The development of a new model to measure plasma-, red cell- and blood volume and its applications in refining the Athlete Biological Passport and improving volume management in hospitals

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This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Human Sciences (Exercise and Sport Science)
2018
Declaration

I, Louisa M. Lobigs, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material that has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

The research involving human data reported in this thesis was approved by the University of Western Australia Human Research Ethics Committee (RA/4/8714), the Shafallah Medical Genetics Center Ethics Committee (2013-002), and the Australian Institute of Sport Ethics Committee (20151001).

Written consent has been received and archived for the research involving participant data reported in this thesis.

This thesis contains published work, which has been co-authored.

Louisa M. Lobigs

PhD Candidate
Statement of contributions

This thesis contains published work, all of which has been co-authored. The bibliographical details of the work are presented on the title page for each paper. The work involved in designing the thesis outline was performed by Louisa M. Lobigs (candidate). The experimental design was planned and developed by the candidate, in consultation with Dr Yorck Olaf Schumacher and Associate Professor Peter Peeling (the candidate’s academic supervisors). Statistical support was provided by Dr Pierre-Edouard Sottas and Dr Ken Sharpe (co-authors). All participant recruitment and management was carried out by the candidate. The candidate drafted the original thesis chapters as well as papers arising from this thesis that have been published or prepared for future publication. Dr Yorck Olaf Schumacher, Associate Professor Peter Peeling and Professor Brian Dawson provided guidance on data collection, data analysis and all drafts associated with the thesis until the examinable version was finalised.

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Dr Yorck Olaf Schumacher
External Supervisor
Executive summary

The haematological module of the Athlete’s Biological Passport (ABP) has advanced anti-doping strategies by providing an indirect approach to identify blood manipulations.\(^1\) The ABP adaptive model monitors specific markers of erythropoiesis longitudinally to develop individualised reference intervals. However, the ABP relies on a number of concentration-based markers. Consequently, variations in plasma volume can confound the results of volumetric ABP markers. Plasma volume fluctuations are influenced by environmental factors such as altitude,\(^2\) heat and exercise,\(^3,4\) posture,\(^5\) and fluid intake,\(^6\) or, can be artificially expanded (illicitly) with compounds such as Hydroxyethyl Starch (HES), Dextran, Desmopressin, Glycerol or Mannitol.\(^7\) Volume fluctuations have previously been shown to influence the results of an individual’s ABP profile.\(^6\) Currently, the biological variance associated with plasma volume shifts cannot be quantified within the ABP paradigm. Therefore, the primary aim of this thesis was to develop a blood test, capable of predicting plasma volume, with the capacity to correct ABP profiles for volume shifts. This was achieved by an initial review of the confounding effects of natural plasma volume shifts (at altitude) on ABP biomarkers followed by three original investigations.

The preliminary meta-analysis (Study 1) of this thesis describes the influence of altitude dose (in Kilometre Hours; km.hr = altitude (m)/1000 x hr)\(^8\) on common biomarkers of erythropoiesis, Haemoglobin Concentration ([Hb]), Reticulocyte Percentage (RET\%) and the OFF-score.\(^9\) From the raw data of 17 altitude studies, separate linear mixed effects analysis was performed. Within the first two days at altitude [Hb] increased, being a rapid volumetric response mechanism in reaction to reduced arterial oxygen content;\(^10\) however, this response was only observed at natural altitude. Subsequent delta [Hb] values increased with altitude dose, reaching a plateau of 0.94 g/dL [95% CI (0.69 to 1.2)] at ~1000 km.hr. The RET\% and OFF-score were the first to identify an erythrocyte response, with respective increases and decreases observed within 100 to 200 km.hr. The response to altitude was complex and a wide range of individual responses was observed, influenced primarily by altitude dose and baseline values (p < 0.05). The observed influence of the initial
plasma volume contraction upon arrival at high altitude identified a need for a volume correction to be established for concentration-based ABP biomarkers.

Study 2 focused on the development of a blood test capable of estimating vascular volumes from the known variability of common biomarkers. A novel approach was applied to identifying an optimal panel of ‘volume descriptive’ biomarkers. During a 6-month investigation 33 healthy males provided a monthly venous sample (serum and whole blood) and performed the CO-rebreathing test (gold-standard control measure for plasma volume), described by Schimdt and Prommer. The variability of 45 serum and whole blood biomarkers (selected based on their propensity to present with low biological variation over time, i.e. CV < 10%) were investigated and matched to the observed plasma volume variation (measured from the CO-rebreathing test). Using Bayesian modelling and multivariate analysis, an optimal set of 8 and 15 biomarkers was identified, capable of describing 68 and 69% of plasma volume variance, respectively. The final multi-parametric model contained a weighting function to allow for isolated abnormalities in single biomarkers. Following on from these findings, Studies 3 and 4 aimed to validate the plasma volume model within the ABP.

The first validation study (Study 3) was performed in parallel to Study 2. At month 6, on completion of the CO-rebreathing test and venous sample collection (described above), 33 male participants performed a 30 min maximum stress test on a cycle ergometer, designed to promote a maximal plasma volume shift. Venous samples were collected immediately post- and 1 hr post-exercise. The [Hb] and OFF-score results from the 6 months of testing and additional exercise challenge results were entered into the adaptive model of the ABP to produce individual profiles. The plasma volume correction (using the set of 8 plasma volume biomarkers) was applied to the ABP adaptive model and a comparison was made of the number of atypical passport findings (ATPFs) both with and without the correction. The frequency of ATPFs was considerably reduced post-exercise with the inclusion of the plasma volume correction. Importantly, the number of ATPFs recorded without exercise did not change with or without the plasma volume correction. Therefore, the sensitivity of the ABP is improved with the plasma volume correction with no impact on the specificity (frequency of false positives).
The final validation study (Study 4) applied altitude as a stimulus for a natural plasma volume shift. Here, 34 endurance athletes (11 females, 9 males) underwent a 21-day Live High: Train Low altitude training camp (14 hr/day at 3000m). Venous samples were collected twice before altitude, at days 3, 8 and 15 at altitude and 1, 7, 21 and 42 days post-altitude. Individual [Hb] and OFF-score values were entered into the ABP adaptive model with and without the plasma volume correction (again, the set of 8 plasma volume biomarkers was applied). With the plasma volume correction the number of [Hb] ATPFs was reduced. However, the OFF-score ATPFs increased with the plasma volume correction, most likely the result of more specific reference limit predictions combined with the altitude-induced increase in red cell production. An augmented level of biological variability was observed in some participants, resulting in a low confidence in the plasma volume prediction (measured by the weighting function of the model). This is a principal component of the plasma volume correction, to ensure the model provides accurate volume predictions and any irregular plasma volume estimations are flagged with a low confidence calculation.

The cumulative results from the four investigations described above were summarised in Study 5 (concluding chapter) in the form of a review. Here, the possible application of the plasma volume correction not only in anti-doping, but also in clinical practice, was highlighted. This final review summarises the findings of this thesis as follows:

- Plasma volume is a major confounding factor within the ABP.
- The development of a simple test to estimate absolute plasma volume has the potential to refine the sensitivity of the concentration-based markers of the ABP by removing the variance component associated with plasma volume fluctuations.
- The novel plasma volume marker can be applied to clinical practice, whereby enhanced knowledge of patient’s vascular volumes has the potential to improve health outcomes.

Further research is required, in particular the development of standardised protocols for the collection, handling, analysis and storage of the novel ‘volume descriptive’ biomarkers, if the system is to be introduced into anti-doping practices in the future. Nevertheless the results presented within this thesis provide a promising advancement to our ability to monitor vascular volumes with ease and accuracy.
References:


Acknowledgements

This thesis has taken me to many corners of the world and allowed me to pursue my passions, cycling and research, in unison. I will begin by thanking the PhD itself for taking me out of my comfort zone and opening my mind. This research was supported by an Australian Government Research Training Program (RTP) scholarship.

This work began in the Middle East at Aspetar Hospital in Doha, Qatar. There are many people from Aspetar who need to be thanked. First and foremost my supervisor, Olaf, you have always encouraged me to pursue my passions, and I wish to express my utmost gratitude for your support. Second, thank you to Ryan, Jock, Julien, and Matt. Fighting the heat, dust, and endless flat roads were always enjoyable when I had your wheels to follow. Finally, the legends of room 214, Arnlaug, Arnhild, Tone, Andreas, Nicol, Nathan, Tessa, Kirsty, Angela, Anders, Hermien, Guus and the whole Aspetar gang, you were a joy to work with in those initial years.

