variance. It is this variance across the participants that requires attention in order to differentiate a universal trend that could be useful in finally understanding the downstream effects of r-HuEPO. The level of modelling as can be seen from the results is complicated and requires further development in order to successfully remove the day to day "noise level" without compromising the overall integrity of the data set. An option to consider based on this participant variance is "Metabotyping"[1] which allows for metabolically linked subjects to be grouped and then analysed for desired response. Example of this can be seen in Fig 9 [A] and [B].

In general, the LC-MS method utilized exhibited good selectivity and sensitivity given the untargeted approach selected.

[A] Metabolite A — Normal Fluctuation; PRE, TREATMENT, POST

[B] METABOTYPE GROUP — A, B, C, D

Figure 9. [A] After modelling attempts the red arrows indicate potential normal fluctuation across phases. However two subjects show opposite response during post phase (from Post1 → Post2). This supports the notion of potential metabotype grouping [B].

References

1. Krug S., Kastenmüller G., Stückler F., et al. The dynamic range of the human metabolome revealed by challenges. *FASEB J.* 26, 2607-2619 (2012)
2. Saude E.J., Adamko D., Rowe B.H., Marrie T., and Sykes B.D. Variation of metabolites in normal human urine. *Metabolomics* DOI 10.1007/s11306-007-0091-1 (2007)
3. De Livera A.M., Dias D.A., De Souza D., et al. Normalizing and integrating metabolomics data. *Anal. Chem.* 84, 10768-10776 (2012)
4. Rubio-Aliaga I., De Roos B., Duthie S.J., et al. Metabolomics of prolonged fasting in humans reveals new catabolic markers. *Metabolomics* 7, 375-387 (2011)

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Defining the training effect of prolonged exercise in combination with recombinant human erythropoietin (r-HuEPO) treatment in healthy trained males.

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Abstract

The aim is to identify the metabolic variability and response of prolonged training as well as its combined effect with r-HuEPO treatment (50 IU.kg^{-1} body mass every two days for 4wk) via an untargeted LC-MS method in 18 healthy males. Blood and urine LC-MS data were processed via MzMatch and subjected to multivariate analysis. Raw data highlights variability of free carnitine and acetyl carnitine (normalising factor), that signals a change in the pathway of energy/lipid metabolism. PCA/OPLSDA analysis of individual responses of normalized plasma data reveals a clear differentiation between phases compared to raw data. RBC S-plot analysis reveals a possible dietary effect on metabolome.

Background & Aims

Recombinant human erythropoietin (r-HuEPO; glycoprotein) is often subject to abuse by athletes due to desired performance enhancing effects despite being banned by the World Anti- Doping Agency (WADA). In order to identify a possible **population-based testing programme** for r-HuEPO in sport; the training element (stressor) in this homogenous group of individuals must be understood in terms of its unique impact on the metabolome [1].

Hence, study **A** aims to identify possible **effect of r-HuEPO in combination with prolonged training** in the urine, plasma and red blood cell (RBC) matrix and pilot study **B** aims to specifically isolate the metabolic markers of aerobic exercise. Taking into consideration this training element [2] could further aid in **minimizing part of the inter-individual variability** observed due to the robustness of each unique metabolome.

Methodology

A r-HuEPO Study

Participants and Experimental Design (Sample Collection)

20 physically active men (mean ± SD, age: 26.5 ± 5.0 yr, body mass: 74.8 ± 7.7, height: 179.5 ± 5.4 cm) participated in this study. The subjects were regularly engaged in predominantly endurance-based activities, such as running, cycling, swimming, triathlon and team sports. They were requested to maintain their normal training but were abstained from official sporting competition for the duration of the research protocol Fig 1.

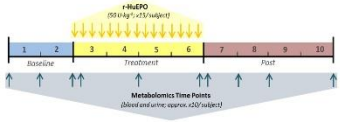


Figure 1. Venous blood (for RBC and plasma) and urine samples were obtained at baseline (three samples over 2 weeks prior to the first r-HuEPO administration), during r-HuEPO administration (on days 2, 14, and 28) and for 4 weeks after r-HuEPO administration (approx. on days 30, 35, 43, 57).

Data Acquisition and Analysis

RBC, Plasma (18 subjects: 174 samples) and urine (19 subjects: 181 samples) were acquired on an Accela HPLC coupled to an Exactive Orbitrap (Thermo Fisher Scientific) in both positive and negative mode. The separation was performed on a ZIC-pHILIC column (HiChrom, Reading UK) in binary gradient mode set at 50,000 resolution (controlled by xcalibur version 2.1.0; Thermo Fisher Corporation). LC-MS data files were processed using mzMatch and analysed via tools available through Ideom; an excel template with macro enabled features for user-friendly processing of metabolomics data. Simca p (Umetrics) was utilised for multivariate statistical analysis (PCA, OPLS-DA and S-plot).

B Pilot Exercise Study

Participants and Experimental Design (Sample Collection)

4 healthy physically active men participated in this study. The subjects were regularly engaged in activities, such as running, long-distance walking and cycling. Fig 2. highlights experimental protocol.

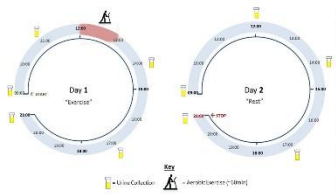


Figure 2. Urine samples were obtained at the following approximate times: 9:00 (Pre1; first thing in the morning), 11:00 (Pre2), 14:00 (Post3), 17:00 (Post4) and 21:00 (Post5) with a visit to the gym between 12:00- 13:00 where subjects had to perform some form of aerobic exercise (treadmill, bike so on) for approximately 1 hour. Day 2 was a rest day with no physical activity and urine samples were collected at the following approximate times: 9:00 (Post6; first thing in the morning), 12:00 (Post7), 14:00 (Post8), 17:00 (Post9) and 21:00 (Post10).

Data Acquisition and Analysis

Urine LC-MS data (N:37) were acquired with the same aforementioned method and processed with mzMatch and analysed with Ideom and Simca p (see *Part A* for details).

Results

A r-HuEPO Study

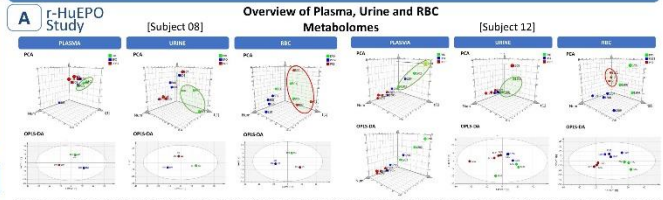
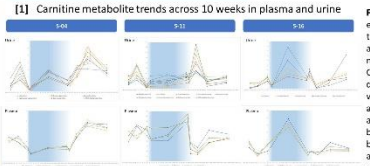


Figure 3. PCA analysis of plasma (N: ~600), urine (N: ~700) and RBC (N: ~800) metabolome in subject 8 and 12 illustrates most discernable differentiation between pre (circled) and post-phases. However, OPLS-DA creates clear clustered groups of the three phases in subject 8 yet not as prominent in subject 12, highlighting the effect of inter-individual variability. No normalisation techniques have been applied. *Similar variations in trend separation can be seen in all investigated subjects, subject 8 and 12 used as examples.*

[1] Carnitine metabolite trends across 10 weeks in plasma and urine



[2] Plasma raw data normalised to Acetyl-Carnitine

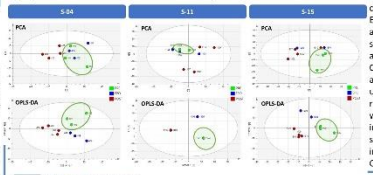


Figure 4. **[1]** Illustrates an example of the metabolite trends of varying carnitines across the 10 week experimental period of study A. Carnitines were clustered dependent on their pattern and variations can be seen not only across the subjects but also across the two different biological matrices. Shaded blue area highlights r-HuEPO administration phase. Each carnitine has been normalised against the sum for that carnitine across the samples. Each subject shows variations across the time-points, subjects shown here have been utilised as examples. **[2]** Acetyl-Carnitine, one of the most abundant carnitines, was utilised to normalise the plasma raw data in order to evaluate whether there would be improvements in phase separation. All subjects showed improvements in both PCA and OPLS-DA.

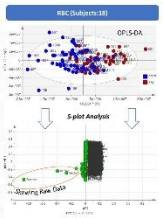


Figure 5. OPLS-DA of raw RBC metabolome across all subjects separates pre from post-phase. S-plot analysis indicates that there are metabolite markers which skew the characteristic "S shape" of this analysis; Creatine, Stachydrine and Betaine. This indicates an abundant dietary effect on the metabolome.

B Pilot Exercise Study

Figure 6. The PCA of the pilot urine samples across the 4 subjects does not immediately differentiate between the phases. However, OPLS-DA clearly clusters the three phases. PRE = samples 1 and 2; 1POST = samples 3, 4 and 5; 2POST = samples 6, 7, 8, 9 and 10.

Figure 7. 4 putative metabolites from the nucleotide metabolism and the steroid and steroid derivatives metabolism showed a marked response to the aerobic exercise as can be seen on 3POST time-point. The steroid conjugates in particular show a diurnal effect however the aerobic exercise is still noticeable. Bar charts show mean (intensity) ± SD. No normalization techniques were used.

Discussion & Conclusion

r-HuEPO seems to have an effect on the urine, plasma as well as the RBC metabolome as can be seen from the PCA and OPLS-DA. However the increased inter-individual variability shown by, for example, the diversity in clustering of time-points, creates barriers towards developing a population-based testing programme. Over the 10 week testing period there are abundant markers such as Creatine (to be expected) as well as Betaine and Stachydrine which are derived by the diets of the subjects. This further emphasises the need to introduce some element of control in spite of the exploratory nature of the study (A). The free carnitines and acyl carnitines have been associated with exercise due to their link to fatty acid oxidation. In study A there are clustered patterns emerging across the 10 weeks which vary across the subjects. Whether there is a direct link to r-HuEPO is unsure however the subjects showed significant performance improvements via 3km time trial and $\dot{V}O_{2max}$ testing [3], in response to r-HuEPO in study A. Whether there is a downstream metabolic improvement in energy related efficiency or fatty acid oxidation is only speculation. In the pilot study B, on the other hand, there are marked responses to aerobic exercise in relation to nucleotide metabolism and steroid conjugates which could have potential positive implications towards health. We wish to expand on the pilot study in order to further investigate these findings for suitability and success of different exercise intensities/ regimes for health and fitness.

References

1. Krug S., Kastenmüller G., Stückler F., et al. The dynamic range of the human metabolome revealed by challenges (2012), *FASEB J.* 26, 2607–2619
2. Christensen B., Nellemann B., Larsen M.S., et al. Whole body metabolic effects of prolonged endurance training in combination with erythropoietin treatment in humans: a randomized placebo controlled trial (2013), *Am J Physiol Endocrinol Metab.* 305(7):E879-89
3. Durussel J., **Daskalaki E.**, Anderson M., et al. Haemoglobin mass and running time trial performance after recombinant human erythropoietin administration in trained men. (2013) PLoS ONE; 8(2):e56151. DOI:10.1371/journal.pone.0056151

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Appendix v. 18 Selected Genes from Final Report of the Gene-Microarray Approach

Table S1. 18 selected genes from final report "a gene- microarray approach to the detection of r-HuEPO". Using an additional 2 fold-change threshold in the set of genes commonly differentially expressed during the last 2 weeks of rHuEPO administration and at 2 and 4 weeks after the last rHuEPO injection, 18 genes were significantly up-regulated during rHuEPO and subsequently down-regulated after administration.

Symbol	Name	Function
ADIPOR1	adiponectin receptor 1	Mediate increased AMP kinase, PPAR-a ligand activities, fatty-acid oxidation and glucose uptake.
ALAS2	aminolevulinate, delta-, synthase 2	Catalyzes the first step in the heme pathway
CA1	carbonic anhydrase I	Carbonic anhydrases are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide. They participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid. CA1 encodes a cytosolic protein which is found at the highest level in erythrocytes. (Genecards.org)
DPYSL5	dihydropyrimidinase-like 5 or collapsin response mediator protein 5 (CRMP5)	CRMPs may have a function in different cellular and molecular events of brain development, myelination, and adult neuronal plasticity.
HBD	haemoglobin, delta	The delta and beta genes are normally expressed in the adult: two alpha chains plus two beta chains constitute HbA, which in normal adult life comprises about 97% of the total haemoglobin. Two alpha chains plus two delta chains constitute HbA-2, which with HbF comprises the remaining 3% of adult haemoglobin. (ghr.nlm.nih.gov).
HBE1	haemoglobin, epsilon 1	The epsilon globin gene is normally expressed in the embryonic yolk sac: two epsilon chains together with two zeta chains (an alpha-like globin) constitute the embryonic haemoglobin Hb Gower I; two epsilon chains together with two alpha chains form the embryonic Hb Gower II. Both of

		these embryonic haemoglobins are normally supplanted by fetal, and later, adult haemoglobin. (Genecards.org)
HBM	haemoglobin, mu or HBAP2	The human alpha globin gene cluster located on chromosome 16 spans about 30 kb and includes seven loci: 5'- zeta - pseudozeta - mu - pseudoalpha-1 - alpha-2 - alpha-1 - theta - 3'. This gene has an ORF encoding a 141 aa polypeptide which is similar to the delta globins found in reptiles and birds. This locus was originally described as a pseudogene; however, it is currently thought to be a protein-coding gene. (Genecards.org)
LMNA	lamin A/C	The *LMNA* gene provides instructions for making several slightly different proteins called lamins. The two major proteins produced from this gene, lamin A and lamin C, are made in most of the body's cells. Lamins A and C are essential scaffolding (supporting) components of the nuclear envelope. (ghr.nlm.nih.gov). The early view of the lamins as a static nuclear exoskeleton is giving way to the view that nuclear lamins form dynamic polymers that comprise a variety of structures intimately involved in nuclear processes such as replication and transcription.
LOC100131726	HCC-related HCC-C11_v3	
LOC100288842	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5 pseudogene	
RNF213	ring finger protein 213	A genome-wide association study identified RNF213 as the first gene associated to Moyamoya disease in which blood flow to the brain is restricted due to arterial stenosis. It has been suggested that RNF213 is involved in a novel signaling pathway in intracranial angiogenesis.
SELENBP1	selenium binding protein 1	It has been demonstrated that the expression of the selenium binding protein 1 gene was increased in the blood and brain of patients with schizophrenia. Although the functional role of SELENBP1 is not well

		understood in the brain, selenium-binding proteins have been shown to co-localize with g-actin at the growing tips of SY5Y neuroblastoma cells, which indicates the potential for SELENBP1 to be associated with the growth and remodeling of neuritis.
SERPINA13	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 13 (pseudogene)	**The truncated human SERPINA13 would not be functional at least as a proteinase inhibitor since it loses the active loop which is critical for the proteinase inhibition activity. The original biological function, which is presumably lost in humans, of the ancestral SERPINA13 is unknown.** Polymorphisms in LMNA and near the SERPINA cluster (closest to SERPINA13) gene are associated with cognitive function in a UK but not in Chinese elderly population.
SLC4A1	solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)	Band 3 is the major integral glycoprotein of the erythrocyte membrane. Band 3 has two functional domains. Its integral domain mediates a 1:1 exchange of inorganic anions across the membrane, whereas its cytoplasmic domain provides binding sites for cytoskeletal proteins, glycolytic enzymes, and haemoglobin. (Genecards.org).
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	Although the function of alpha-synuclein is not well understood, studies suggest that it plays an important role in maintaining a supply of synaptic vesicles in presynaptic terminals. (ghr.nlm.nih.gov). May be involved in the regulation of dopamine release and transport. (Genecards.org). Several lines of evidence suggest an involvement of SNCA in familial and sporadic Parkinson's disease.
STRADB	STE20-related kinase adaptor beta	STRADB is a pseudokinase which, in complex with CAB39/MO25, binds to and activates STK11/LKB1. (Genecards.org). AMPK is a protein kinase cascade that plays an important role in regulating energy homeostasis. The first report of an upstream regulator came when it was discovered that STK11, in complex with STRAD and the scaffolding protein MO25, can phosphorylate and activate AMPK. Subsequently, it was demonstrated that

		STK11 can phosphorylate the T-loop of 12 other AMPK related human kinases. (atlasgeneticsoncology.org).
TESC	tescalcin	Essential for the coupling of ERK cascade activation with the expression of ETS family genes in megakaryocytic differentiation. (Genecards.org). During differentiation and maturation of megakaryocytes (thrombocytes), the expression of tescalcin is dramatically increased upon sustained activation of ERK1/2. Furthermore, tescalcin was shown to be a critical factor in megakaryocytic differentiation that is necessary for coupling MEK/ ERK cascade with expression of ETS family transcription factors.
TPRA1	transmembrane protein, adipocyte associated 1 or GPR175	Regulates cell division of early mouse embryos.