Next stop, Lausanne in Switzerland. Thank you Pierre-Edouard for teaching me the ropes of the Biological Passport and being such an integral part of this thesis. The shores of Lac Lemond were one of the most stunning places in the world to analyse data.

The next stage of the journey took me back home to Australia. Thank you to my Aussie supervisors, Pete and Brian, at UWA for taking me on as a long-distance student. Your ongoing support and encouragement to complete this thesis whilst also traveling with cycling and basing myself +3,400 km away has been incredible. In Canberra, thank you Chris Gore and the AIS physiology department for giving me a supportive environment to work in during my second year. And of course my family, although I barely had the time to visit, you have always been a phone call away and always reminded me of my Suomen Sissu when life felt tough. Thank you to my second family, the Holden Women’s Cycling team, Graeme, Jessie, Julien, Miranda, Dash, Kimbers, Mamma K, GB, Stretch, there are many, many more; Thank you for your consistent support and belief in me. You have all been my outlet from the PhD and have made my own cycling journey so special. And finally, Ryan, no words will do justice for your constant support. I love you with my heart and soul.
In my third year I was back to Europe with the support of the Amy Gillett Foundation (AGF). A big thank you to the AGF for the opportunity to race in Europe with the Australian National Team and for supporting my joint academic and sporting ambitions. In 2016, I wrote and raced my way through Italy, France, Czech Republic, Belgium, Germany, Sweden, Norway, and Holland. Thank you Martin, Leanne, Ray, Jess, Jenelle, Dash, and Kimbers (again) for keeping the emergency chocolate and trashy tunes close at hand to get me through those demanding 18 hour van drives and 2000 km of Spring Classics racing on top of the thesis writing!

I will finish with a big thank you to good city planning; to all the useful power point locations, ergonomic study desks, and free wifi! Thank you to the numerous libraries, coffee shops, airports, railway stations, and community spaces for supplying the essentials for studying on the road. And a special thank you to those spaces that also came with a beautiful view.

The journey has been extraordinary. The people, the places and the experience will always be close to my heart.

“No man can live this life and emerge unchanged. He will carry, however faint, the imprint of the desert...”

- Wilfred Thesiger

Now, onwards to the next expedition.
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<th>Description</th>
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<tbody>
<tr>
<td>ABP</td>
<td>Athlete’s Biological Passport</td>
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<td>ABPS</td>
<td>Abnormal Blood Profile Score</td>
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<td>AIC</td>
<td>Akaike’s Information Criterion</td>
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<td>ALB</td>
<td>Albumin</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>ALT</td>
<td>Alanine Aminotransferase</td>
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<td>ATPF</td>
<td>Atypical Passport Finding</td>
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<td>BASO</td>
<td>Basophils</td>
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<td>Ca</td>
<td>Calcium</td>
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<td>CAS</td>
<td>Court of Arbitration of Sport</td>
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<tr>
<td>CERA</td>
<td>Continuous Erythropoietin Receptor Activator</td>
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<tr>
<td>CHOL</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride</td>
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<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
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<tr>
<td>$^{51}$Cr</td>
<td>Radioactive Chromium</td>
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<td>CRE</td>
<td>Creatinine</td>
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<td>ESA</td>
<td>Erythropoiesis Stimulating Agent</td>
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<td>Free Thyroxine</td>
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<td>GGT</td>
<td>Gamma-Glutamyl Transferase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Hb</td>
<td>Haemoglobin</td>
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<td>Haemoglobin Mass</td>
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<td>Percent Carboxyhaemoglobin</td>
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<td>Haematocrit</td>
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<td>HDL</td>
<td>High-density Lipoprotein</td>
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<td>Hydroxyethyl Starch</td>
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<td>hGH</td>
<td>Human Growth Hormone</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-Inducible Factor</td>
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<tr>
<td>$^{131}$I or $^{125}$I</td>
<td>Radioactive Iodine</td>
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<td>IGF-1</td>
<td>Insulin-Growth Factor 1</td>
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<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>Km.hr</td>
<td>Kilometre Hours</td>
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<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
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<tr>
<td>LFR</td>
<td>Low Fluorescence Reticulocytes</td>
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<tr>
<td>LHTH</td>
<td>Live High: Train High</td>
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<td>LHTL</td>
<td>Live High: Train Low</td>
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<tr>
<td>LYMPH</td>
<td>Lymphocytes</td>
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<tr>
<td>MCH</td>
<td>Mean Corpuscular Haemoglobin</td>
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<td>MCHC</td>
<td>Mean Corpuscular Haemoglobin Concentration</td>
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<tr>
<td>MCV</td>
<td>Mean Corpuscular Volume</td>
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<tr>
<td>MFR</td>
<td>Medium Fluorescence Reticulocytes</td>
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<tr>
<td>Mg</td>
<td>Magnesium</td>
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<td>MONO</td>
<td>Monocytes</td>
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MPV  Mean Platelet Volume
Na   Sodium
NEUT Neutrophils
PCA Principal Component Analysis
PCT Plateletcrit
P_LCR Platelets Large Cell Ratio
PLT Platelets
proANP Pro-Atrial Natriuretic Peptide
PDW Platelet Distribution Width
RBC Red Blood Cell Count
RBC_Hb Red Blood Cells Haemoglobin Content
RDW.CV Red Cell Distribution Width Coefficient of Variation
RDW_SD Red Cell Distribution Width Standard Deviation
RET_Hb Reticulocytes Haemoglobin Content
RET# Reticulocyte Number
RET% Reticulocyte Percentage
RPE Rating of Perceived Exertion
rHuEPO Recombinant Human Erythropoietin
sTfR Soluble Transferrin Receptor
sqrt(RET%) Square-root Transformed Reticulocyte Percentage
T3 Triiodothyronine
T4 Thyroxine
Tfn Transferrin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
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<tr>
<td>TP</td>
<td>Total Protein</td>
</tr>
<tr>
<td>UCI</td>
<td>Union Cycliste Internationale</td>
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<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cells</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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Publications arising from this thesis

This thesis contains published work that has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below. A statement for each publication that clarifies the contribution of the student to the work is also provided.


Student’s Contribution: 90%


Student’s Contribution: 90%


Student’s Contribution: 90%


Student’s Contribution: 90%
Peer-reviewed conference proceedings


*A step towards removing plasma volume variance from the athlete’s biological passport: the use of biomarkers to describe vascular volumes from a simple blood test.*

Invited speaker at the annual symposium of the Japanese Society of Physical Fitness and Sports Medicine, Ehime, Japan.


*A step towards removing plasma volume variance from the athlete’s biological passport: the use of biomarkers to describe vascular volumes from a simple blood test.*

Oral presentation at the annual congress of the European College of Sports Science, Essen, Germany. Awarded Young Investigators Award 3rd place.


*Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data*

Oral presentation at the annual Canberra Health Annual Research Meeting, Canberra, Australia.
Introduction
Chapter 1: Introduction

1.0 Background

The immense pressures imposed upon athletes to win competitions, overcome an injury rapidly or advance by a level within one’s sport, can tempt some individuals to supplement training with illegal doping practices. These practices can enhance an athlete’s biochemistry and physiology beyond natural levels and provide an unfair performance advantage with the risk of possible adverse health outcomes. To counteract illegal doping, the Athlete’s Biological Passport (ABP) was introduced in 2008 by the World Anti-Doping agency (WADA) in order to indirectly detect doping by identifying unusual patterns in key biomarkers within an athlete’s haematology, steroid or endocrinal profiles. The haematological module of the ABP detects illicit blood manipulation through the longitudinal analysis of key markers of erythropoiesis. Bayesian statistics are utilised to develop individualised reference ranges for each marker, producing a unique haematological profile, where each athlete is his/her own reference.[1] Since 2008, the ABP has become an integral anti-doping tool, and has been responsible for the direct sanctioning of more than 100 athletes.[2] Furthermore, use of the ABP has seen the number of athletes found positive for erythropoiesis stimulating agents’ increase by ~400% across multiple sports (internal communications). Within this thesis, reference to the ABP specifically refers to the haematological module of the biological passport.