Appendix vi. Supplementary Table

Table S2. Continued list of metabolomics-based articles since 1999 with main study aim to investigate effect of exercise on different human bio-fluid metabolomes.

Study	**Instrument**	**Exercise Test**	**Subject Characteristics** (N, Gender, Age, Test Groups)	**Sampling Time-Points**	**Ref.**
Baranowski, et al.	**HPLC** [C18 reversed-phase, Varian Inc. OmniSpher 5, 4.6 × 150mm column]	**48hr Ultramarathon Race**	**7, Male 45.4 ± 9.2yrs** One Group- runners with weekly distance of 83 ± 32km and training history 5 ± 2yrs	3hr pre-race, after 24hr of running, 10min post-race as well as 24 and 48hr of recovery.	(Baranowski et al., 2014)
Green, et al.	Ion- pair reversed-phase **HPLC**	**Session 1-** $\dot{V}O_{2peak}$ [progressive cycle test to fatigue] **Session 2-** randomised into Group 1: 30min @ M & H Group 2: 60min @ M & H [M: 69.8% ± 1.8% $\dot{V}O_{2peak}$ H: 85.7% ± 1.2% $\dot{V}O_{2peak}$] 10 consecutive days of training per condition, 2wks break between conditions. **TESTS:** 1. Progressive test, before (1wk) and after (3-4 days) training. [Power output > incrementally every 2nd min until subject not able to maintain cadence of 60rpm]	**15, Male, 21 ± 1.7yrs** 2 Groups- recreationally active	Before and after 1st training program at rest, pre-exercise and 45min of exercise and at fatigue. Final sample following training was taken at same absolute time as pre- training. Second training program at rest, not during exercise. Following the 2nd training program samples taken during exercise and at the same times as during the first program following training.	(Green et al., 2013b)

		2. Prolonged Test, after (5-6 days) progressive test, following training @ 2 days recovery, and 1-2 days prior to progressive test. [Same work rate @ 60% $\dot{V}O_{2peak}$ until v.e.]			
Green, et al.	Ion- pair reversed-phase **HPLC**	**Session 1**- $\dot{V}O_{2peak}$ [progressive cycle test to fatigue] **Session 2**- randomised into 30min @ 59.9% $\dot{V}O_{2peak}$, or 60min @ 59.9% $\dot{V}O_{2peak}$ 10 consecutive days of training per condition, 2wks break between conditions. **TESTS:** 1. Progressive test, before (1wk) and after (3-4 days) training. 1. 4min @ 25W then 2. 15W increments in power output every 2nd min until subject not able to maintain cadence of 60rpm] 2. Prolonged Test, after (5-6 days) progressive test, following training @ 2 days recovery, and 1-2 days prior to progressive test. [Same work rate @ 60% $\dot{V}O_{2peak}$ until v.e.]	**7, Male, 22 ± 2yrs** One Group- active but untrained [initial average $\dot{V}O_{2peak}$ 44.4 ± 1.4 $ml.kg^{-1}.min^{-1}$]	Pre-exercise, at 45min of exercise and at fatigue. Final sample following training was taken at same absolute time as pre training. Pre-training at rest and prior to 2nd training program.	(Green et al., 2013a)
Røsjø, et al.	**HPLC**	**Group 1**- Maximal bicycle exercise stress test 1. Start test at 100W 2. >50W every 4min until v.e.	**Group 1- Male (N:7; 45 ± 5yrs) and Female (N:2)**	Group 1- Pre- and post-exercise Group 2- Pre-course and at day 3, 5 and 7	(Røsjø et al., 2013)

		Group 2- Military range course [24hr of continuous physical activities with total energy expenditure of >25MJ/day, also subjected to sleep and energy deprivation (max ~2500-3400kJ per subject)]	[Combination of well-trained and sedentary] **Group 2- 8, Male, 25 ± 1yr** [Norwegian military academy students]		
Spirlandeli, et al.	**HPLC** [10 µ C18 reverse-phase column (4.6 × 25 cm)]	**Running based Anaerobic Sprint Test (RAST)** performed twice 1. 20min warm-up of habitual stretching and running exercises 2. 6 bouts of 35m sprints w/ 10s intervals between sprints [2min rest between RAST protocols]	**8, Male, 18.6 ± 1.2yrs** One Group- healthy well-trained soccer players	Pre-exercise, immediately post- and 1hr post-exercise	(Spirlandeli et al., 2014)
Bye, et al.	**^{1}H-MR**	**Session 1-** $\dot{V}O_{2max}$ Test, treadmill [Ramp protocol which incl. keeping speed constant and increase inclination by 2% every second minute]	**Nord-Trøndelag Health Study (HUNT3)** Subset → **Fitness Study** **218, Male and Female, 45 – 59yrs, Healthy** Group 1- 108, low $\dot{V}O_{2max}$ Group 2- 110, high $\dot{V}O_{2max}$	Pre-exercise sample	(Bye et al., 2012)
Heyman, et al.	**LC-APCI-MS**	**Session 1-** Incremental Maximal Exercise test [ergometric bicycle, 80W start and > 40W every 3min until v.e.] **Session 2-** Familiarisation Test **Session 3-** Experimental Test 1. 15min seated and at rest 2. 60min @ 55% W_{max}	**11, Male, 23.3 ± 5.1yrs** One Group- healthy well-trained cyclists	During seated rest, post- 60min exercise, post- TT and after 15min recovery	(Heyman et al., 2012)

		3. Cycle @ pre-determined work load equal to 30min @ 75% W_{max} (TT) 4. 15min recovery [Same protocol in session 2 as 3 separated by 1wk]			
Krug, et al.	**(FIA)-MS/MS** **NMR** [plasma lipoprotein] **^{1}H-MR** [urine] **PTR MS** [breath] **(FIESI-ICR-FT)/MS**	**6 experimental conditions (challenges)** across 4 days. [DAY 1] Condition 1: fasting, overnight + 28hr post 8am lab visit [DAY 2] Condition 2: standard liquid diet, fiber-free formula, 12 noon **[DAY 3]** Condition 3: oral glucose tolerance test, 8am post overnight fast **Condition 4: Exercise Test** [DAY 4] Condition 5: lipid rich test diet Condition 6: cold pressure test **Exercise Condition:** 30min bicycle ergometer training @ power level of individual anaerobic threshold	**15, Male, 27.8 ± 2.9yrs** One Group- healthy	Specific to exercise condition: **Plasma** pre-exercise, 15 and 30min during-exercise, 45min, 1hr, 1.5hr and 2hr post-exercise **Exhaled breath condensate** at 0, 1hr and 2hr **Urine** at 0 min and 2hr	(Krug et al., 2012)
Baranowski, et al.	**HPLC** [C18 reversed-phase column (Varian, OmniSpher 5, 4.6 · 150 mm)]	**Session 1-** $\dot{V}$O2max, graded exercise test [cycloergometer] 1. 1W.kg^{-1} of body weight for 3min 2. >0.75W.kg^{-1} every 3min with v.e. **Session 2-** Group 1: 30min @ 70% $\dot{V}O_{2max}$ Group 2: 60min @ 70% $\dot{V}O_{2max}$	**20, Male** Group 1 (N:10)- 20.4 ± 0.7yrs, untrained with 47.3 ± 3mL.kg^{-1}.min^{-1} $\dot{V}$O2max Group 2 (N:10)- 21.1 ± 0.9yrs, endurance trained with 57.3 ± 6.5mL.kg^{-1}.min^{-1} $\dot{V}$O2max	Pre- exercise, during 30 and 60min (Group 2) of exercise and 30min of recovery	(Baranowski et al., 2011)

		[Session 1 and 2 separated by 6-7 days]			
Green, et al.	**HPLC**	**Session 1-** $\dot{V}O_{2peak}$ [progressive cycle test to fatigue, before and after training] 1. 4min loadless cycling 2. Continuous ramp increae in power output until subject could not maintain 60rpm **Session 2-** Standardised cycle test before (1wk) and after (24-48hr) training 1. 3min @ 25W (x2) 2. Power output increased to pre-established level (equivalent to ~62% $\dot{V}O_{2peak}$) for 1min 3. 60min rest 4. 3min @ 25W 5. 15min @ ~62% $\dot{V}O_{2peak}$ **Training-** 2h/day of cycling for 5 consecutive days @ 62% $\dot{V}O_{2peak}$	**9, Male, 20.8 ± 0.70yrs** One Group- healthy, not engaged in regular exercise [initial average $\dot{V}O_{2peak}$ 47.8 ± 1.7 $ml.kg^{-1}.min^{-1}$]	Two samples from each leg at each pre- and post- training exercise sessions. Samples taken at rest and at 10, 60, and 180 s of exercise. For dependent variables samples taken at warm up (Session 2 point 1.), after Session 2 point 2., then at 10 and 180s of exercise during point 5 (Session 2).	(Green et al., 2009)
Green, et al.	Ion- pair reversed-phase **HPLC**	**Session 1-** $\dot{V}O_{2peak}$ [progressive cycle test to fatigue on cycle ergometer,4wks before training protocol] **Training-** 3 days of exercise @ ~60% $\dot{V}O_{2peak}$ (until fatigue or maximum 120min) followed by 3 days of recovery [exercise day 1 was repeated 3-4wks prior to 6 day protocol]	**12, Unknown, 19.2 ± 0.27yrs** One Group- healthy, untrained and not engaged in regular exercise [initial average $\dot{V}O_{2peak}$ 44.8 ± 2.0 $ml.kg^{-1}.min^{-1}$]	At exercise days 1 and 3 pre- and post- exercise. Post samples taken immediately after cessation. Plus during recovery day 1, 2 and 3. 7 in total samples from different sites, taken in duplicate at each incision point.	(Green et al., 2008)

Menshikova, et al.	**HPLC**	**12wk training program** **Session 1-** $\dot{V}O_{2max}$ test, graded cycle exercise test [pre- and post- training program] **Session 2-** [4-6 exercise sessions per wk, 3 supervised) Wk 1-4: 30min @ heart rate corresponding to 50-60% $\dot{V}O_{2max}$ Wk 5-8: 40min same intensity Wk 9-12: 40min @ ~70% $\dot{V}O_{2max}$	**8, Male and Female, 67.3 ± 0.6yrs** Healthy sedentary	Pre- and post- training program	(Menshikova et al., 2006)
Okura, et al.	**HPLC**	**20wk training program** **Session 1-** $\dot{V}O_{2max}$ test, progressive maximal exercise test to v.e. [pre- and post- training program] **Session 2-** 3 exercise sessions per wk for 20wks 1. training began at heart rate corresponding to 55% $\dot{V}O_{2max}$ for 30min 2. progressed to 75% $\dot{V}O_{2max}$ for 50min in the last 6wks	**HERITAGE Family Study 730, Male and Female** Black males (N:90), 32.9 ± 12.3yrs Black females (N:159), 33 ± 11.4yrs White males (N:236), 36.2 ± 14.9yrs White females (N:245), 35.1 ± 14.1yrs	Pre- and post- training program	(Okura et al., 2006)
Aguil*ó*, et al.	**HPLC** [Nova Pak, C18, 3.9 x 150 mm column and Waters Spherisorb 5µm	**Stage 3 of the "Setmana Catalana 2000"**, consisting of 171km mountainous terrain [part of a 5 day competition for professional cyclists, Spain]	**8, Male, 23.8 ± 0.9yrs** One Group- professional cyclists [$\dot{V}O_{2max}$ of 80.2 ± 1.6 mL.kg^{-1}.min^{-1}]	Morning of the 3rd stage, immediately post-exercise, 3hrs post-exercise and ~15hrs into recovery (following morning)	(Aguiló et al., 2005)

	ODS2, 34.6 x 150mm column]				
Imai, et al.	**HPLC** [Shodex-Asahipak ES-502N column (10 x 0.76cm i.d., DEAE form)]	**5 Day Kendo training camp** [2 and half hr session in the morning following by a 3hr session in the afternoon]	**Group 1- 30, Male, 20 ± 1.1yrs**, elite kendo athletes **Group 2- 20, Male, 22.1 ± 1.8yrs**, controls	1st day of training pre-practice and on 5th day w/in 40min of end of practice	(Imai et al., 2002)
Karatzaferi, et al.	**HPLC-UV** [reversed-phase]	**Session 1-** 10s max effort @ 120 crank rpm on an isokinetic cycle ergometer	**2, Male and Female, 32 and 25yrs** healthy	Rest and immediately post-exercise	(Karatzaferi et al., 2014)
Zhao, et al.	**HPLC** [Hibar Li-chrosphere 100 CH-18/2 (Merck; 240 x 4 mm)]	**Session 1-** 1. 30s 'all-out' sprint, cycle ergometer 2. 10min rest in supine position	**7, Male, 23.9 ± 2.3yrs** One Group- healthy, active, non-specialised trained	Plasma- 2min, 5min and 10min post-exercise Muscle (*vastus lateralis*) extracts- rest, immediately post-exercise, 5min and 10min post-exercise	(Zhao et al., 2000)
Green, et al.	Ion- pair reversed-phase **HPLC**	**Session 1-** $\dot{V}O_{2peak}$ [progressive cycle test to fatigue on cycle ergometer, 1-2 days before training protocol] **Session 2-** 30 min of cycling at power output relative to predetermine values, at day 0 of training, day 3 of training (24-30hrs after 3rd day exercise) and at day 6 (24-36hrs after last training exercise session). **Training-** 6 consecutive days of cycling. *Group 1:* 127W ± 7.3W, 69% $\dot{V}O_{2peak}$ for 2hrs or until fatigue	**14, Male** Group 1 (N:7)- age 21.6 ± 1.0yrs, $\dot{V}O_{2peak}$ 41.0 ± 1.3 $ml.kg^{-1}.min^{-1}$ Group 2 (N:7)- age 21.0 ± 0.63yrs, $\dot{V}O_{2peak}$ 51.4 ± 0.90 $ml.kg^{-1}.min^{-1}$ [Healthy with no regular exercise]	Pre- and at 3 and 30min of exercise (Session 2). 9 in total, taken in duplicate at each incision.	(Green et al., 1999)

		Group 2: 159W ± 4.5W, 66% $\dot{V}O_{2peak}$ for 2hrs or until fatigue			

Abbreviations: $\dot{V}O_{2max}$: maximal oxygen consumption; W: Watts; WR_{peak}: peak work rate; v.e.: volitional exhaustion; rpm: revolutions per minute; VT: Ventilator threshold; HR: heart rate; TT: time trial.

Appendix vii. Supplementary Table

Table S3. Continued metabolite details as presented from the exercise metabolomics literature and identified through a metabolomics-based technology.

Study	**Sample Tested** (Plasma:P; Erythrocytes:E; Urine:U; Serum: S; Muscle Extract:ME;Breath:B; Whole Blood:WB)	**Metabolite Pathways** (↓: reduced post- exercise, ↑: increased post-exercise)	**Ref.**
Baranowski, et al.	P and E	Ceramide Sphingosine Sphinganine Sphingosine- 1- phosphate, ↓**(P)** Sphinganine- 1- phosphate, ↓**(P)**	(Baranowski et al., 2014)
Green, et al.	ME (*Vastus Lateralis*)	ATP ADP AMP IMP, ↓ **with training during exercise**	(Green et al., 2013b)
Green, et al.	ME (*Vastus Lateralis*)	ATP ADP AMP IMP, ↓ **trend response to training**	(Green et al., 2013a)
Røsjø, et al.	P	Adrenaline Noradrenaline, ↑ **Group1**	(Røsjø et al., 2013)
Spirlandeli, et al.	P	Malondialdehyde, ↑	(Spirlandeli et al., 2014)
Bye, et al.	S	Free Choline, ↑ **in Group 1 *vs.* Group 2** Phosphotidylcholine, ↓ **in Group 1 *vs.* Group 2** Unsaturated fatty acids, ↓ **in Group 1 *vs.* Group 2** Triglycerides, **correlation with free choline**	(Bye et al., 2012)
Heyman, et al.	P	Anandamide, ↑ 2-arachidonoylglycerol *N*-palmitoylethanolamine, ↑ *N*-oleylethanolamine, ↑	(Heyman et al., 2012)
Krug, et al.	P, U and B	Lactate, ↑ **(P)** Acyl-carnitines, ↑ **(P)**	(Krug et al., 2012)
Baranowski, et al.	P and E	Sphingosine Sphinganine, ↑ **(E) in endurance trained** Sphingosine-1-phosphate, ↑ **(P) in untrained** Sphinganine-1-phosphate, ↑ **(P) in untrained and ↑ (E) in endurance trained** Ceramide, ↓ **(E) in untrained**	(Baranowski et al., 2011)
Green, et al.	ME (*Vastus Lateralis*)	ATP ADP AMP	(Green et al., 2009)
Green, et al.	ME (*Vastus Lateralis*)	ATP AMP ADP IMP, ↑ **during exercise of training day 1**	(Green et al., 2008)
Menshikova, et al.	ME (*Vastus Lateralis*)	Cardiolipin, ↑ NADH oxidase, ↑ Succinate oxidase, ↑	(Menshikova et al., 2006)

Okura, et al.	P	Total Homocysteine, ↓ **post training in individuals with hyperhomocysteinemia,** ↑ **in individuals that were in normal range**	(Okura et al., 2006)
Aguil*ó*, et al.	WB and P	Liposoluble vitamins (P) Carotenoids (P) Glutathione disulphide, ↑ **(WB)**	(Aguiló et al., 2005)
Imai, et al.	S	Mercaptalbumin fraction, ↓ **post training**	(Imai et al., 2002)
Karatzaferi, et al.	ME (*Vastus Lateralis*)	ATP IMP Phosphocreatine, ↓ **in all fibres** Creatine	(Karatzaferi et al., 2014)
Zhao, et al.	P and ME (*Vastus Lateralis*)	ATP, ↓ IMP, ↑	(Zhao et al., 2000)
Green, et al.	ME (*Vastus Lateralis*)	ATP AMP ADP IMP, **3 days training ↓ exercise levels of IMP**	(Green et al., 1999)

Abbreviations: HGPRT: hypoxanthine-guanine phosphoribosyl-transferase; ADP: adenosine 5' diphosphate; AMP: adenosine monophosphate; IMP: inosine monophosphate; ATP: adenosine 5' triphosphate; THR: threshold training distribution

Appendix viii. MzMatch Processing Settings with Script for Pilot Exercise Study

```
library(rJava)
library(mzmatch.R)
mzmatch.init()


setwd()
mzXMLfiles <- dir(full.names=TRUE,pattern="\\.mzXML$",recursive=TRUE)
outputfiles <- paste(sub(".mzXML","",mzXMLfiles),".peakml",sep="")


xset <- xcmsSet(mzXMLfiles, method='centWave', ppm=2, peakwidth=c(5,100),
snthresh=3, prefilter=c(3,1000), integrate=1, mzdiff=0.001,
verbose.columns=TRUE,
fitgauss=FALSE, nSlaves=16)

## Afterwards you split resulting xset by filenames and process it with
peakml file writer.

xsets <- split (xset,xset@filepaths)

peakMLparallel <- function(x)
{
library(mzmatch.R)
mzmatch.init ()
xset <- xsets[[x]]
PeakML.xcms.write.SingleMeasurement
(xset=xset,filename=outputfiles[x],ionisation="detect",addscans=20,writeReje
cted=FALSE,ApodisationFilter=TRUE)
}


if (length(xsets)==length(mzXMLfiles))
{

## Create a snow cluster for writing peakml files
cl <- makeCluster (8, type="SOCK")
clusterExport (cl,list=c("xsets","outputfiles"))
system.time(clusterApply(cl,1:length(outputfiles),peakMLparallel))
stopCluster(cl)
} else
{
cat ("xcms set does not contains peaks for all mzXML samples.")
}


MainClasses <- dir ()
dir.create ("combined_RSD_filtered")
dir.create ("combined_RSD_rejected")
dir.create ("combined")
for (i in 1:length(MainClasses)){FILESf <- dir
(MainClasses[i],full.names=TRUE,pattern="\\.peakml$",recursive=TRUE)
OUTPUTf <- paste ("combined/",MainClasses[i],".peakml",sep="")
if(length(FILESf)>0){mzmatch.ipeak.Combine
(i=paste(FILESf,collapse=","),v=T,rtwindow=30,o=OUTPUTf,combination="set",pp
m=5,label=paste(MainClasses[i],sep=""))
RSDf <- paste ("combined_RSD_filtered/",MainClasses[i],".peakml",sep="")
REJf <- paste ("combined_RSD_rejected/",MainClasses[i],".peakml",sep="")
if(length(FILESf)>1)
mzmatch.ipeak.filter.RSDFilter(i=OUTPUTf,o=RSDf,rejected=REJf,rsd=1,v=T)
else file.copy(OUTPUTf,RSDf)}}
INPUTDIR <- "combined_RSD_filtered"
```

```
FILESf <- dir (INPUTDIR,full.names=TRUE,pattern="\\.peakml$")
mzmatch.ipeak.Combine(i=paste(FILESf,collapse=","),v=T,rtwindow=30,
o="combined.peakml",combination="set",ppm=5)
mzmatch.ipeak.filter.NoiseFilter
(i="combined.peakml",o="combined_noisef.peakml",v=T,codadw=0.8)
mzmatch.ipeak.filter.SimpleFilter(i="combined_noisef.peakml",
o="combined_sfdet.peakml", mindetections=3, v=T)
mzmatch.ipeak.filter.SimpleFilter(i="combined_sfdet.peakml",
o="combined_highintensity.peakml", minintensity=1000, v=T)
PeakML.GapFiller(filename = "combined_highintensity.peakml", ionisation =
"detect", Rawpath = NULL, outputfile = "highintensity_gapfilled.peakml", ppm
= 0, rtwin = 0)
mzmatch.ipeak.sort.RelatedPeaks (JHeapSize=5000,
i="highintensity_gapfilled.peakml", v=T,
o="mzMatch_output.peakml",basepeaks="mzMatch_basepeaks.peakml",ppm=5,rtwindo
w=6)
annot <- paste("relation.id,relation.ship,codadw,charge")
mzmatch.ipeak.convert.ConvertToText (JHeapSize=5000,
i="mzMatch_output.peakml", o="mzMATCHoutput.txt",v=T,annotations=annot)

# Processing finished!     Now extracting chromatogram images for each peak.
chromdir <- "chromatograms"
dir.create(chromdir)
PeakMLData <- PeakML.Read("mzMatch_output.peakml",Rawpath=NULL)
peakIDlist <- c(1:length(unique(PeakMLData$peakDataMtx[,10])))
sampnames <- PeakMLData$sampleNames
sampleslist<-c(1:max(PeakMLData$peakDataMtx[,9]))
groupsets <- max(PeakMLData$peakDataMtx[,11])
if (groupsets!=1) {samplegroups <- PeakMLData$phenoData} else {samplegroups
<- sampnames}
classnumbers <- samplegroups
for (i in 1:length(unique(samplegroups))){classnumbers <-
sub(unique(classnumbers)[i], i, classnumbers)}
for (a in 1:length(peakIDlist)){peakID <- peakIDlist[a]
hits <- which(PeakMLData$peakDataMtx[,10]==peakID)
intslist <- vector ("list")
rtlist <- vector ("list")
for (i in 1:length(hits)){intslist[[i]] <-
PeakMLData$chromDataList[[hits[i]]][2,]
rtlist[[i]] <- (PeakMLData$chromDataList[[hits[i]]][3,]) / 60 }
maxint <- max(unlist(intslist))
minrt <-  min (unlist(rtlist))
maxrt <- max (unlist(rtlist))
samplenumbers <- PeakMLData$peakDataMtx[hits,9]
myfilename <- paste(getwd(),"/",chromdir,"/",peakID,".png",sep="")
png(myfilename, width = 350, height = 300)
lw = 0.2
par(fig=c(0,1-lw,0,1))
plot (1,1,xlab="RT (m)", ylab="Intensity", pch="", xlim=c(minrt,maxrt),
ylim=c(0,maxint))
for (i in 1:length(hits)){if (PeakMLData$peakDataMtx[hits[i],9] %in%
sampleslist==TRUE){
points (rtlist[[i]], intslist[[i]], type="l", col=classnumbers[samplenumbers
[i]])}}
par(fig=c(0,1,0,1))
lpos <- par("usr")[2]-(lw /(1-lw ))*(par("usr")[2]-par("usr")[1])
legend (lpos, par("usr")[4], text.col=unique(classnumbers[sampleslist]),
unique(samplegroups[sampleslist]), cex=0.8,xpd=TRUE)
dev.off()}


## Rename file based on folder name
RENAME<-paste(basename(getwd()),".txt",sep="")
file.rename("mzMATCHoutput.txt",RENAME)
```

```
## Rename file based on folder name
RENAME<-paste(basename(getwd()),".peakml",sep="")
file.rename("mzMatch_output.peakml",RENAME)

### folder renaming
OLDNAME<-getwd()
RENAME<-paste(getwd(),"-done",sep="")
setwd()
file.rename(OLDNAME,RENAME)
```

Appendix ix. IDEOM Settings for Pilot Exercise Study

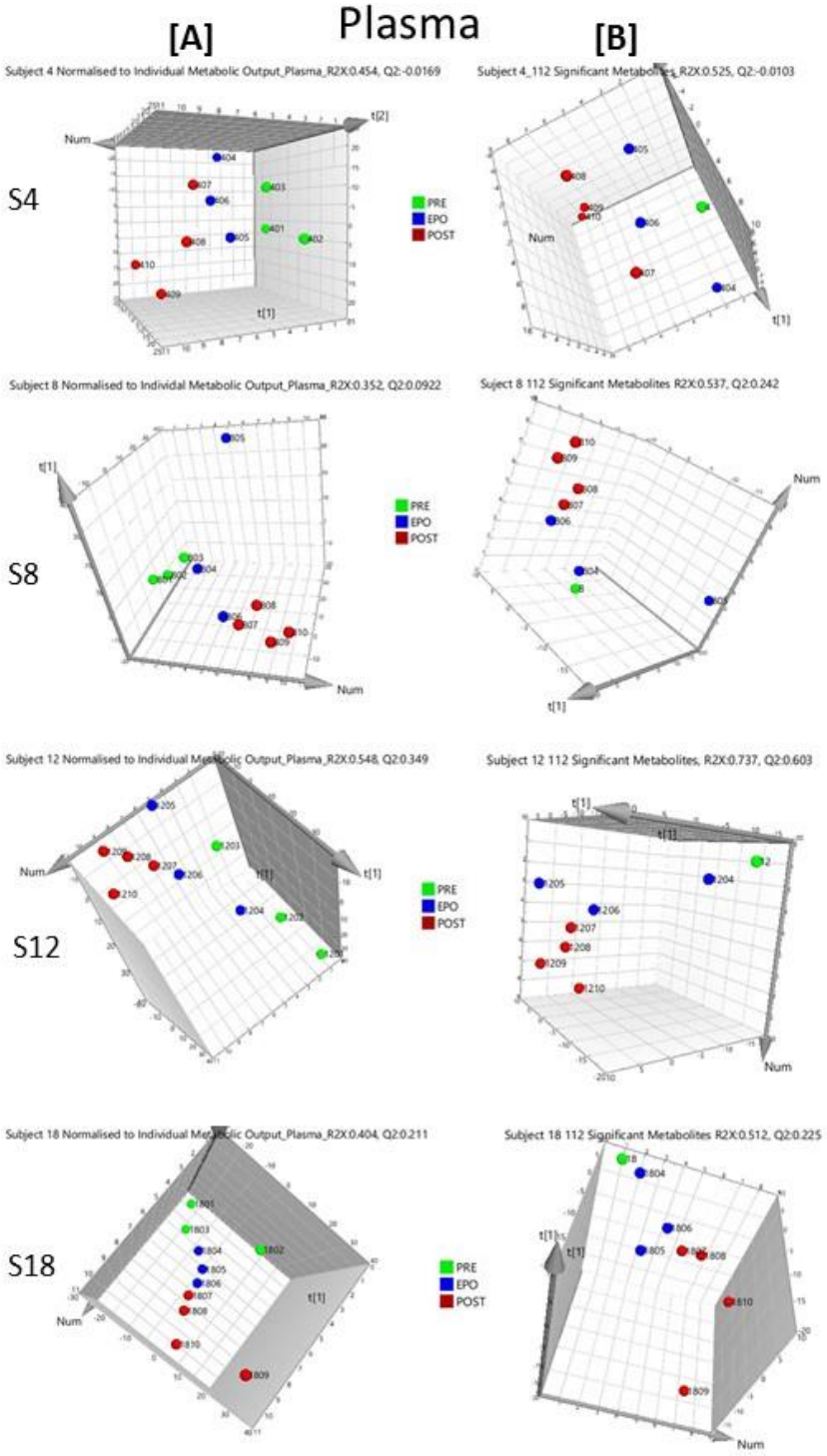
Table S4**.** IDEOM settings.

Category	Parameter	Input
XCMS (Centwave)	Method (file type)	mzXML
	Ppm	2
	Peak width (min)	5 s
	Peak width (max)	100 s
	S/N* threshold	3
	Pre-filter (# points)	3
	Pre-filter (intensity)	1000
	M/z difference	0.001
MzMatch	Mzmatch grouping RT~ window	0.5 min
	Mzmatch grouping m/z ppm	5 ppm
	Relative Std Dev (RSD) filter	0.80#
	Noise filter (CODA-DW¥)	0.80
	Intensity filter (LOQα)	1000
	Minimum detections #	3
	RT window for related peaks	0.10 min
IDEOM	RT for id of authentic standards	5.0%
	RT for id for calculated RT	50.0%
	Ppm for mass identification	3.0 ppm
	Ignore related peaks before RT	0.0 min
	RT window for complex adducts	0.50 min
	RT window for duplicate peaks	1.00 min
	RT window for shoulder peaks	2.0 min
	Intensity ratio for shoulder peaks	5 to 1
	Intensity limit duplicate peaks	1%
	r^2 limit for duplicate peaks	0.99
	Preferred database	HumanDB (hsaβ)

Input details for settings in IDEOM version 19 excel file. * signal to noise; ~ retention time; # generous; ¥ Durbin-Watson criterion; α limit of quantification; β homo sapiens.