The ability to differentiate between natural biological variation and variation that stems from blood manipulation is central to the success of the ABP. Each haematological variable has inherent between- and within-individual variation. Between-individual variation is a function of age, sex, genetics and analytical bias. In contrast, within-individual variation is associated with sample analysis and, for the volumetric ABP markers, plasma volume shifts.[3] The ABP paradigm makes adjustments for heterogeneous factors (age, sex and ethnicity), and uses an athlete’s internal reference range for each marker, thereby removing the majority of between-individual variance components.[1] The adoption of standardised protocols for sample collection, transport, analysis and storage further reduces the variance associated with analytical bias (incorporated within inter-individual variance).[4-6]

Despite the reduced variability achieved with the use of stable haematological markers and carefully controlled measurement standards, numerous environmental factors have the potential to influence markers of the ABP and consequently confound a passport profile. For example, a number of markers within the ABP are calculated as a concentration in blood,
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such as Haemoglobin Concentration ([Hb]), and are thus influenced by fluctuations in plasma volume. As a result, a dehydrated athlete can present with elevated [Hb], and an adverse passport finding, as the direct result of a plasma volume contraction.\textsuperscript{[7,8]} Plasma volume fluctuations accounts for a major proportion of uncontrolled variability associated with volumetric ABP markers. It is thus essential that naturally occurring confounders are well documented, and that our knowledge of such haematological fluctuations is expanded.

1.1 Statement of the problem

The influence of plasma volume fluctuations on concentration-based markers within the ABP paradigm is of immediate concern. Plasma volume can be influenced by numerous environmental factors such as altitude,\textsuperscript{[9]} heat and exercise,\textsuperscript{[7,10]} posture,\textsuperscript{[5]} and fluid intake.\textsuperscript{[8]} Furthermore, plasma volume can be artificially expanded (illicitly) with compounds such as Hydroxyethyl Starch (HES), dextran, desmopressin, glycerol or mannitol.\textsuperscript{[11]} Such fluctuations of vascular volumes have previously been shown to influence the results of an individual’s ABP profile.\textsuperscript{[8]} Plasma volume fluctuations represent the most substantial source of biological variance within the volumetric markers of the ABP. It is thus imperative that new analytical approaches are established to circumvent this ‘plasma volume problem’ within the ABP.

1.2 Thesis outline

This thesis investigates the variance associated with natural plasma volume fluctuation in volumetric markers of the ABP. Based on findings that a number of chemistry markers have the capacity to describe plasma volume;\textsuperscript{[12]} a novel approach to reducing the influence of plasma volume fluctuations within the ABP is proposed and evaluated.

1.2.1 Preliminary investigation

The preliminary investigation explores the fluctuations observed in biomarkers of erythropoiesis associated with altitude training. Athletes who train in hypoxic environments often experience an acute plasma volume contraction upon arrival at altitude,\textsuperscript{[9]} followed by
increased erythropoiesis stimulated by the low oxygen environment.\textsuperscript{[13]} The initial plasma volume contraction may influence the volumetric haematological markers ([Hb] and the OFF-score (OFF-score = [Hb] (g/dL) \times 10^{-60} \times \sqrt{\text{Reticulocyte Percentage (RET%)}}) and, in turn, the variance of the parameters if measured during this period.\textsuperscript{[9]} These haematological changes, however, have not yet been systematically quantified. Therefore, the initial investigation explores the peripheral blood changes observed during- and post-altitude training in an attempt to understand the confounding effect of a natural plasma volume shift on volumetric markers. The results of this investigation highlight the need to account for plasma volume shifts within the current ABP paradigm in order to improve the systems specificity.

\subsection*{1.2.2 Principal investigation}

The principal investigation develops and evaluates a new model to estimate vascular volumes. The model uses a panel of common ‘volume descriptive’ biomarkers. These markers are observed longitudinally and their individual variance estimations are compared to the variation in plasma volume, which is estimated with the CO-rebreathing method (the current gold-standard tracer technique to calculate vascular volumes).\textsuperscript{[14]} The primary aim is to identify an optimal panel of biomarkers capable of describing plasma volume. The proposed approach, which only requires a blood sample, has the potential to trump the more complex tracer techniques, such as the CO-rebreathing method, which are unsuitable in an anti-doping testing scenario. The identified plasma volume marker is then applied to the ABP paradigm to correct the predicted reference intervals for plasma volume variations during: a) an exercise-induced and, b) an altitude-induced plasma volume shift. It is hypothesised that the inclusion of this novel plasma volume correction within the current ABP paradigm will improve the specificity of the calculated reference ranges by reducing the confounding effects of plasma volume fluctuations on the ABP’s volumetric markers.

\subsection*{1.2.3 Research aims}

The primary aims of this thesis were to:

1) Define the fluctuations associated with peripheral blood markers during a sojourn at altitude.
2) Specify the influence of the natural plasma volume contraction observed during the initial days at altitude on volumetric ABP markers.

3) Identify a panel of ‘volume descriptive’ biomarkers capable of describing absolute vascular volumes.

4) Apply the newly developed marker for vascular volumes to the haematological module of the ABP with the aim to reduce the influence of plasma volume shifts.

1.3 Study aims and hypotheses

Outlined below are the specific aims and hypotheses of each study within this thesis:

Study 1 (Chapter 3) - The athlete’s response to hypoxia: A meta-analysis of the influence of altitude exposure on key biomarkers of erythropoiesis

Aims:

1) Present the observed changes during- and post-altitude exposure, in primary ABP variables ([Hb], Reticulocyte Percentage (RET%), and the OFF-score).

2) Investigate the acute haematological adaptations and associated vascular volume fluctuations during the first days at altitude.

Hypothesis:

During the initial days at altitude a natural plasma volume contraction will increase the concentration-based blood markers: [Hb], Haematocrit (HCT) and the OFF-score. These changes will be followed by a second, more gradual, increase in all markers of erythropoiesis.

Study 2 (Chapter 4) - The use of biomarkers to describe plasma-, red cell- and blood volume from a simple blood test

Aim:

1) Identify an optimal panel of common biomarkers capable of estimating red cell, plasma and blood volumes.
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Hypothesis:

A multi-parametric approach will be required, involving a number of volume
descriptive biomarkers. The selected biomarkers should be involved in various
biological processes in order to reduce the impact of external factors (other than
plasma volume fluctuations) on vascular volume estimations.

Study 3 (Chapter 5) - A step towards removing plasma volume variance from the
Athlete’s Biological Passport: The use of biomarkers to describe vascular volumes from
a simple blood test

Aims:

1) Apply the novel plasma volume marker (described in Chapter 2) to the Bayesian
network of the ABP.

2) Using exercise as a natural stimulus to contract plasma volume, demonstrate the
ability of the plasma volume correction to allow for extreme shifts in plasma volume
in the ABP’s reference calculations.

Hypothesis:

With the introduction of the plasma volume correction, the component of biological variance
associated with volume fluctuations will be reduced, thereby enhancing the sensitivity of the
ABP reference calculations. The impact of the plasma volume correction is assumed to be
most significant in situations where plasma volume has influenced the haematological
sample, for example during an exercise-induced plasma volume contraction.

Study 4 (Chapter 6) - Validation of a blood marker for plasma volume in endurance
athletes during a live high: train low altitude training camp

Aim:

1) To validate the novel plasma volume correction (described in Chapters 4 and 5)
during a 2 week sojourn at a simulated altitude of 3000m.
Hypothesis:

The initial ascent to altitude will prompt a natural plasma volume shift. With the application of the plasma volume correction, the calculated ABP reference intervals will allow for this volume shift. The improved specificity of the ABP reference limit calculations with the plasma volume correction will not impact on the sensitivity of the ABP; in other words there will be no change to the number of false positives.

1.4 Significance of the research

1.4.1 Equality in sports and athlete health

The ability to reduce the confounding impact of plasma volume fluctuation has the potential to significantly improve the accuracy and specificity of the ABP. This in turn, may further reduce the prevalence of blood manipulation in sports, thereby protecting athlete equality and preserving athlete health and wellbeing.\cite{1,2}

1.4.2 Blood sampling in sports science

The development of a simple blood test to determine vascular volumes may also provide a useful tool in the field of sports science research. Accurate and simple monitoring of an athlete’s hydration status during extreme exercise or heat/altitude training may be used to ensure optimal performance. A simple test to estimate vascular volume may also provide opportunities to expand our understanding of the thermoregulatory responses to extreme heat.