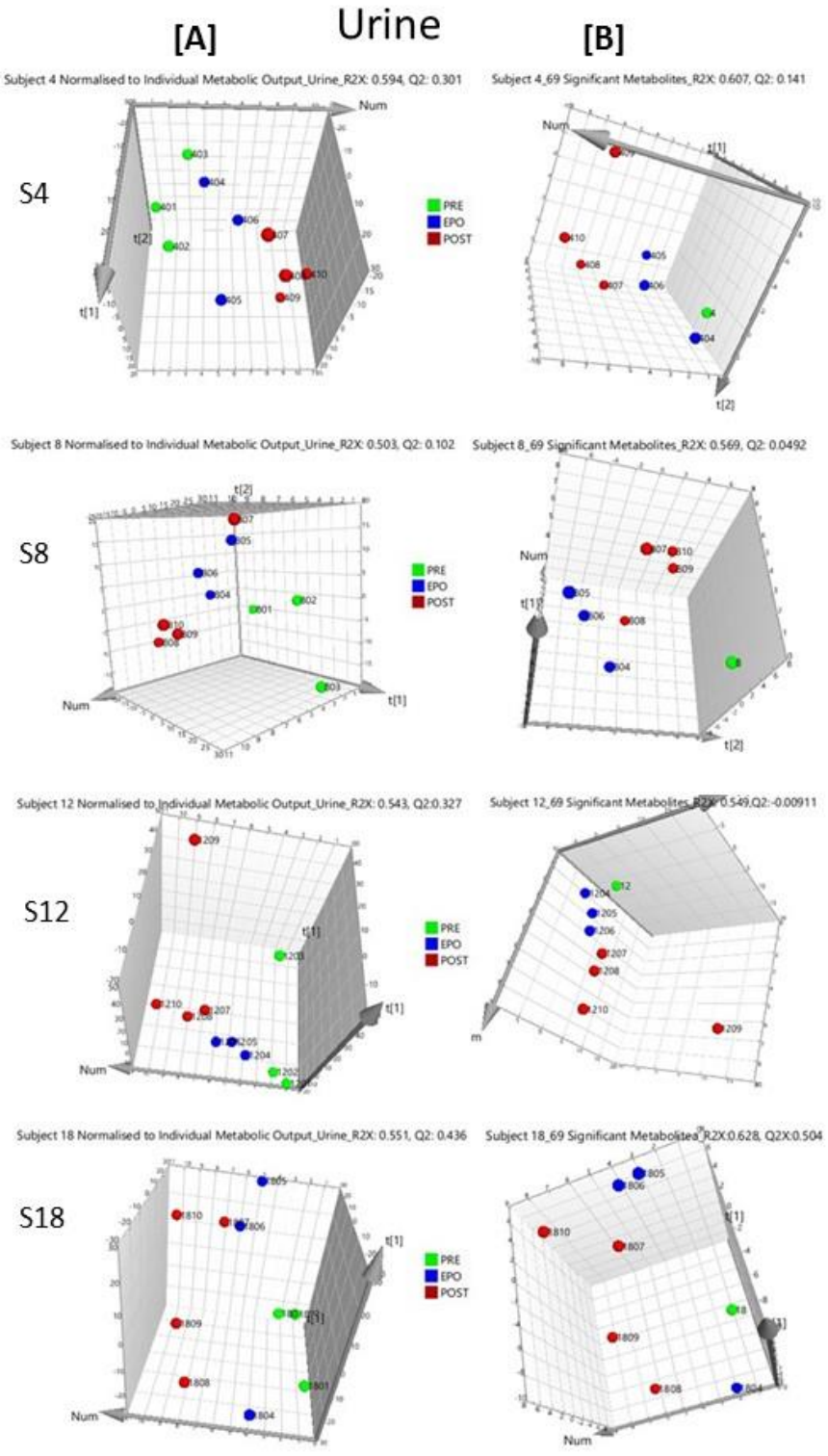
Appendix x. Individual Plasma Profiles

Plasma profiles of subject 4, 8, 12 and 18 with [A] all filtered putative metabolites and [B] significant putative metabolites with averaged PRE values.



Appendix xi. Individual Urine Profiles

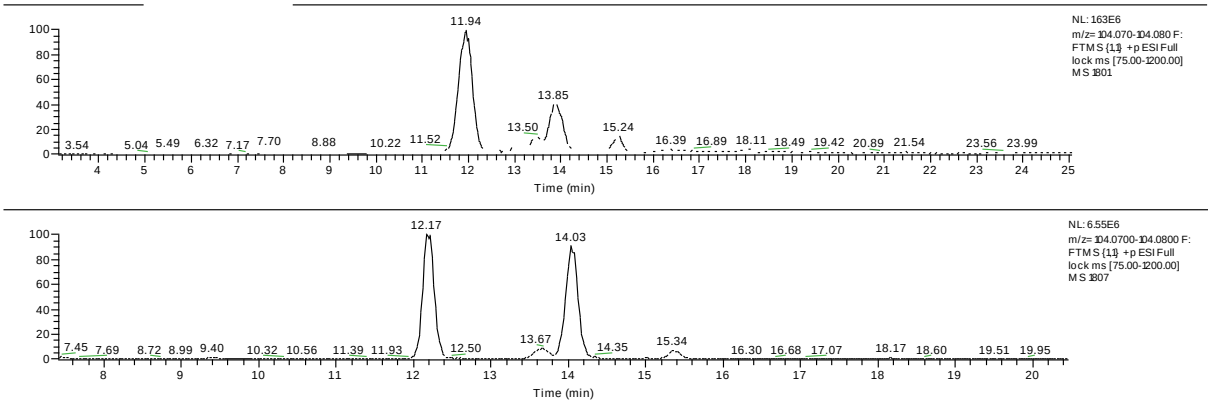
Subject 4, 8, 12 and 18 with [A] all filtered putative metabolites and [B] significant putative metabolites with averaged PRE values.



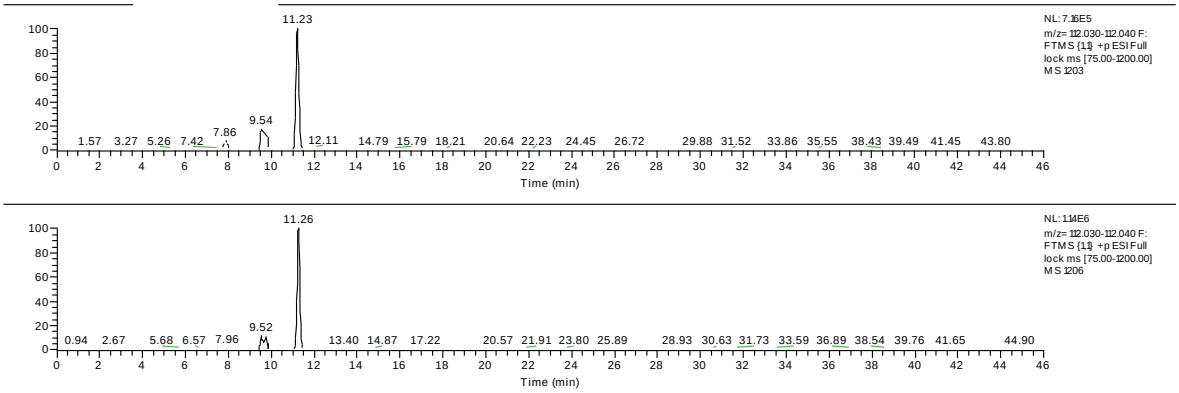
Appendix xii. Extracted ion chromatograms for the most significant urinary markers of the effects of r-HuEPO

Metabolites of traces 3, 4, 5, 6, and 8 have been associated with exercise (Daskalaki et al., 2015a, 2015b).

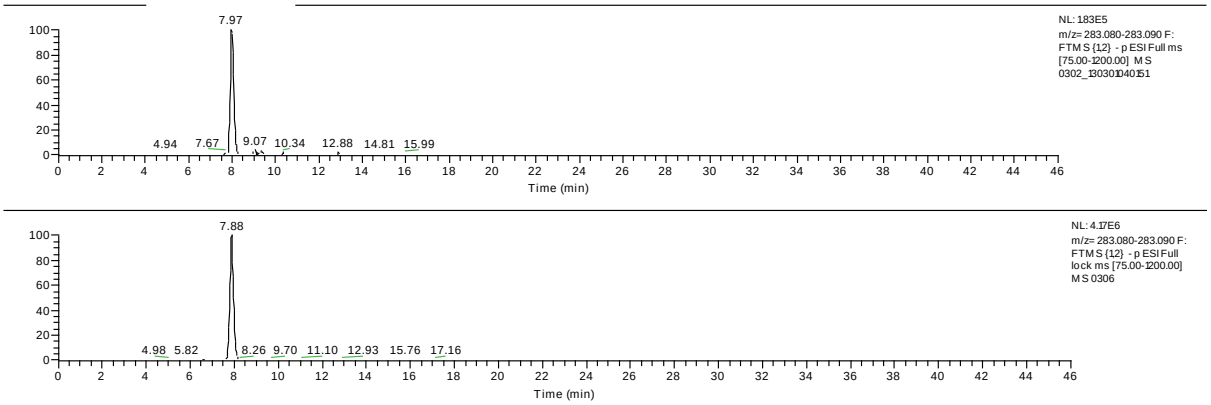
(1) 4-Aminobutanoate (Mass:103.0632, RT:14.03; ID:HMDB00112)



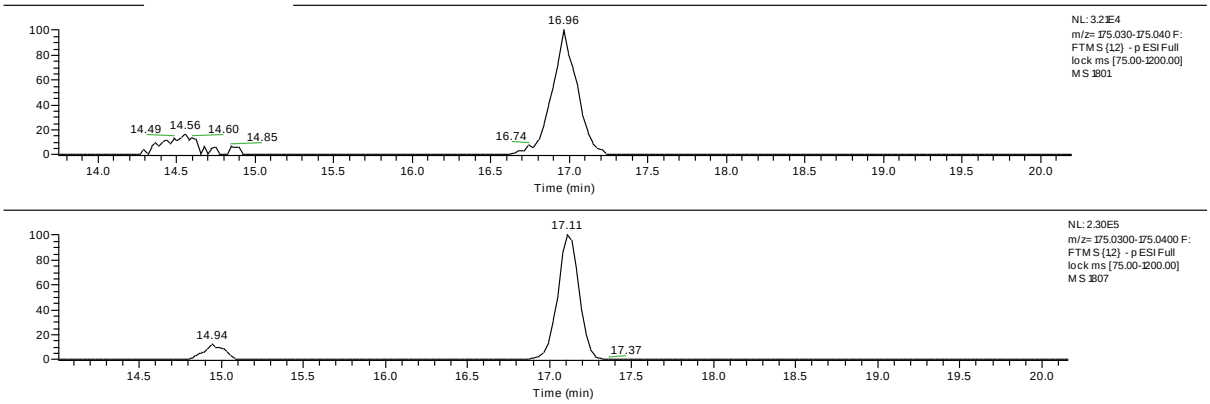
(2) Pyrrole-2-carboxylate (Mass:111.032; RT:11.3; ID:HMDB04230)



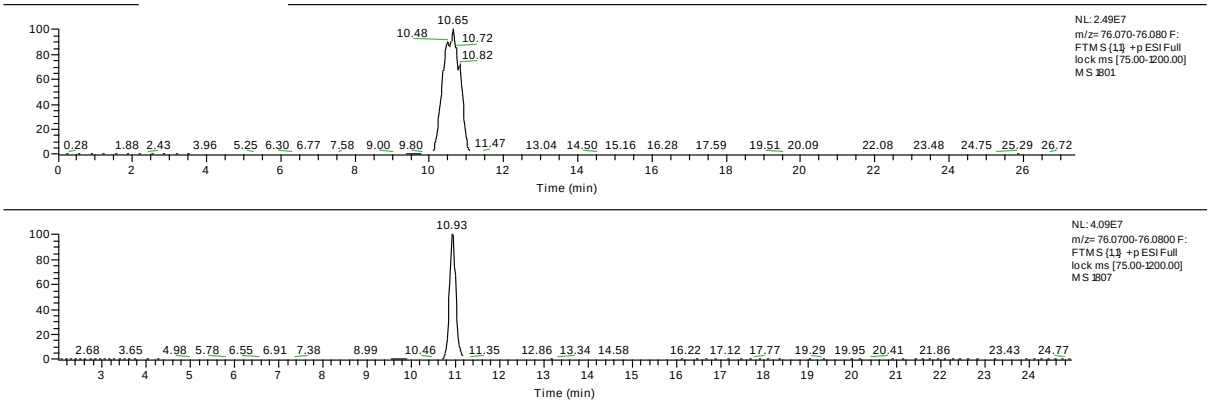
(3) p-Cresol glucuronide (Mass:284.090; RT:7.9; ID:HMDB11686)



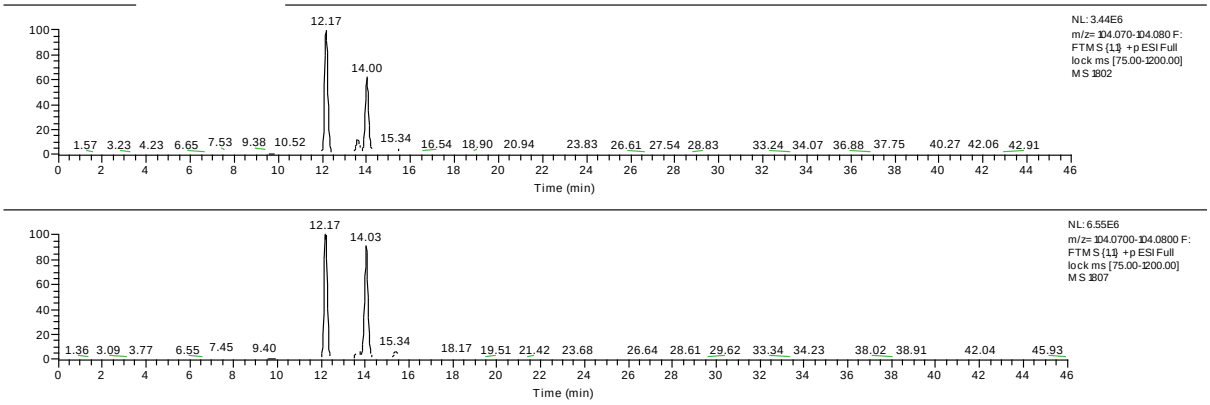
(4) N-Carbamoyl-L-aspartate (Mass:176.04, RT:17.13; ID:HMDB00828)



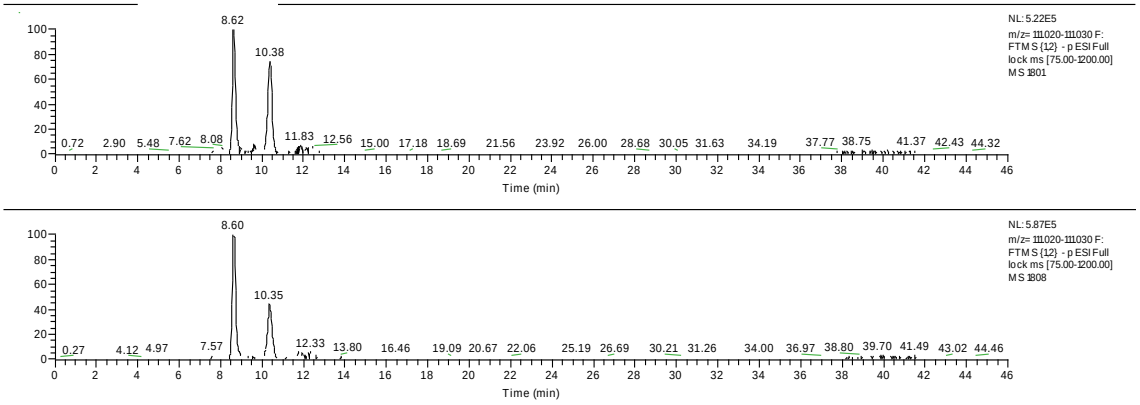
(5) Trimethylamine N-oxide (Mass:75.069; RT:10.91; ID:HMDB00925)



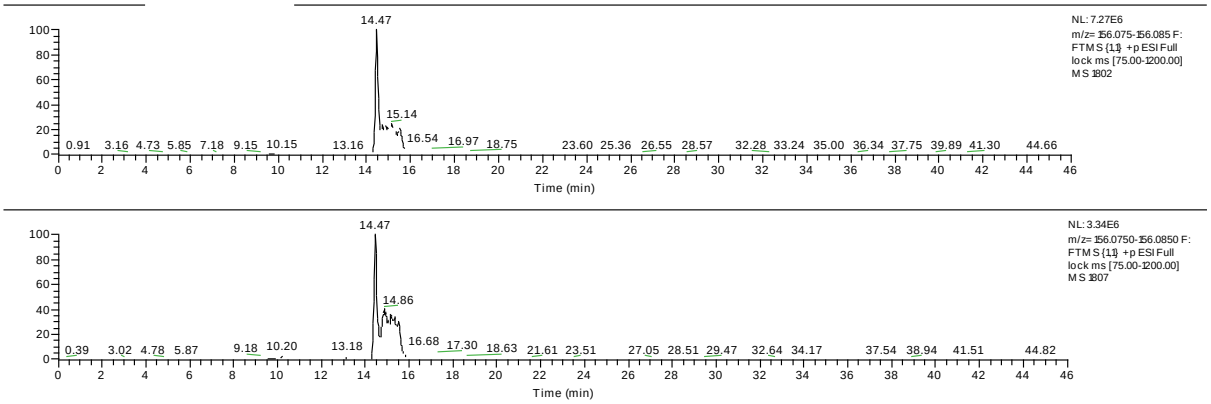
(6) N,N-Dimethylglycine (Mass:103.063; RT:12.2; ID:HMDB00092)



(7) Uracil (Mass:112.027; RT:8.6; ID:HMDB00300)



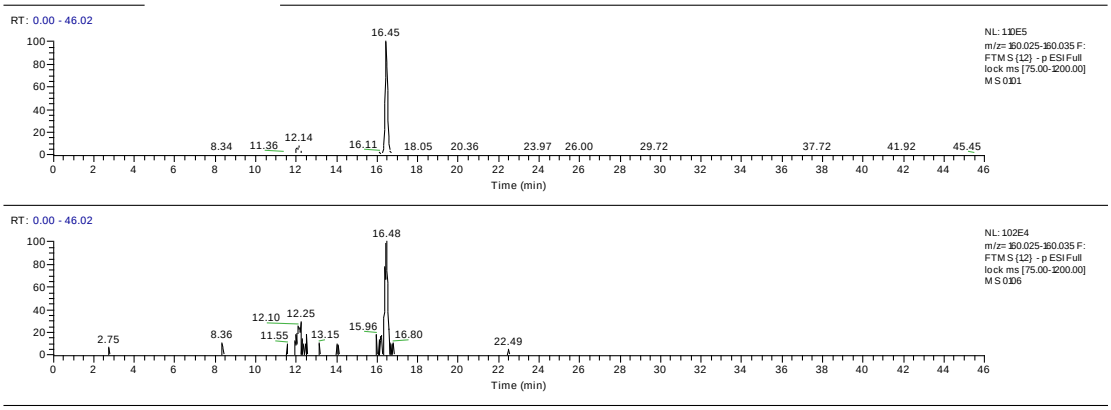
(8) L-Histidine (Mass:155.070; RT:14.50; ID:HMDB00177)



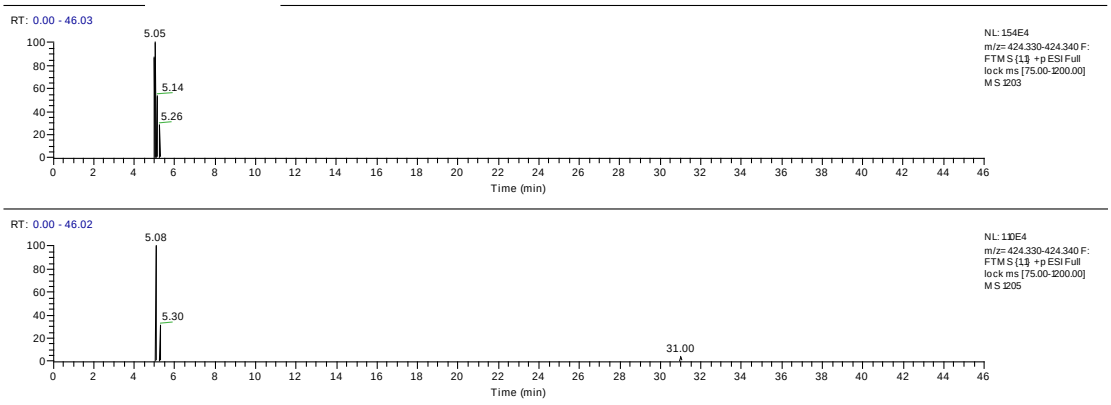
Appendix xiii. Extracted ion chromatograms for the most significant plasma markers of the effects of r-HuEPO

Metabolites of traces 4, 6, 7 and 8 have been associated with exercise (Daskalaki et al., 2015a, 2015b).

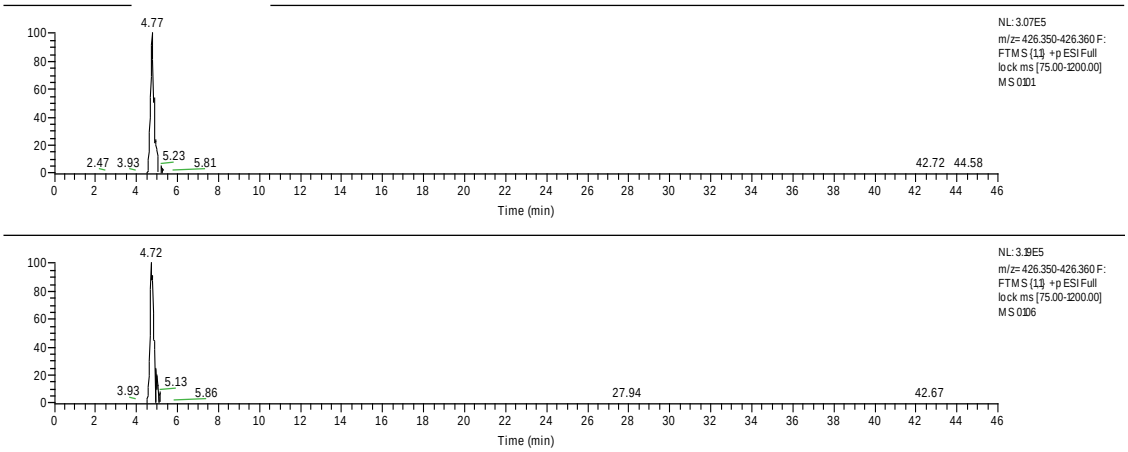
(1) N-Formyl-L-aspartate (Mass:161.0325; RT:16.4, ID:HMDB60495) *Subject 1*



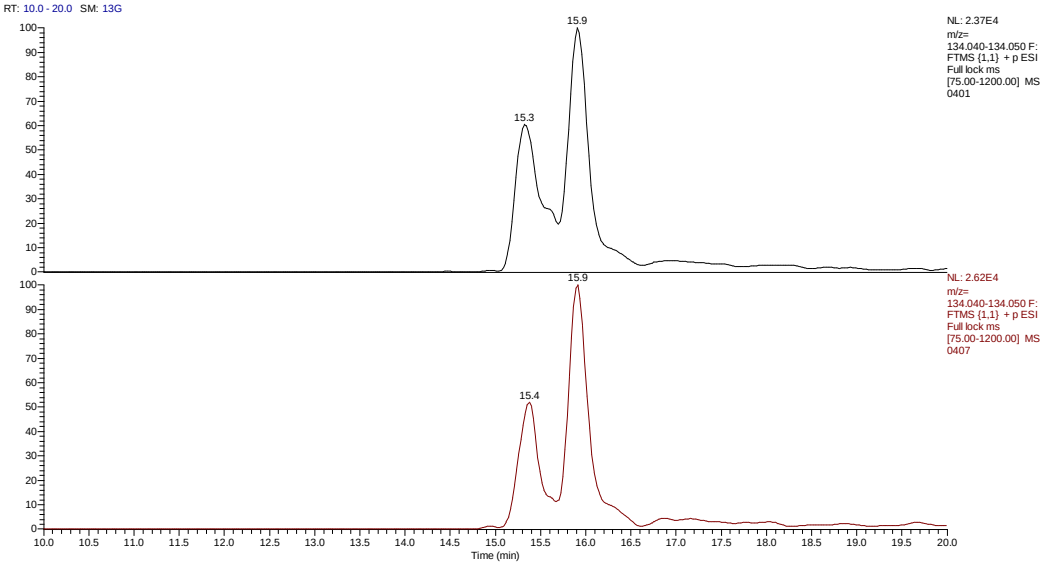
(2) Linoelaidyl carnitine (Mass:423.334; RT:5.0; ID:HMDB06461) *Subject 12*



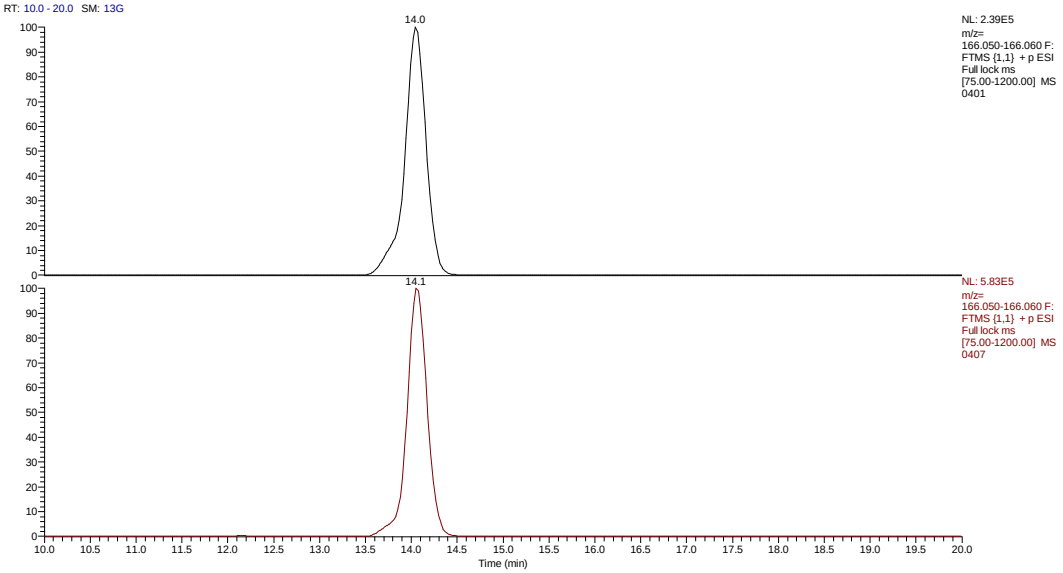
(3) Elaidic carnitine (Mass:425.350; RT:4.7; ID:HMDB06464)



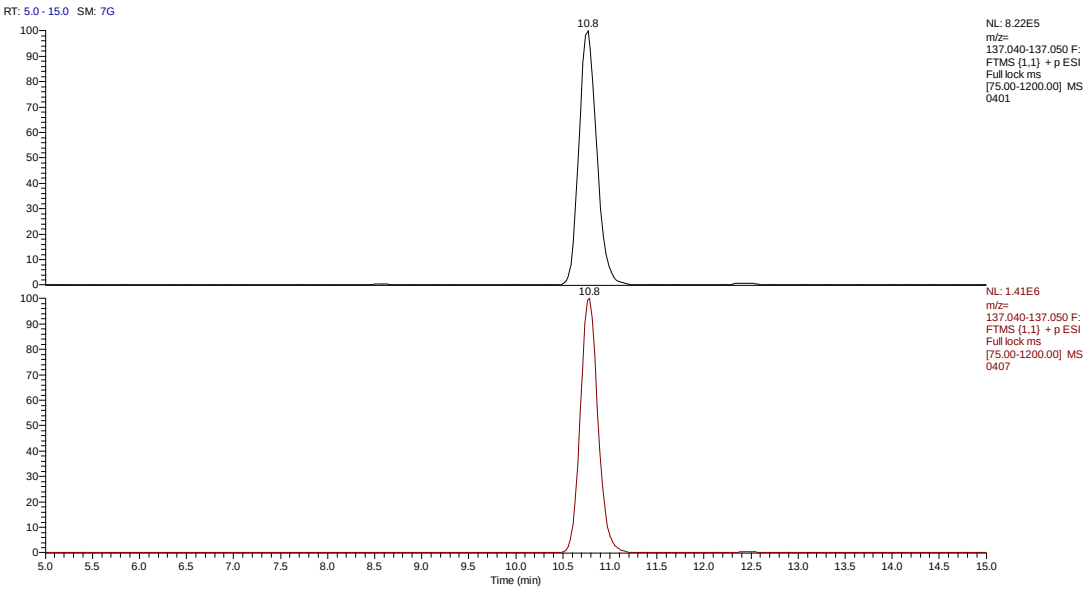
(4) L-Aspartate (Mass:133.038; RT:15.5; ID:HMDB00191)



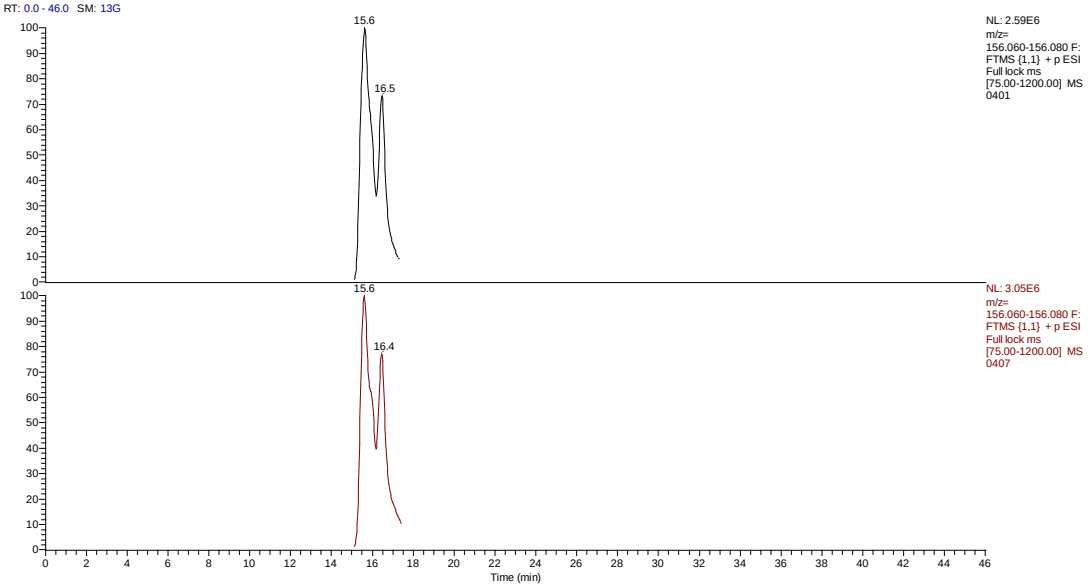
(5) L-Methionine S-oxide (Mass:165.046; RT:14.0; ID:HMDB02005)



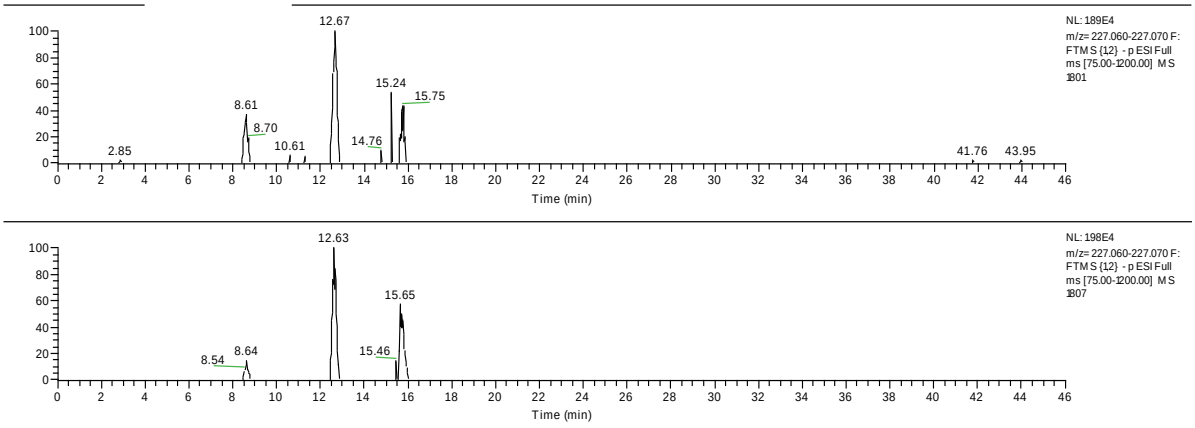
(6) Hypoxanthine (Mass:136.039; RT:10.77; ID:HMDB00157)



(7) L-Histidine (Mass:155.069; RT:16.03; ID:HMDB00177)