1.4.3 Patient monitoring in hospitals

Precise volume management is fundamental to the health outcomes of critically ill patients, especially those suffering from heart and kidney failure.\cite{15} However, there is currently no simple test available to estimate absolute vascular volumes. A blood test that can accurately measure plasma-, red cell- and blood-volumes (as developed in this research) would provide an innovative and much needed tool to successfully monitor the vascular volumes of critically ill patients. This may help reduce hospital morbidity and mortality rates.
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1.5 References


Chapter 1: Introduction


Literature review
Chapter 2: Literature review

2.0 Foreword

In a thesis presented as a series of papers, the University of Western Australia guidelines do not include a requirement for a traditional “Review of Literature” chapter, as each experimental paper will include relevant research findings in the text, particularly in the “Introduction” and “Discussion” sections. Consequently, what follows here, in Chapter 2, is a succinct review of background information relevant to the topics investigated in the experimental papers that follow (i.e. Chapters 3-6), but without unnecessarily duplicating and repeating the specific research findings from the literature pertinent to each study.
2.1 Background

Since the first demonstration in the 1970’s that blood transfusions significantly improve endurance performance, blood doping has challenged both the integrity of sports and the health of the athlete.\textsuperscript{[1]} The prevalence of blood doping was curbed, initially, by the introduction of the “no start rule” in 1997, with specified upper limits for Haemoglobin Concentration ([Hb]: for males 17.0 g/dL and females 16.0 g/dL), and Haematocrit (HCT: 50%), and then with the urine test for Recombinant Human Erythropoietin (rHuEPO) introduced in 2000. Dishonest athletes have nevertheless maintained the upper hand, avoiding detection through the continuous development of novel, unspecified performance enhancing compounds, minimising the measurable level of banned substance within the body or masking the effects of doping. It was not until 2008, with the introduction of the Athlete Biological Passport (ABP), that anti-doping agencies held the capacity to detect doping, despite anticipated advances in the doping substance or method.

Blood doping refers to the process of increasing the number of circulating red blood cells, either by blood transfusion or the use of erythropoiesis stimulants, in order to enhance oxygen delivery and thus aerobic performance.\textsuperscript{[2,3]} Such techniques originate from clinical practice. The first homologous blood transfusion was performed in 1795 and the technique is now widely used in hospitals to treat anaemia and improve a patient’s physiological reserve (aerobic capacity). More recently, rHuEPO, Continuous Erythropoietin Receptor Activator (CERA) and Hypoxia-Inducible Factor (HIF) agents were developed with the similar goal of manipulating [Hb] levels and improving oxygen delivery in hospital patients, all of which have been abused by dishonest athletes in order to gain a competitive advantage.\textsuperscript{[4]}

Blood manipulation techniques result in tell-tale shifts in markers of erythropoiesis, and it is these shifts that are considered within the ABP paradigm. The ABP markers include (but are not limited to); HCT, [Hb], Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Volume (MCV), Red Blood Cell Count (RBC), Reticulocyte Number (RET#), Reticulocyte Percentage (RET%) and the OFF-score (a statistical model sensitive to decelerated and accelerated erythropoiesis; OFF-score = [Hb] (g/dL) × 10 – 60 × √RET%).\textsuperscript{[5]} The ABP uses an indirect detection approach by monitoring markers of altered erythropoiesis over time and applying decision algorithms to identify abnormal profiles that suggest a blood manipulation.\textsuperscript{[5-8]} The ABP’s adaptive model develops individualised reference ranges focusing on the physiological changes occurring within an athlete (intra-individual biomarker variation) rather than on differences between athletes (inter-individual biomarker variation).
Chapter 2: Literature review

The system is based on a global Bayesian inference approach,[9] which essentially “learns” with each blood sample collected, thus an individual’s confidence interval for each marker becomes increasingly narrow over time.[10,11] The ABP approach follows a paradigm shift currently materialising in forensic identification science, where assumptions of absolutist certainty are becoming out-dated, and are being replaced by more defensible probabilistic reasoning.[12] Following the success of the blood passport,[13] the World Anti-Doping Agency (WADA) has recently introduced the steroidal module of the ABP, and is currently also developing an endocrinological module. Hereafter, mention of the ABP refers specifically to the blood module.

Classical markers of the ABP, [Hb] and HCT, respond to almost all forms of blood doping. However, the major limitation of these markers is that they are concentration-based and heavily influenced by shifts in plasma volume.[11] Plasma volume rapidly fluctuates as a physiological adaptation to physical exercise, heat stress, altitude, microgravity, illness, and posture.[13,14,15] A number of ABP markers are used in a clinical setting and the use of [Hb] to define anaemia can also be problematic as fluctuations in plasma volume may confound results.[16] The methods currently available to determine plasma volume and correct for volumetric fluctuations are often time-consuming, cumbersome, unreliable, or involve the use of tracers. Of note, it is important to consider that no viable method currently exists to correct concentration-based blood markers for plasma volume shifts within an anti-doping setting. Currently, it is the role of the anti-doping expert to defend the analytical techniques that have led to the prosecution of an athlete through forensic reasoning. Experts must consider the pre-analytical and analytical aspects of an athlete’s sample, such as environmental influences, pathological conditions, and the potential of a doping scenario (Figure 2.1).[17] Some ABP doping cases presented before the Court of Arbitration of Sport (CAS) are currently vulnerable to the “plasma volume expansion claim”, whereby athletes are using natural plasma volume fluctuations to explain an abnormal profile. Moreover, methods such as micro-dosing have narrowed the disparity between natural and unnatural biological variation. Consequently, improved knowledge of haematological marker variation and plasma volume shifts (both natural and artificial), occurring at all stages of the athlete’s competition cycle, is vital in order to improve the efficacy of the ABP. In addition, the concept of individualised haematological profiles has the potential to improve health assessment within the emerging field of personalised medicine. Therefore, improved knowledge of haematological and vascular volume fluctuations is a central theme to the research presented here, with applications not only in the field of anti-doping, but also public health care.
Figure 2.1. **Overview of the ABP paradigm.** Circles contain factors that may influence blood volumes and/or blood values.
2.2 The development of individualised haematological profiles

2.2.1 History

Differentiating between exogenous and endogenous substances in a doping case can be difficult, and in some instances, impossible.\cite{18,19} Consequently, the introduction of the ABP has directed the focus towards the physiological outcomes of doping rather than direct detection. In 1999, Audran et al. demonstrated the influence of rHuEPO administration on a number of haematological markers such as RET%, Erythropoietin (EPO) and Soluble Transferrin Receptor (sTfR).\cite{20} However, in isolation these markers do not have the discriminating power to identify rHuEPO abuse with certainty. In 2001, Parisotto et al. proposed a multiparametric approach.\cite{7} Here, indirect markers of altered erythropoiesis with specificities and sensitivities optimised for rHuEPO detection were identified and combined to develop the OFF-score. This measure combines [Hb] and RET% to specifically detect rHuEPO abuse; the score is also sensitive to slow, long-acting erythropoietic agents such as Insulin-Growth Factor 1 (IGF-1), Human Growth Hormone (hGH) and rHuEPO microdosing. The OFF-score remains amongst the best indirect blood doping markers. In 2003, a second generation of multiparametric markers was developed,\cite{5} and in 2006, specific pattern classification techniques\cite{21} were used to combine all the data contained in a single athlete’s blood profile to develop the Abnormal Blood Profile Score (ABPS).\cite{8} The ABPS comprises 12 indirect markers that reflect altered erythropoiesis. Advances in Bayesian mathematical modelling then allowed for the use of an athlete’s previous results to establish individualised basal levels,\cite{9,22} thereby shifting away from the use of population means to the development of a model where the athlete is his/her own reference.\cite{6} An individualised model for the longitudinal monitoring of a combination of key markers of erythropoiesis was hence created, utilising probabilistic inference techniques to evaluate the available scientific evidence (Figure 2.2).\cite{12,23} A number of further developments provide the foundations to the successful methodology behind the ABP:

1. The consideration of heterogeneous factors such as sex, age and ethnicity in the ABP adaptive model.\cite{5,24,25}
2. Standardisation of blood collection, transport, analysis, storage and inter-instrument bias.\cite{26,27}
3. The accreditation of specific anti-doping laboratories (WADA accreditation and/or ISO 17025).
4. The inclusion of information on the prevalence of doping in specific populations under consideration.\cite{12}
Figure 2.2. Screenshot of the ABP’s adaptive model. A) Normal Passport (female); B) Abnormal Passport (male). Blue lines indicate the athlete’s scores, red lines indicate the predicted reference interval for that individual athlete. Athlete scores outside the predicted reference interval are flagged as suspicious, and it is the role of the expert to use forensic reasoning to determine the cause of the abnormal profile. Modified from: Sottas et al. (2010). [11]
2.2.2 Statistics of the ABP adaptive model

The statistics behind the adaptive model follows that of Bayesian reasoning \cite{28} where the direction of thought is opposite to that of causality. For example, an athlete injects rHuEPO into their system (the cause) resulting in an increase in [Hb] (the effect). The adaptive model links effect to cause to predict whether an observed increase in [Hb] is the result of rHuEPO abuse or the result of natural variation. This is expressed as:

\[
P(D|M) = \frac{P(M|D) \cdot P(D)}{P(M)}
\]

Where \( D \) is a dichotomic variable with two states, doped and non-doped, and \( M \) is a continuous variable representing the value of a given blood marker, for example [Hb]. Accordingly, \( P(D|M) \) represents the probability of an athlete being in one state of \( D \) (e.g. doped) as a function of the blood marker value, \( M \) (e.g. [Hb] 15.0 g/dL). So, \( P(M|D) \) represents the probability of measuring a value for blood marker, \( M \), knowing the athlete is in a state of \( D \).\cite{11} In the current adaptive model, decision rules are exclusively based on the capacity of a blood marker to correctly identify a negative, non-doped case (the specificity) with the underlying assumption that the athlete is non-doped.