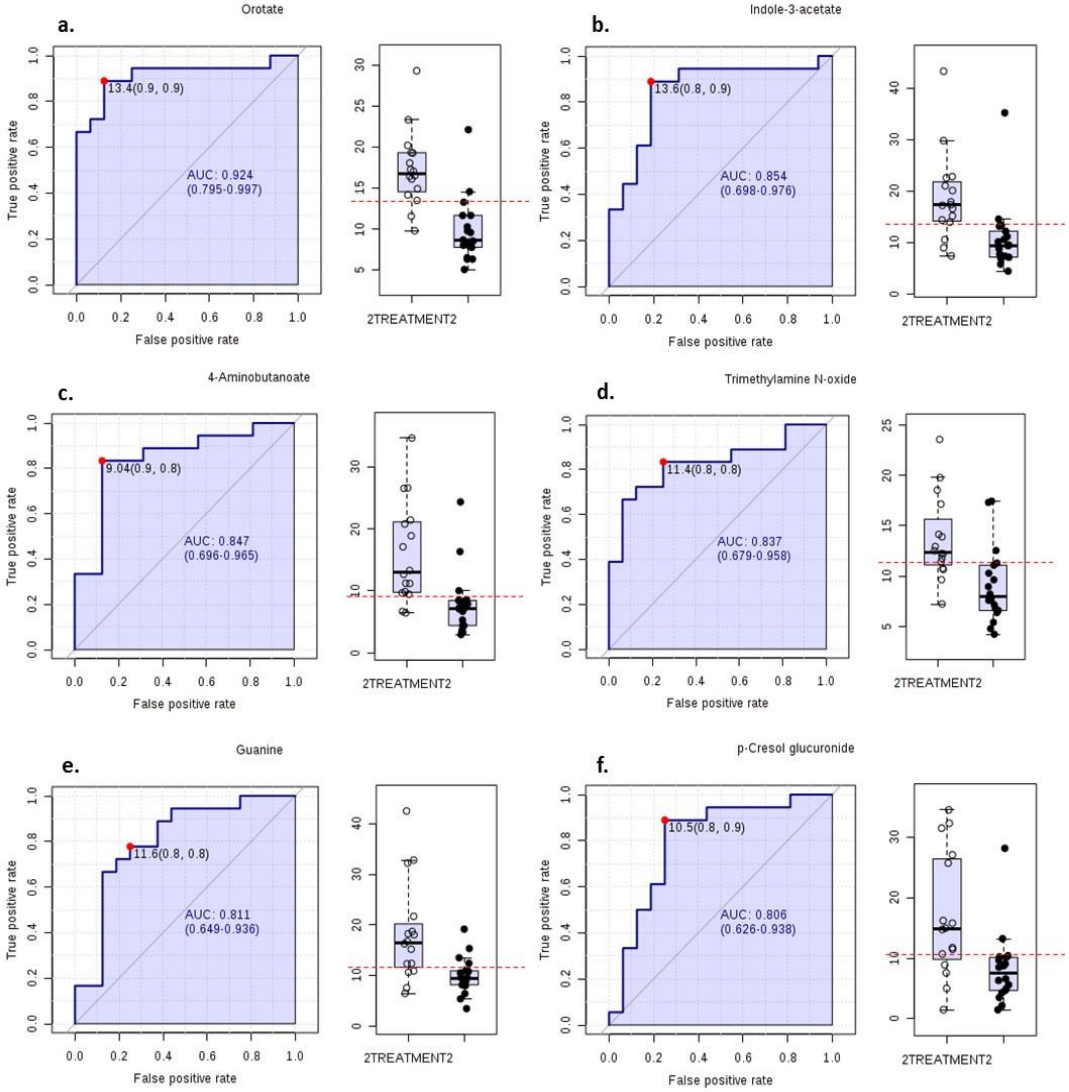


(8) Deoxyuridine (Mass:228.075; RT:8.60; ID:HMDB00012)