Expanding from the core equation (stated above), the ABP evaluates the evidence associated with a given value for blood marker, \( M \), using a longitudinal approach where two variables, the expected mean (\( \mu \)) and standard deviation (\( \sigma \)) of a sequence of \( M \) values are defined (Figure 2.3). A number of confounding factors influence blood marker \( \mu \) and \( \sigma \), including sex, age,\cite{29} ethnicity,\cite{22,30} sport, illness, altitude, environment, time of day, posture and competition phase (in/out of competition).\cite{31} The ABP accounts for a number of these heterogeneous factors in the Bayesian network as fixed (sex, ethnic origin, sport) or time-varying (age and haematology analyser) factors.\cite{12} By accounting for these factors the total variance associated with marker, \( M \), is reduced and allows for the focus to shift to intra-individual variance components.
Figure 2.3. The ABP bayesian network. Sex (male, female), ethnicity (Caucasian, Asian, African, Oceanian, Other), sport (endurance, non-endurance), analyser (Sysmex, Bayer Advia, other, although the Sysmex is now the preferred analyser of use in accredited anti-doping laboratories), age (< 19 years, 19-24 years, > 24 years), D (doped, non-doped). Modified from: Sottas et al. (2010)\textsuperscript{[11]}
2.2.3 Components of biomarker variance

A main requirement for a biomarker to be suitable for longitudinal follow-up is that intra-individual variation is less than inter-individual variation.\(^{32}\) For the primary marker, [Hb], inter-individual variance is associated with genetics (ethnicity), sex, age and analytical bias, while intra-individual variance is associated with analytical variation (test-retest analytical uncertainty), plasma volume shifts and changes in Haemoglobin Mass (HbM). The ABP adjustments for heterogenous factors (age, sex and ethnicity) and the use of an athlete as his/her own reference remove the inter-individual variance components. The standardisation of sample collection, transport and analysis removes across-instrument analytical bias. However, intra-individual variance components (in particular, variance associated with plasma volume) remain the major contributors to [Hb] variance within the current ABP paradigm. A primary goal in the current ABP paradigm is to reduce the variance associated with plasma volume, resulting in the potential to greatly improve the specificity of volumetric ABP blood markers, such as [Hb].

2.2.4 Applying individualised reference limits to hospital patient

As dishonest athletes have applied blood manipulations techniques, originally developed in the clinic, to improve their own athletic performance, anti-doping detection techniques (namely the development of individualised reference ranges for target biomarkers) can be re-applied back to the clinic to improve patient monitoring. The use of biological markers to set targets for the treatment of critically-ill patients is extremely challenging, primarily due to the deranged physiology observed that is often not uniform across all patients with a similar diagnosis. Health targets for specific biomarkers are still set for patients based on population-based references, despite the obvious flaws associated with a population-based approach. A prime example is the use of serum Creatinine (CRE) as the most common biomarker to assess renal dysfunction, where up to 60% of total kidney function can be lost before CRE values fall outside the recommended, population-based, reference ranges (45-90 umol/L for females and 60-110 umol/L for males). Recently, Sottas and colleagues have demonstrated that personalised reference ranges, established using a Bayesian network (that allows for confounders such as gender and age), hold the capacity to greatly improve the sensitivity and specificity of CRE as a marker for renal dysfunction.\(^{34}\) In a population of 50 patients with stage 2 or 3 Chronic Kidney Disease (CKD), a loss of 10 to 50% of renal function (obtained
from a direct measurement of glomerular filtration rate and clearance rate) was detected in only 7 patients when traditional CRE reference ranges were used, however, the Bayesian approach detected 38 patients when using a specificity of 99% (< 1 in 100 chance of occurring naturally). Early detection of renal dysfunction greatly improves the preservation of the healthy nephrons and reduces the need for subsequent dialysis; this may be achieved through an individualised reference approach.[34] Of course, the Bayesian approach may be more suitable for “at risk” patients only, as it requires an initial healthy value to develop a predicted reference range (calculated from pre-set between- and within-subject variations of a given biomarker). This first estimated reference interval relies heavily on population-based values, however, as a patient produces more test results for this given biomarker, the Bayesian model relies less on population-based inferences, and eventually takes solely within-subject variance into account to estimate a specific and individualised reference range. Additionally, the Bayesian models allow for additional levels to be applied whereby confounding factors such as age, sex or genetic predisposition related to a particular biomarker can be introduced, further improving the specificity of the model. This individualised approach has obvious benefits in a setting where patient physiology is not uniform. The concept of individualised reference calculations therefore has applications beyond anti-doping, and as such, future research of the ABP paradigm should also consider any possible clinical implications.

2.3 Vascular volume response to exercise, environment and illness and the implications on volumetric biomarkers

2.3.1 Exercise and hydration status

The ABP relies primarily on concentration-based haematological markers, such as [Hb], which are measured in a suspension of blood plasma and therefore are sensitive to shifts in plasma volume.[11,14] The only ABP variable immune to plasma volume is RET% as any change (natural or manipulated) affects the ratio of mature and immature erythrocytes equally. Plasma volume responds rapidly to physical exercise, heat stress, hydration status, altitude, microgravity, trauma and sickness.[14,15,36-38] Haemoconcentration is regulated by fluid loss, the redistribution of blood flow and/or capillary exchange dynamics (influenced by oncotic pressure, mediated by the concentration of plasma proteins e.g. albumin). Previous investigations have shown strenuous exercise and elevated metabolic demands to induce a
progressive increase in extracellular water, in particular, plasma volume.\textsuperscript{[38-42]} Such changes in plasma volume occur due to a loss of fluid through sweat and shifts to the interstitial space, usually followed by an over-compensation (increase in plasma volume) on cessation of exercise. An increase in plasma volume results in a decrease in concentration-based blood markers, termed haemodilution.\textsuperscript{[43]} Although the blood volume response to exercise has been well described, our understanding of the influence that these shifts have on key haematological variables is limited. The primary concern is that changes in key ABP markers as a consequence of a plasma volume shift may breach the range of predicted biological intra-individual variability, resulting in a false-positive doping violation. Moreover, anecdotal evidence suggests that hyperhydration can be used to mask an altered haematological profile, resulting from the use of a prohibited substance. This was demonstrated by Bejder and colleagues, who found that the ingestion of 1000 mL of water masked rHuEPO abuse and reduced the identification of atypical OFF-score values from \(~49\%\) (control group, \(n = 10\)) to \(~11\%\) (hyperhydration group, \(n = 10\)), decreasing the sensitivity of the ABP.\textsuperscript{[43]} Currently, anti-doping samples are collected 2 hr post competition (or exercise) in order to circumvent the influences of exercise-induced fluctuations in plasma volume. Athletes have nevertheless made claims that dehydration was the cause of an abnormal ABP profile, and since there is no method currently available within an anti-doping setting to quantify the influence of plasma volume shifts, there is a clear a risk that athletes may be using methods as simple as hyperhydration to mask a doping offence.

2.3.2 Altitude

Among several environmental factors that may influence a haematological profile, altitude exposure is considered a primary confounding variable. Altitude training is a popular (legal) method to enhance red cell mass prior to competition.\textsuperscript{[2]} However, altitude training can result in a similar haematological profile to that of rHuEPO abuse.\textsuperscript{[44-46]} Plasma volume is also known to contract upon high altitude exposure, and fluctuations in concentration-based haematological markers have been observed within the first few hours of an ascent.\textsuperscript{[47]} An increased rate of erythropoiesis and red cell mass occur as the altitude sojourn continues, with the level of response dependent on the individual, the altitude, the time spent at altitude and the use of natural or artificial hypoxic environments. Importantly, altitude has not been found to systematically result in an atypical passport finding (breaching 99\% reference limits).\textsuperscript{[48]}
However, the athletes under investigation in the 2014 paper by Garvican-Lewis \cite{48} were also competing in a cycling tour, which most likely prompted an exercise-induced decrease in [Hb] during the investigation. Given the potential confounding nature of altitude exposure on the blood markers of the ABP, it was initially proposed that the effect of altitude be introduced directly into the ABP software; however, the idea was abandoned due to its complexity. Nevertheless, hypoxic training continues to be a major confounding factor that is not adequately weighted within the ABP. To complicate matters further, dishonest athletes have also been known to manipulate their blood whilst at altitude, using the natural haematological fluctuations induced by the hypoxic environment to mask doping.\cite{49} This shifts responsibility to ‘the expert’ to make an appropriate judgement on the plausible influence altitude may have on any athlete that comes under question.\cite{46,50} To this end, further research into the initial volumetric response to an ascent to high altitude and subsequent increase in red cell production at altitude in relation to the ABP is required in order to improve anti-doping sample analysis.

2.3.3 Volume management in hospital patients

In clinical practice, clinicians use [Hb] as an indicator of anaemia (< 12.0 g/dL in non-pregnant females and < 13.0 g/dL in males),\cite{51} yet [Hb] can be a misleading measure as it is dependent on both the total HbM (a true determining factor of anaemia) and total plasma volume. A recent study from Otto and colleagues has demonstrated that [Hb] does not necessarily reflect the true HbM of a patient due to fluctuations in plasma volume.\cite{16} This investigation identified no correlation between HbM and [Hb] in patients with liver disease or heart failure because plasma volume explained the majority of the variance observed in [Hb]. The authors advocated the implementation of tests to measure HbM and plasma volume in routine clinical assessments.\cite{16} Furthermore, successful volume management, and therefore knowledge of absolute vascular volumes, is fundamental to achieving positive health outcomes in numerous critical care scenarios. Rivers and colleagues demonstrated that maintenance of adequate intravascular volume is a key part of successful septic shock therapy.\cite{52} In addition, Schrier identified adequate renal perfusion and intra-vascular volume as vital in the prevention and therapy of acute kidney injury in intensive care units.\cite{53} In chronic heart failure patients, hypervolaemia is often the stimulus for acute decompensated heart failure and a worsening of the patient’s condition, which requires reducing fluid
levels. Conversely, unsuccessful volume management resulting in fluid overload is associated with a higher incidence of non-recovery of renal function in patients starting renal replacement therapy. The diagnosis and treatment of anaemia or hypovolaemia is thus a core concept in pre-operative patients and the critically ill to maintain organ perfusion and intravascular volumes, yet no rational treatment decision can be reached without direct knowledge of blood volumes. Therefore, a simple tool to estimate plasma volume fluctuation (and correct [Hb] measures) not only has application in the field of anti-doping for the correct identification of a blood manipulation, but also has the potential to improve volume management and patient monitoring in hospitals.

2.4 Current methods to determine vascular volumes

2.4.1 Rationale behind vascular volume measurements in anti-doping, clinical practice and sports science

Blood volume estimation is a primary measure in exercise physiology and clinical investigations. In exercise physiology, measures of blood volume are commonly utilised to monitor body fluid redistribution during exercise, thermoregulatory stress, contributions of plasma volume and [Hb] to oxygen transport and performance, and altitude adaptation. Furthermore, plasma volume plays a crucial role in many physiological processes, and is an important variable for many clinical decisions. Deviations from normovolaemia occur in response to many physiological and pathological insults, and these deviations of plasma volume can be of profound importance for blood pressure regulation and organ perfusion by altering vascular, cardiac, renal, hepatic, immune, and endocrine endpoints. Restoration of tissue perfusion and oxygen delivery is the ultimate goal following circulatory collapse, and in order to reach a successful outcome, accurate and reliable information regarding the patient’s volume status must be readily available. However, there is currently no practically applicable method to accurately measure plasma volume.

Two approaches to estimate blood volumes currently exist. The first method utilises observed changes in concentration-based markers, [Hb] and HCT, to estimate a change in plasma volume. The second approach requires the use of tracers to calculate absolute blood volumes.
2.4.2 The Dill and Costill equation

In 1974, Dill and Costill published a study identifying the use of [Hb] and HCT values to estimate changes in plasma volume.\[^{68}\] The Dill and Costill equation is extensively used in exercise physiology to estimate haemoconcentration during exercise:

\[
\Delta \text{plasma volume(\%)} = 100 \times \left[ \frac{[\text{Hb}] \left( \frac{\text{g}}{\text{dL}} \right)_{\text{pre}}}{[\text{Hb}] \left( \frac{\text{g}}{\text{dL}} \right)_{\text{post}}} \right] \times \left( \frac{100 - \text{HCT(\%)}_{\text{post}}}{100 - \text{HCT(\%)}_{\text{pre}}} - 1 \right)
\]

Both [Hb] and HCT are commonly used in hospitals to estimate a change in plasma volume (not absolute volume). Although simple to apply, this technique is prone to false red blood cell volume estimations when plasma volume is either rapidly expanded or reduced, a common manifestation in the critically ill.\[^{69}\] The equation also assumes HbM is stable and therefore is impractical in certain scenarios, for example during the testing of athletes at altitude, or in hospital patients after the administration of rHuEPO. Although useful in monitoring plasma volume changes, the Dill and Costill equation highlights the shortcoming of [Hb] and HCT as markers for the ABP; that is, both measures are highly sensitive to plasma volume shifts.

2.4.3 Tracer methods – Evon’s Blue dye, radio-isotopic and CO-rebreathing methods

Tracer methods are based on the dilution of an administered compound in the circulation. The use of tracers allows direct estimation of plasma volume by labelling plasma proteins, for example, Evans Blue dye \[^{70}\] or albumin labelled with radioactive Iodine (\(^{131}\)I or \(^{125}\)I).\[^{71}\] Alternatively, methods employing radioactive Chromium (\(^{51}\)Cr)-labelled red blood cells \[^{72}\] or the carbon monoxide (CO) rebreathing technique, where CO binds to Hb,\[^{73}\] provide estimations for red cell volume, from which, plasma volume and blood volume can be calculated.

Evon’s Blue dye has a high affinity to plasma proteins. The technique has been used extensively to measure plasma volume and is considered an ideal marker for plasma space. However, the method has sustained criticism around its validity, with Gore et al. calculating
the mean error of measurement (adjusted to 1 day between trials and expressed as a coefficient of variation) to be 6.7% (90% CI 4.9 to 9.4%). Of note, it is the variable loss of the dye from the vascular space that results in an overestimation of plasma volume, which is a major contributor to the relatively large measurement error. Further, the method is unreliable when plasma is turbid or contaminated by Hb.

The \textit{radioactive iodine-labelled albumin} method involves the collection of a venous blood sample that is subsequently labelled with radioactive iodine and re-injected. Venous samples are then taken at three specific time points, and plasma volume is estimated by dividing the standard concentration of radio-labelled albumin with the concentration of radioactivity in the post-plasma samples. The shortcoming of the method is that blood volume cannot be allowed to change markedly over the course of the 30 min equilibration period. Although the method is recommended in a clinical setting, estimates are problematic with an increase in plasma albumin extravasation rates during inflammation, for example. The inherent danger of radioactive material must also be considered.

The \textit{BVA-100} blood volume analyser (Daxor Corporation, New York City, NY) is a simplified, pre-packaged, semi-automated kit used to measure blood volume in the clinic. BVA-100 assesses the dilution of radioactive iodine-tagged albumin following injection into a patient, estimating plasma volume from centrifuged blood. Blood volumes may then be estimated with subsequent measures of HCT. This relatively new technique has been welcomed in the clinic, yet remains costly and relies on radioactive material.