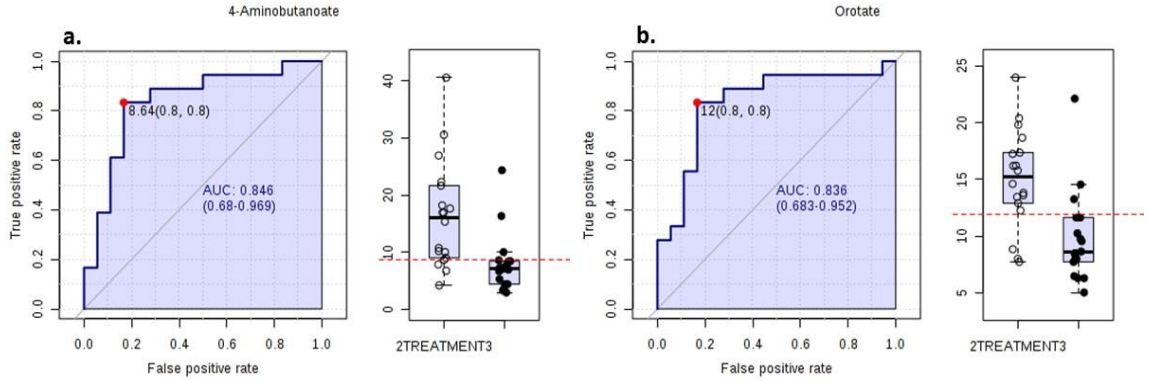


Appendix xiv. ROC Curve Analysis Plots for Urinary Markers with an AUC > 0.8

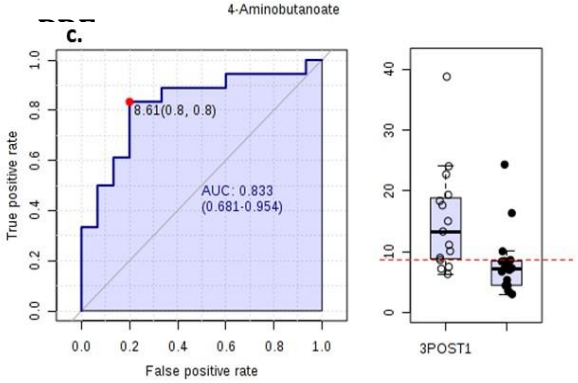
EPO2 *vs.* PRE



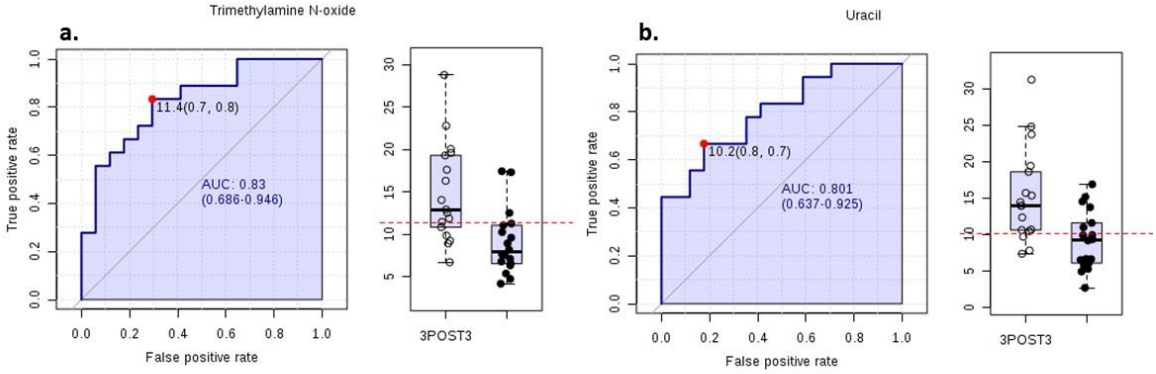
EPO3 *vs.* PRE



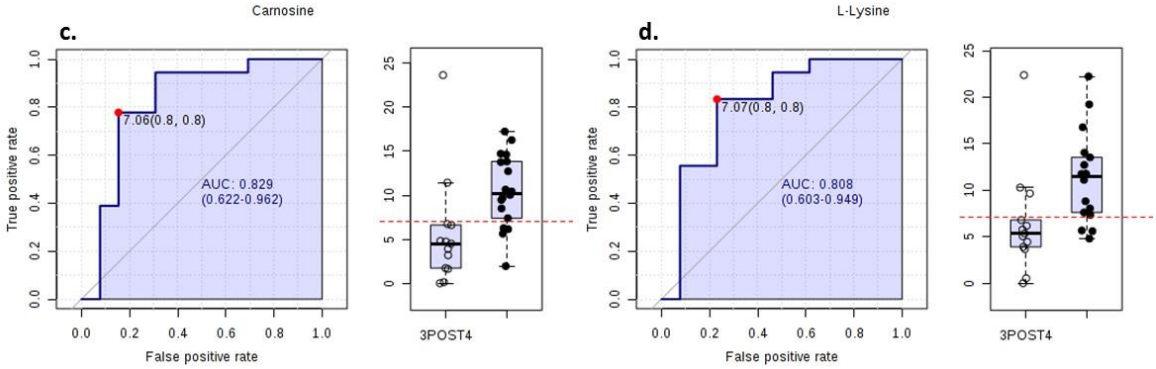
POST1 *vs.*



POST3 *vs.* PRE

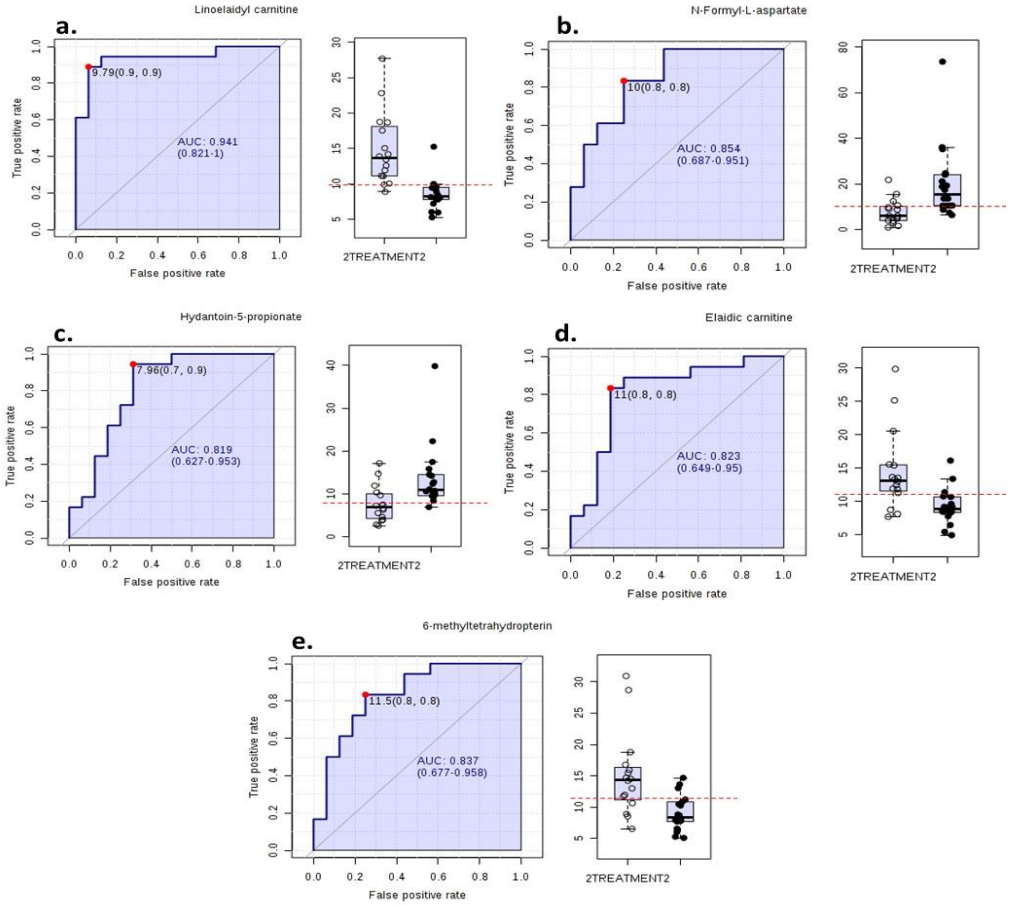


POST4 *vs.* PRE

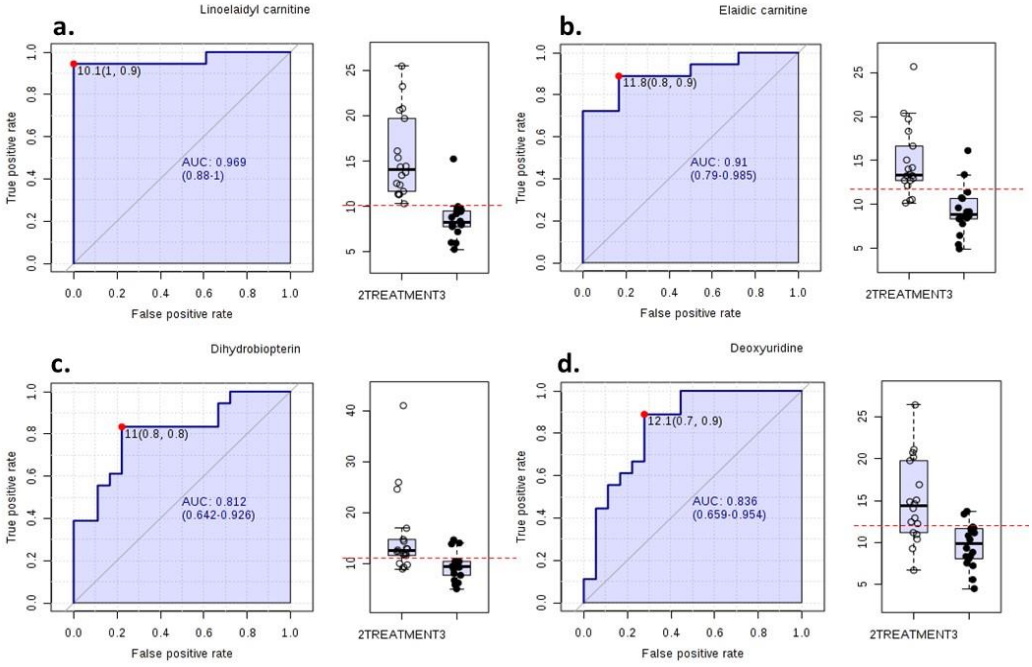


Appendix xv. ROC Curve Analysis Plots for Plasma Markers with an AUC > 0.8

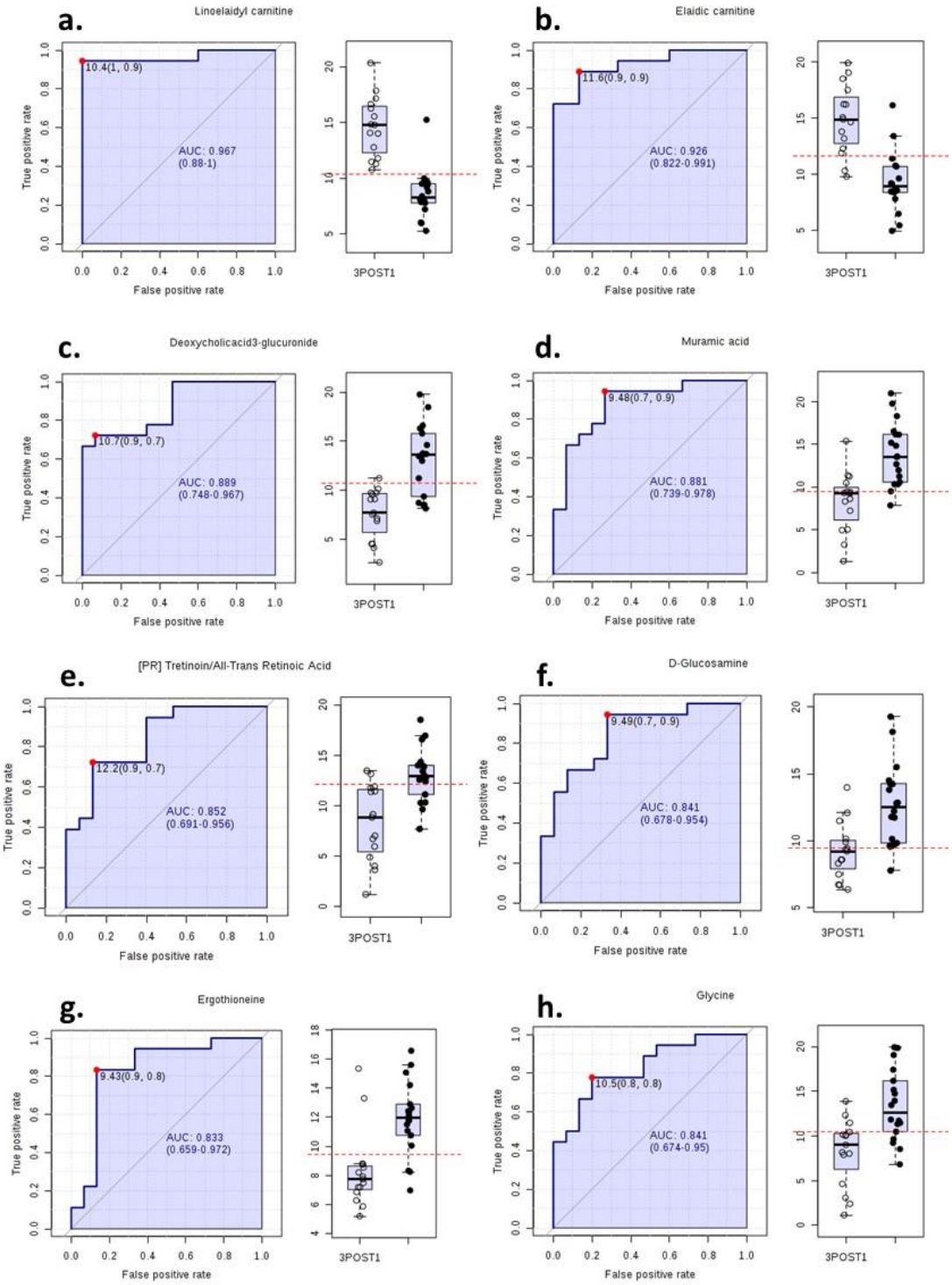
EPO2 *vs.* PRE

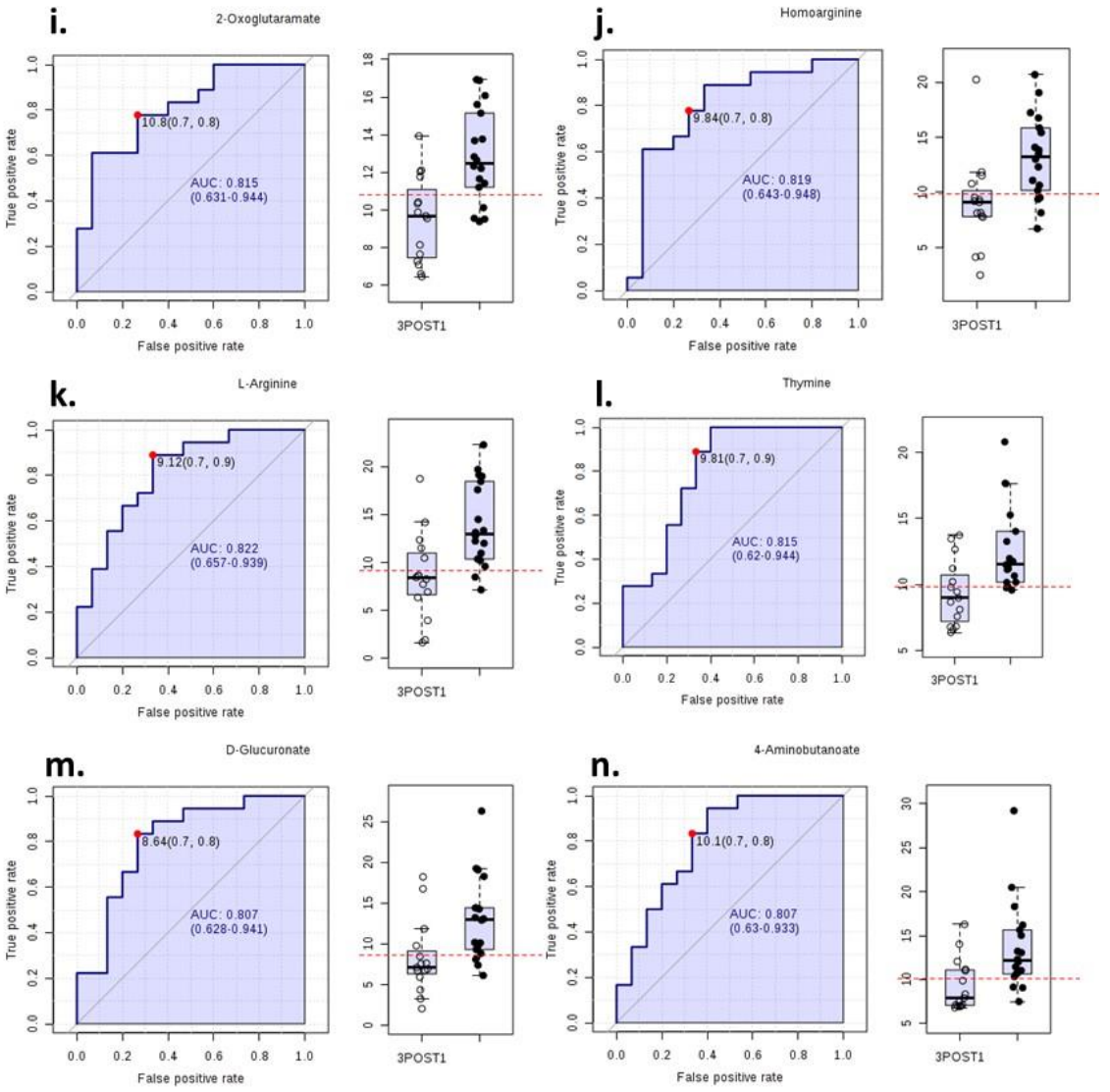


EPO3 *vs.* PRE

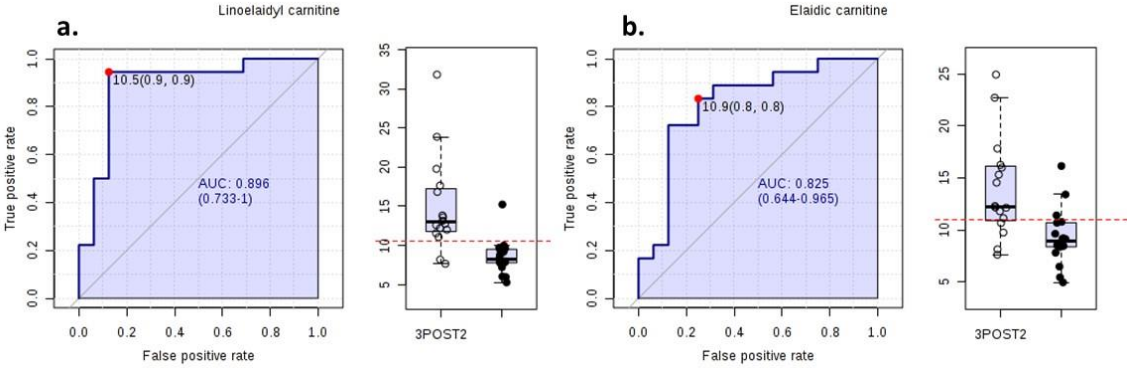


POST1 *vs.* PRE

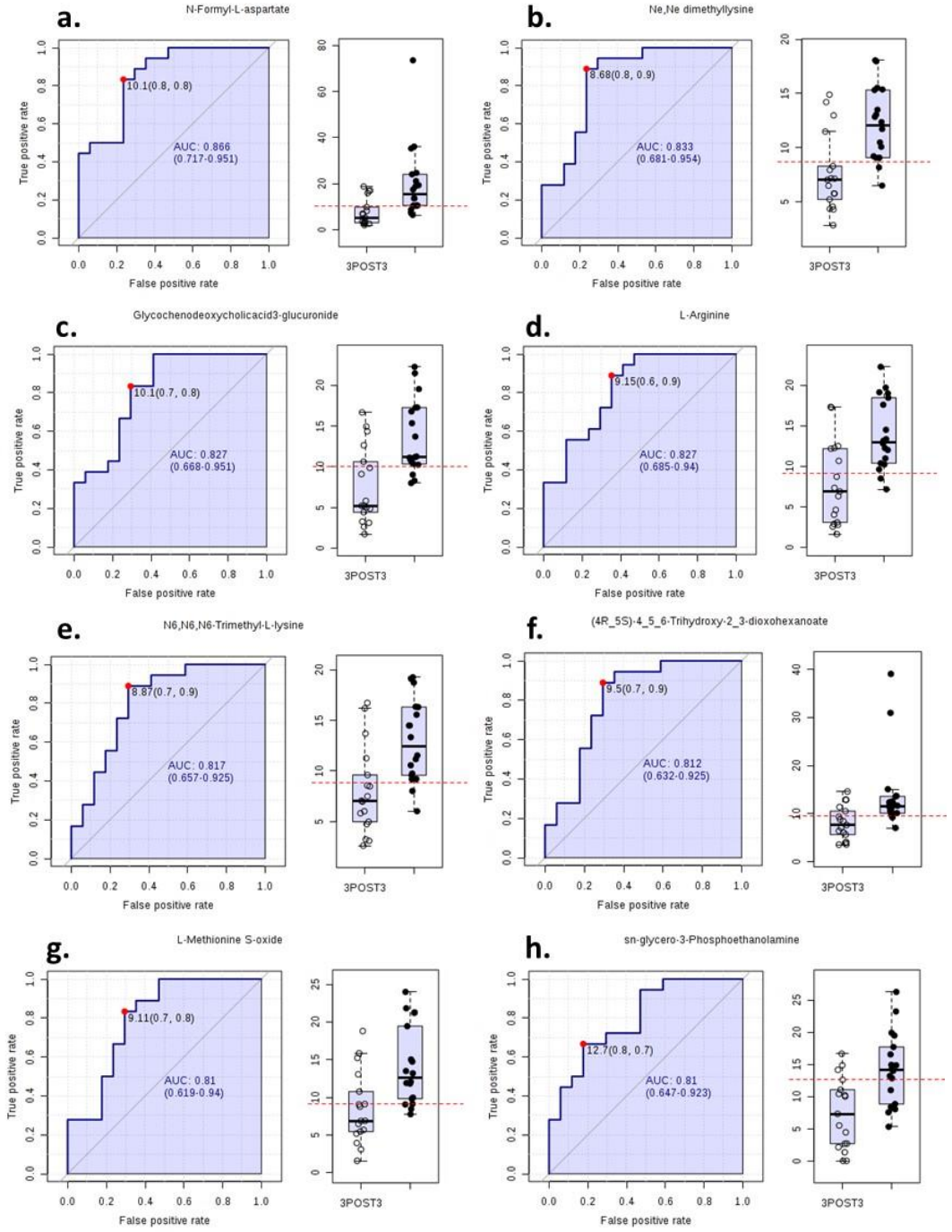

a.
Linoelaidyl carnitine
10.4(1, 0.9)
AUC: 0.967
(0.88-1)
b.
Elaidic carnitine
11.6(0.9, 0.9)
AUC: 0.926
(0.822-0.991)
c.
Deoxycholicacid3-glucuronide
10.7(0.9, 0.7)
AUC: 0.889
(0.748-0.967)
d.
Muramic acid
9.48(0.7, 0.9)
AUC: 0.881
(0.739-0.978)
e.
[PR] Tretinoin/All-Trans Retinoic Acid
12.2(0.9, 0.7)
AUC: 0.852
(0.691-0.956)
f.
D-Glucosamine
9.49(0.7, 0.9)
AUC: 0.841
(0.678-0.954)
g.
Ergothioneine
9.43(0.9, 0.8)
AUC: 0.833
(0.659-0.972)
h.
Glycine
10.5(0.8, 0.8)
AUC: 0.841
(0.674-0.95)
True positive rate
False positive rate
3POST1


i.
2-Oxoglutaramate
10.8(0.7, 0.8)
AUC: 0.815
(0.631-0.944)
True positive rate
False positive rate
3POST1
j.
Homoarginine
9.84(0.7, 0.8)
AUC: 0.819
(0.643-0.948)
k.
L-Arginine
9.12(0.7, 0.9)
AUC: 0.822
(0.657-0.939)
l.
Thymine
9.81(0.7, 0.9)
AUC: 0.815
(0.62-0.944)
m.
D-Glucuronate
8.64(0.7, 0.8)
AUC: 0.807
(0.628-0.941)
n.
4-Aminobutanoate
10.1(0.7, 0.8)
AUC: 0.807
(0.63-0.933)

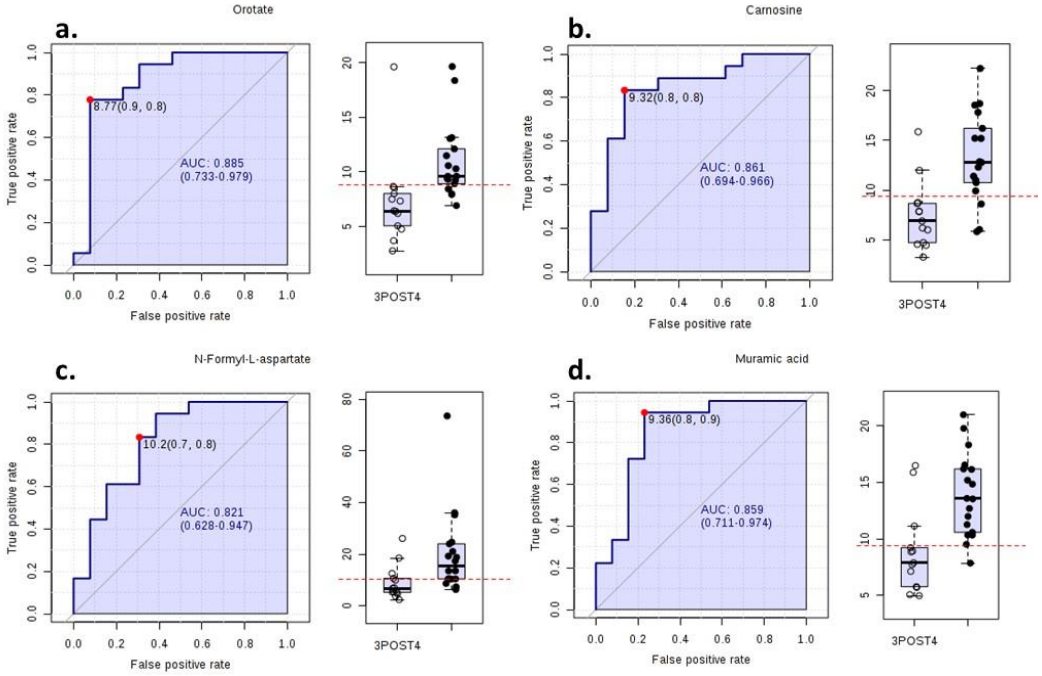
POST2 *vs.* PRE


a.
Linoelaidyl carnitine
10.5(0.9, 0.9)
AUC: 0.896
(0.733-1)
True positive rate
False positive rate
3POST2
b.
Elaidic carnitine
10.9(0.8, 0.8)
AUC: 0.825
(0.644-0.965)

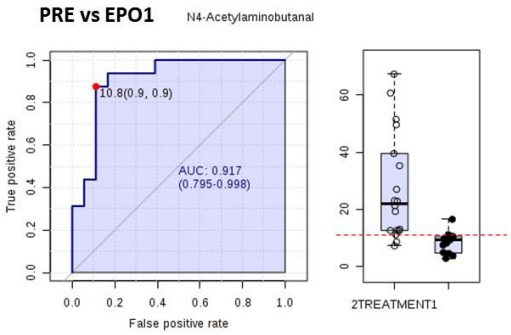
POST3 *vs.* PRE

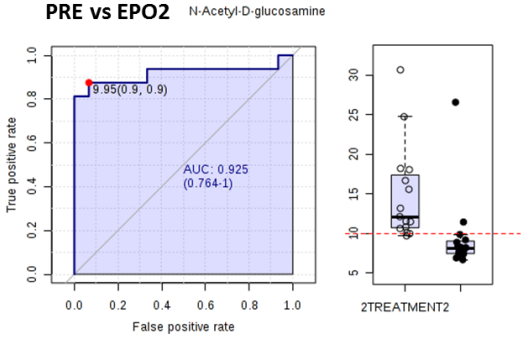

a.
N-Formyl-L-aspartate
10.1(0.8, 0.8)
AUC: 0.866
(0.717-0.951)
True positive rate
False positive rate
3POST3
b.
Ne,Ne dimethyllysine
8.68(0.8, 0.9)
AUC: 0.833
(0.681-0.954)
c.
Glycochenodeoxycholicacid3-glucuronide
10.1(0.7, 0.8)
AUC: 0.827
(0.668-0.951)
d.
L-Arginine
9.15(0.6, 0.9)
AUC: 0.827
(0.685-0.94)
e.
N6,N6,N6-Trimethyl-L-lysine
8.87(0.7, 0.9)
AUC: 0.817
(0.657-0.925)
f.
(4R_5S)-4_5_6-Trihydroxy-2_3-dioxohexanoate
9.5(0.7, 0.9)
AUC: 0.812
(0.632-0.925)
g.
L-Methionine S-oxide
9.11(0.7, 0.8)
AUC: 0.81
(0.619-0.94)
h.
sn-glycero-3-Phosphoethanolamine
12.7(0.8, 0.7)
AUC: 0.81
(0.647-0.923)