The use of $^{51}$Cr labelled erythrocytes is the gold-standard method for blood volume estimations on the basis of “\textit{reliability, reproducibility and ease of use in routine clinical [practice]}” in accordance with recommendations from the International Committee for Standardisation in Haematology. The technique involves the collection of venous blood, labelling with $^{51}$Cr and reinjection, followed by an equilibration period. The method has one of the lowest measurement errors among those available for estimations taken one day apart (along with the CO-rebreathing technique) with a coefficient of variation of 2.8% (90% CI 2.4 to 3.2%). The technique does, however, systematically underestimate whole blood volume, as erythrocytes in venous blood are concentrated relative to their numbers in the arteries and capillaries, and suffers from the drawback of requiring the use of radioactive material.
The optimised CO-rebreathing method was introduced by Schmidt and Prommer in 2005.\textsuperscript{[78]} The method requires approximately 30 min to complete and involves the rebreathing of a weight-adjusted level of CO by the subject for two minutes to ensure binding of CO to Hb. Carboxyhaemoglobin content is measured before and after the rebreathing manoeuvre using a small capillary sample. From this HbM and blood volumes are calculated, as follows:

\[
\text{Red cell volume} = \frac{\text{Hb mass (g)}}{\text{MCHC} \times 100}
\]

\[
\text{Blood volume} = \frac{\text{Hb mass (g)} \times 100}{[\text{Hb}] \div 0.91}
\]

\[
\text{Plasma volume} = \text{Blood volume} - \text{Red cell volume}
\]

\[
\text{Mean Corpuscular Haemoglobin Concentration (MCHC)} = \frac{[\text{Hb}]}{\text{HCT} \times 100}
\]

The technique requires only one venepuncture and/or a few capillary samples, and is less invasive compared to the alternative tracer methods mentioned above. The CO-rebreathing technique has a low measurement error for estimations taken one day apart, with a coefficient of variation of 2.2% (90% CI 1.4 to 3.5%).\textsuperscript{[74]} Recent studies have suggested that this technique be introduced into the anti-doping paradigm.\textsuperscript{[79]} However, the introduction of this technique in the anti-doping scenario is hindered,\textsuperscript{[80]} primarily due to lack of quality control, as a human (rather than a machine) performs the test.

Large between-laboratories variations have also been observed for the CO-rebreathing method, as well as for the $^{51}$Cr and Evans Blue dye methods.\textsuperscript{[74]} Generally, tracer methods are associated with numerous sources of error; the primary assumption that a steady state exists \textit{in vivo} is an inherent caveat. Additional sources of error arise from the preliminary measurement (amount of tracer administered), the primary circulatory space measured by the tracer (for example, the albumin space when using the Evans Blue or radioactive-iodine tracers), the reliability and accuracy of the administration of the tracer, potential unquantifiable loss of the tracer (for example, gas leaks when using the CO-rebreathing method), tracer-mixing kinetics, estimation of the volume distribution of the tracer (protein binding changes and dye optical properties), any additional parameters used in the method equation and in the primary and secondary method equations to derive blood measures.\textsuperscript{[74]} Additionally, the invasive nature and the use of radioactive compounds challenge the ethics of utilising most of these methods in the clinic, the research laboratory, or for anti-doping purposes.
In conclusion, current methods to determine blood volumes are mostly inaccurate, time-consuming, cumbersome, costly, invasive, require the handling of complex equipment, and/or involve the use of radioactive material. As a result, clinicians may hesitate to use such methods in settings such as the Intensive Care Unit, and such practices are often uninviting to research participants. In addition, the use of such methods in an anti-doping context are inappropriate for ethical, technical and analytical reasons.

2.4.4 Volume descriptive biomarkers – A novel approach to estimating plasma volume

Utilising ‘volume descriptive’ biomarkers to estimate plasma volume is a novel initiative. One previous investigation\(^{[81]}\) has shown Calcium (Ca), Gamma-Glutamyl Transferase (GGT), Total Protein (TP) and Albumin (ALB) concentrations in blood to correlate well with estimations of plasma volume, calculated using the Dill and Costill equation. The authors suggested these biomarkers, in particular Ca concentration, as an alternative indicator to estimate haemoconcentration during high-intensity exercise.\(^{[81]}\) However, in this study, the Dill and Costill equation provides a standard for plasma volume change, rather than an absolute estimate. Nevertheless, the novel approach is promising, because it presents a simplified approach to estimating blood volumes. Of note, the development of a viable method to estimate absolute plasma volume with ease and confidence will not only impact on the specificity of the ABP, but is also attractive to the clinician with the promise of improved patient care.

In this thesis, a novel method for estimating plasma volume is proposed, where a number of ‘volume descriptive’ serum chemistry markers (with low variance values, < 10%) will be combined in a statistical model with the aim to accurately predict absolute plasma volume.

2.5 Summary

The concept of natural and predictable biomarker variation has led to the development of the ABP as an essentially “timeless” anti-doping tool, able to detect all forms of blood doping through the identification of the physiological changes such practices will elicit. However, a major confounding factor to the current blood module is the use of concentration-based blood markers, influenced by shifts in plasma volume. Further, methods to directly estimate plasma
volume are often cumbersome, time-consuming and present with health risks; thus making them un-suitable in an anti-doping setting. The proposed concept of a panel of ‘volume descriptive’ biomarkers able to accurately estimate absolute plasma volume through a simple blood test has potential to greatly improve the specificity of the concentration-based markers of the ABP. This novel approach to account for plasma volume not only has the potential to advance our current ability to detect illegal blood manipulation in sports, but will also have a broader impact in health care and in exercise physiology research.
2.6 References


Chapter 2: Literature review


The athlete’s response to hypoxia: A meta-analysis of the influence of altitude exposure on key biomarkers of erythropoiesis

This chapter is based on a peer-reviewed paper accepted and published in the American Journal of Haematology:

3.0 Abstract

Altitude training is associated with changes in blood markers, which can confound results of the Athlete’s Biological Passport (ABP). This meta-analysis aims to describe the fluctuations during- and post-altitude in key ABP variables; Haemoglobin Concentration ([Hb]), Square-Root Transformed Reticulocyte Percentage (sqrt(RET%)) and the OFF-score. Individual de-identified raw data were provided from 17 studies. Separate linear mixed effects analyses were performed for delta values from baseline for [Hb], sqrt(RET%) and OFF-score, by altitude phase (during and post). Mixed models were fitted with the hierarchical structure: study and subject within study as random effects. Delta values as response variables and altitude dose (in Kilometre Hours; km.hr = altitude (m) / 1000 x hr), sex, age, protocol and baseline values as fixed effects. Allowances were made for potential autocorrelation. Within two days at natural altitude [Hb] rapidly increased. Subsequent delta [Hb] values increased with altitude dose, reaching a plateau of 0.94 g/dL [95%CI (0.69, 1.20)] at ~1000 km.hr. Delta sqrt(RET%) and OFF-score were the first to identify an erythrocyte response, with respective increases and decreases observed within 100 to 200 km.hr. Post-altitude, [Hb] remained elevated for two weeks. Delta sqrt(RET%) declined below baseline, the magnitude of change was dependent on altitude dose. Baseline values were a significant covariate (p < 0.05). The response to altitude is complex resulting in a wide range of individual responses, influenced primarily by altitude dose and baseline values. Improved knowledge of the plausible haematological variations during- and post-altitude provides fundamental information for both the ABP expert and sports physician.

Key words: Haemoglobin, reticulocytes, OFF-score, biological variation, anti-doping
3.1 Introduction

Altitude training is a well-documented and applied method used to enhance endurance performance. Improvements are achieved, in part, by increases in maximal oxygen uptake, concomitant with a hypoxia-induced elevation of total Haemoglobin Mass (HbM).\(^1\) Altitude training is hence associated with changes in an athlete’s haematological profile, and thereby presents as a confounding factor for not only sports medicine physicians but also within the haematological module of the anti-doping tool, the Athlete Biological Passport (ABP).\(^2\) The ABP paradigm is based on the knowledge that blood manipulations cause characteristic alterations in several markers of erythropoiesis, resulting in a distinctive biological fingerprint. Over the course of an athlete’s sporting career, blood markers are longitudinally monitored within an adaptive model (based on Bayesian inference), which considers previous values and creates individualised reference limits for each key marker. Abnormal patterns within an individual ABP profile (where values exceed the predicted reference limits) are electronically flagged, and are subsequently investigated by an expert panel in view of a potential doping offence. However, a major drawback to the current ABP paradigm is that altitude training can elicit similar haematological fluctuations to those observed from blood doping, including Recombinant Erythropoietin (rHuEPO) abuse.\(^2-5\) Previous attempts to include altitude within the ABP algorithm were abandoned due to complexity. Altitude is currently flagged within the ABP by a set of questions, which asks athletes: 1) if they have sojourned at altitude any time two weeks prior the doping test, 2) the time, place and duration of the altitude exposure, and 3) whether the altitude was simulated or natural. The concept of individualised reference limits has been used within anti-doping practices since 2008. However, the concept may also be applied to (sports) medicine.\(^6\) Elite athletes often present with unique physiologies, and therefore, improved knowledge of individual responses to hypoxia will not only clarify the analysis of an ABP profile influenced by altitude but enhance our ability to monitor an athletes’ health status whilst altitude training.