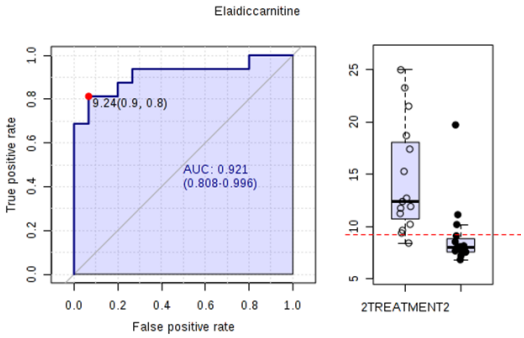
POST4 *vs.* PRE

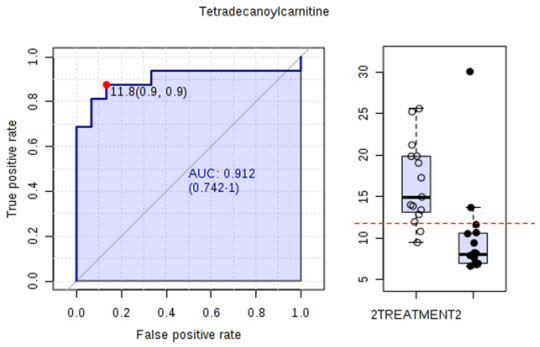

a.
Orotate
8.77(0.9, 0.8)
AUC: 0.885
(0.733-0.979)
True positive rate
False positive rate
3POST4
b.
Carnosine
9.32(0.8, 0.8)
AUC: 0.861
(0.694-0.966)
True positive rate
False positive rate
3POST4
c.
N-Formyl-L-aspartate
10.2(0.7, 0.8)
AUC: 0.821
(0.628-0.947)
True positive rate
False positive rate
3POST4
d.
Muramic acid
9.36(0.8, 0.9)
AUC: 0.859
(0.711-0.974)
True positive rate
False positive rate
3POST4

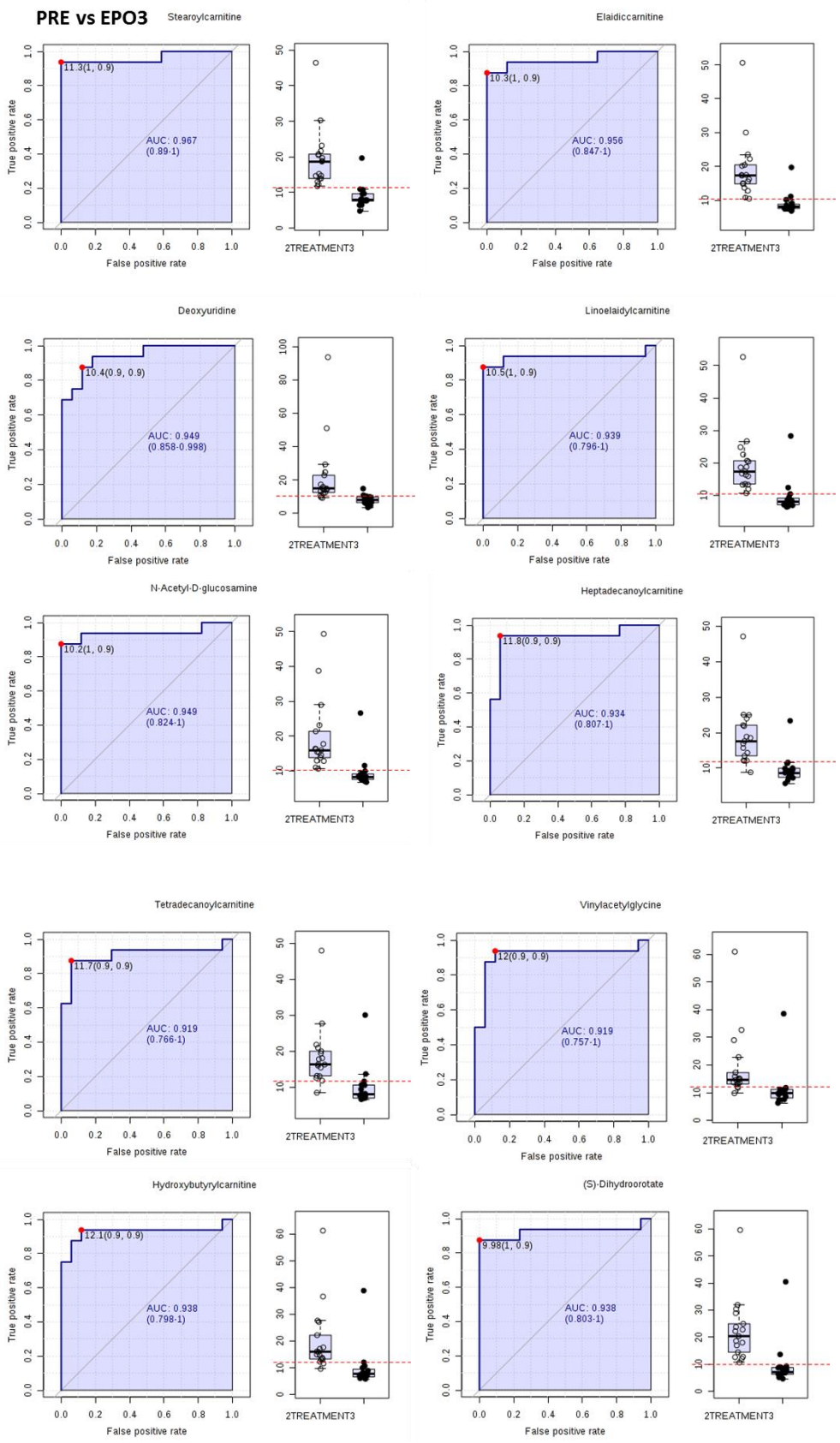
Appendix xvi. ROC Curve Analysis Plots for RBC Markers from Centrifuged Blood Residue with an AUC > 0.9

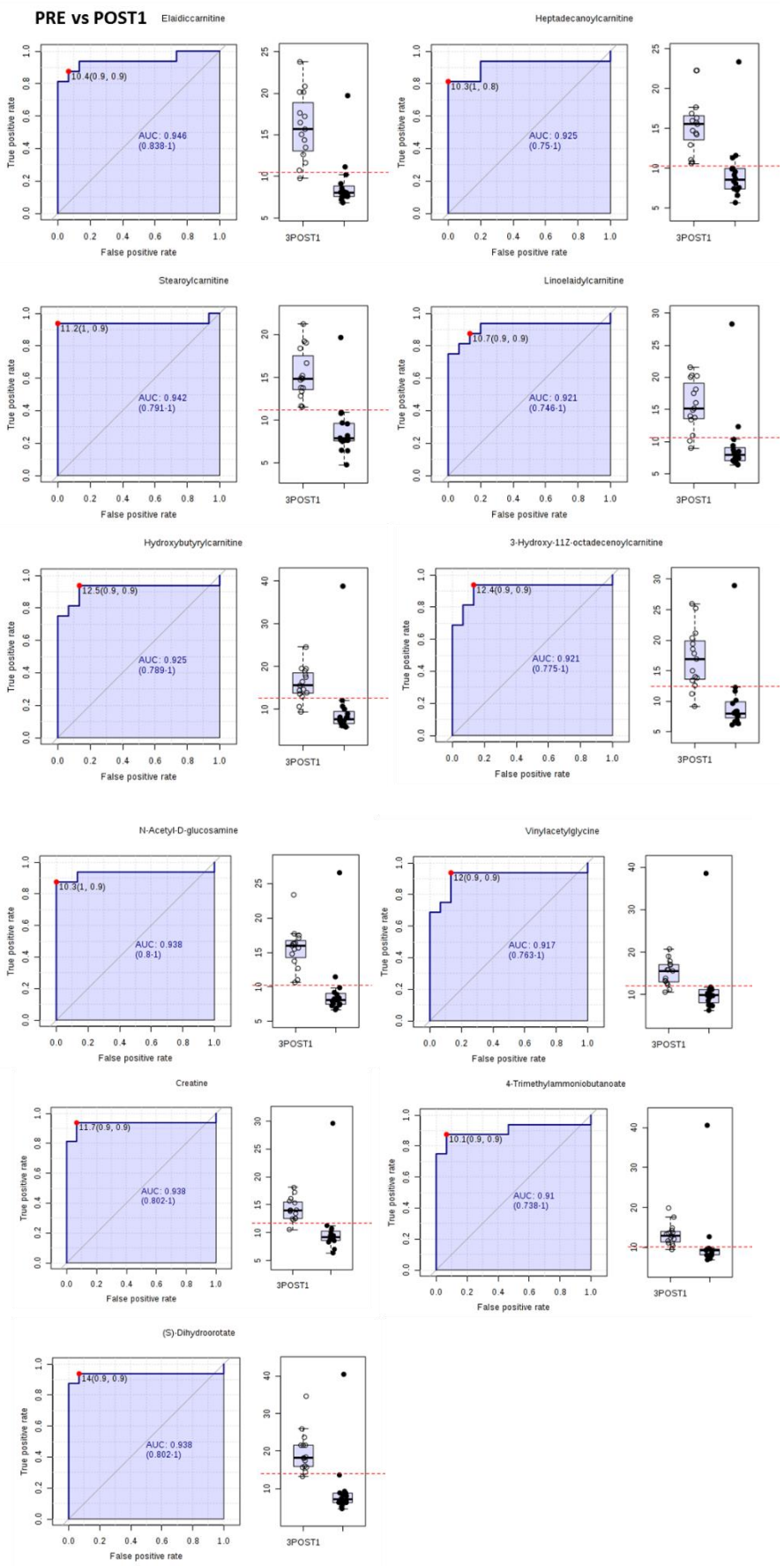


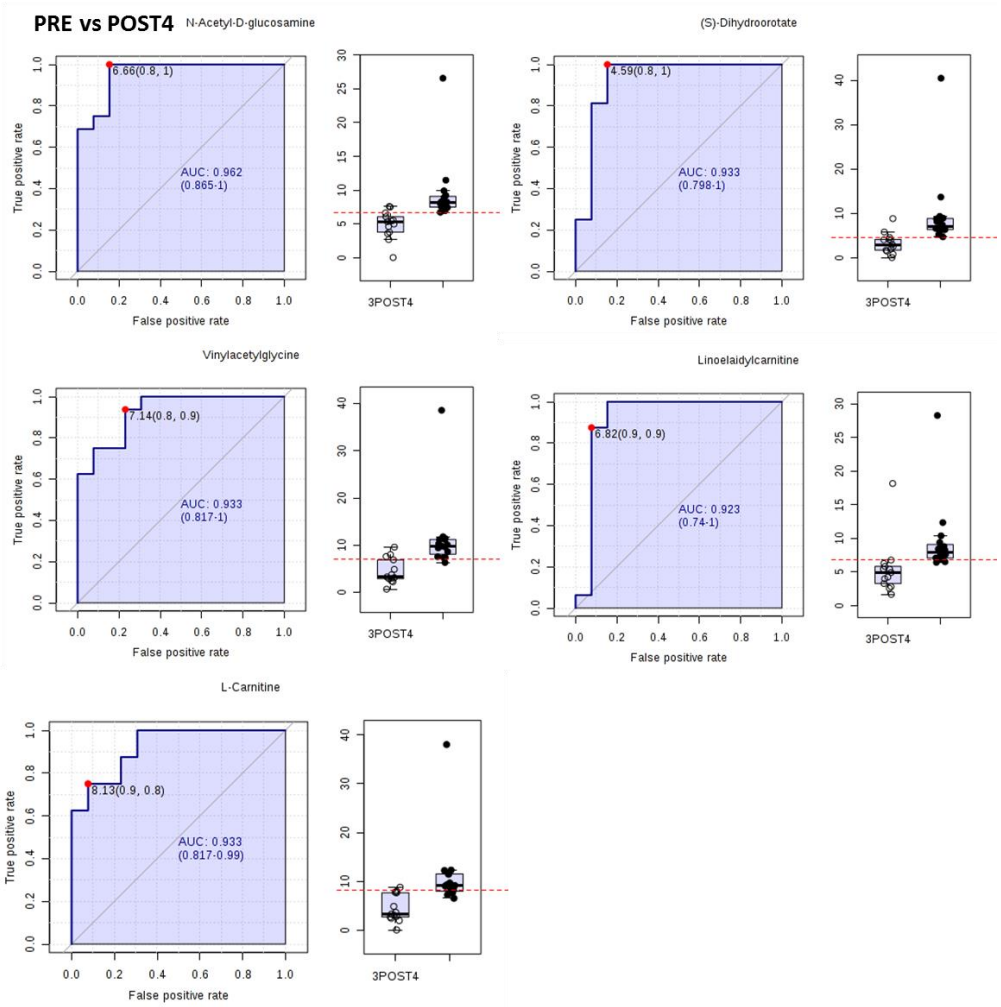







PRE vs EPO3
Stearoylcarnitine
11.3(1, 0.9)
AUC: 0.967
(0.89-1)
Elaidiccarnitine
10.3(1, 0.9)
AUC: 0.956
(0.847-1)
Deoxyuridine
10.4(0.9, 0.9)
AUC: 0.949
(0.858-0.998)
Linoelaidylcarnitine
10.5(1, 0.9)
AUC: 0.939
(0.796-1)
N-Acetyl-D-glucosamine
10.2(1, 0.9)
AUC: 0.949
(0.824-1)
Heptadecanoylcarnitine
11.8(0.9, 0.9)
AUC: 0.934
(0.807-1)
Tetradecanoylcarnitine
11.7(0.9, 0.9)
AUC: 0.919
(0.766-1)
Vinylacetylglycine
12(0.9, 0.9)
AUC: 0.919
(0.757-1)
Hydroxybutyrylcarnitine
12.1(0.9, 0.9)
AUC: 0.938
(0.798-1)
(S)-Dihydroorotate
9.98(1, 0.9)
AUC: 0.938
(0.803-1)
True positive rate
False positive rate
2TREATMENT3


PRE vs POST1
Elaidiccarnitine
10.4(0.9, 0.9)
AUC: 0.946
(0.838-1)
Heptadecanoylcarnitine
10.3(1, 0.8)
AUC: 0.925
(0.75-1)
Stearoylcarnitine
11.2(1, 0.9)
AUC: 0.942
(0.791-1)
Linoelaidylcarnitine
10.7(0.9, 0.9)
AUC: 0.921
(0.746-1)
Hydroxybutyrylcarnitine
12.5(0.9, 0.9)
AUC: 0.925
(0.789-1)
3-Hydroxy-11Z-octadecenoylcarnitine
12.4(0.9, 0.9)
AUC: 0.921
(0.775-1)
N-Acetyl-D-glucosamine
10.3(1, 0.9)
AUC: 0.938
(0.8-1)
Vinylacetylglycine
12(0.9, 0.9)
AUC: 0.917
(0.763-1)
Creatine
11.7(0.9, 0.9)
AUC: 0.938
(0.802-1)
4-Trimethylammoniobutanoate
10.1(0.9, 0.9)
AUC: 0.91
(0.738-1)
(S)-Dihydroorotate
14(0.9, 0.9)
AUC: 0.938
(0.802-1)
True positive rate
False positive rate
3POST1


PRE vs POST4
N-Acetyl-D-glucosamine
6.66(0.8, 1)
AUC: 0.962
(0.865-1)
True positive rate
False positive rate
3POST4
(S)-Dihydroorotate
4.59(0.8, 1)
AUC: 0.933
(0.798-1)
Vinylacetylglycine
7.14(0.8, 0.9)
AUC: 0.933
(0.817-1)
Linoelaidylcarnitine
6.82(0.9, 0.9)
AUC: 0.923
(0.74-1)
L-Carnitine
8.13(0.9, 0.8)
AUC: 0.933
(0.817-0.99)