A sojourn at altitude has been demonstrated to influence haematological parameters through two distinct mechanisms. First, an acute decrease in plasma volume is observed within hours to days of an ascent to high altitude, rapidly increasing Haemoglobin Concentration ([Hb]) and the arterial oxygen content.\(^7,8\) Second, within 7-10 days an increase in red cell mass is likely to be detected, resulting from an increased production of Erythropoietin (EPO) and reticulocytes.\(^8,9\) Exposure to altitude thus results in changes to key ABP variables, primarily, [Hb],\(^10\) Percentage Reticulocytes (RET%),\(^11-13\) and the OFF-score (OFF-score = [Hb]
(g/dL) × 10 − 60√(RET%), where [Hb] and RET% are expected to increase and the OFF-score is expected to decrease.\textsuperscript{[14]} Upon return to sea-level, a progressive down-regulation in erythropoiesis occurs through the re-adaptation of red cell mass to the new environment, (possibly aided by neocytolysis) resulting in a return to baseline levels for [Hb] and the OFF-score and expected decline below baseline levels for RET%.\textsuperscript{[15]} Previously, two principal approaches to altitude training have been rigorously investigated, including; (a) classic altitude training [‘Live High: Train High’ (LHTH)], where athletes live and train at (natural) altitudes, and (b) ‘Live High: Train Low’ (LHTL), where athletes sleep in hypoxia (either natural or simulated) and train at sea level. The minimal daily dose for accelerated erythropoiesis to occur at natural altitude has been suggested to be four weeks at 2000 to 2500 m, and for simulated altitudes (using a LHTL protocol), a minimum of 12-16 hr/day at ≥ 3000 m is required.\textsuperscript{[16,17]}

A scientific review of the haematological changes observed during and after altitude is timely in order to quantify the effect of altitude on peripheral blood markers, exploring the differentiation of response between a natural change and a change caused by doping. This meta-analysis of published altitude experiments aims to present the observed changes in primary variables of the ABP, [Hb], RET%, and the OFF-score, during- and post-altitude, in healthy lowlanders. In addition, a comparison between natural LHTH, and simulated LHTL altitude protocols will be made, as will the influence of sex and age. Improved knowledge of individual haematological responses to altitude will also aid sports physicians to monitor an athlete’s health status.

### 3.2 Methods

#### 3.2.1 Data sources

Up to June 2016 we introduced the following key words and Boolean connectors in PubMed and Google Scholar: (altitude OR hypoxia) AND (erythropoiesis OR haemoglobin OR haematocrit OR haemoglobin mass OR red blood cell volume OR blood volume OR plasma volume). The search was limited to humans with published results of key erythropoiesis markers, [Hb] and or RET% during a sojourn at altitude. The initial search generated 45 suitable references and individual authors were contacted for access to raw data files. Individual de-identified raw data were provided from 19 suitable studies, 8 LHTH\textsuperscript{[9,18-24]} and
Collected data items were restricted to subject age, sex, sport, haematological results pre-, during- and/or post-altitude exposure, elevation (metres), altitude duration (hours), altitude protocol (LHTH or LHTL), supplementation provided and haematology analyser.

3.2.2 Eligibility criteria

Only data published after 2000 on healthy lowlanders whose training history ranged from well-trained to elite were selected (training levels were prescribed within each individual publication). Participants were excluded if exposed to altitude in the two months prior to each respective research start date. Subjects must have been sleeping at altitudes > 1200 m (low altitude) for > 1 week. Venous blood must have been collected pre-, during- and/or post-hypoxic exposure. Studies which involved blood manipulation were not accepted, or only suitable subject groupings were accepted (for example, only control data from Garvican et al. were included). The use of legal supplements (for example, iron supplementation) during the study protocol was accepted, as per the 2017 World Anti-Doping Agency (WADA) prohibited list. Buchheit et al. was removed from the final analysis as the pre-altitude samples were collected within 24 hr of a long-haul plane flight and the [Hb] baseline samples were likely influenced by the dehydrating effects of air travel. Additionally, Garvican-Lewis et al. was removed as this study was performed during a cycling stage race and as exercise-induced plasma volume fluctuations influence [Hb], the effect of altitude vs. exercise could not be differentiated. A single individual result was omitted from Wachsmuth et al. as the subject was known to be ill, which was reflected in the haematological profile. One and three individuals were removed from two separate studies as they were iron deficient prior to the altitude investigation. Finally, four individual values from three studies were influential outliers, most likely the result of analytical error, and removed. A summary of the data collection process is provided in the supplementary material (Appendix A, Figure I). The final analysis was performed on 17 individual altitude investigations (Table 3.1).
3.2.3 Coding of predictor variables

The total hours spent at altitude were calculated from the hours spent per day in hypoxia and the number of days of exposure. This approach allowed for a comparison between LHTH (continuous exposure) and LHTL (intermittent exposure) protocols. Hauser et al. [28] studied the LHTL protocol at both natural and simulated altitude. For simplicity, this LHTL natural group was classified as LHTL without reference to the type of altitude within the final models. [28] All but two studies applied ≥ 14 hr/day in hypoxia, while one study used 11 hr/day [30] and the other 13 hr/day. [28]

In addition to days and hours at altitude, altitude dose for each blood collection time point was calculated as Kilometre Hours (Kilometre Hours (km.hr) = (altitude (m) / 1000) x hours). [36] Kilometre Hours were chosen, as this new metric incorporates elevation as well as hours of exposure. If multiple altitudes were used within a given study, a cumulative km.hr was calculated. For example, subjects slept at 2500 m for 14 hr/day for 7 days, and at 3500 m for 14 hr/d for an additional 7 days, blood was collected at day 14 [km.hr (day 14) = (2.5 x 98 hr) + (3.5 x 98 hr) = 588 km.hr].

Low and high baseline [Hb] were identified by the observed quartiles from our data set for further analysis into the influence of baseline values and subsequent change values. For males, \( \text{Hb}_{\text{Low}} = < 14.0 \) and \( \text{Hb}_{\text{High}} = > 16.0 \) (g/dL). For females, \( \text{Hb}_{\text{Low}} = < 12.9 \) and \( \text{Hb}_{\text{High}} = > 14.4 \) (g/dL).

3.2.4 Statistics

The statistical program R, [17] with the nlme package, [38] was used to perform a linear mixed effects analysis of the relationship between the observed delta values in [Hb], RET% and the OFF-score and various measures of exposure for both the “during” and “post” altitude phases. All analyses for RET% were carried out on the square root scale \( \sqrt{\text{RET} \%} \) in order to stabilise the residual variance. The delta values were calculated separately for each individual subject, as the difference between the baseline value and the subsequent during- or post-altitude value (delta = “subsequent” – “baseline”). Separate linear mixed effects analyses were performed for [Hb], \( \sqrt{\text{RET} \%} \) and OFF-score, by altitude phase, with the post-altitude data restricted to the next 20 days. The reason for this restriction was to ensure that altitude remained the primary environmental factor influencing the post-values, rather than
additional factors such as training volume or tapering for competition. To allow the data from
the different studies to be combined, the mixed models were fitted with the hierarchical
structure: study and subject within study as random effects.

All models were fitted with the delta values as the response variable. Fixed effects included
altitude dose (as study day, hours at altitude or km.hr), sex, age, LHTH vs LHTL, days post-
altitude (for the post-altitude phase) and baseline values of the relevant variable ([Hb],
sqrt(RET%) or OFF-score). Study and subject within study were fitted as random effects.
Where appropriate, an allowance was made for potential autocorrelation and for different
residual variability for males and females. For each combination of response variable and
phase (during- or post-altitude) considered, the most appropriate model was selected based
primarily on its physiological feasibility, and then on its AIC (Akaike’s Information
Criterion; smaller is better) \(^{39}\) and its residual standard deviation (again, smaller is better).
Similar analyses were carried out using the delta values of the three variables in order to
estimate within-subject variability, with the best models per variable and altitude phase
applied.
### Table 3.1. Summary of the altitude studies and participant demographics.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Altitude Mode</th>
<th>Altitude (m)</th>
<th>Duration (hours)</th>
<th>Total Km.hr</th>
<th>Sport</th>
<th>Calibre of Athlete</th>
<th>Participants</th>
<th>Measures per participant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brugniaux 2006</td>
<td>LHTL (3)</td>
<td>2500-3000</td>
<td>252</td>
<td>714</td>
<td>Running</td>
<td>Elite</td>
<td>5 (m)</td>
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<td>1380-3000</td>
<td>504</td>
<td>1002</td>
<td>Race walking</td>
<td>Elite</td>
<td>4 (m), 4 (f)</td>
<td>2</td>
</tr>
<tr>
<td></td>
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<td>504</td>
<td>696</td>
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<td>Elite</td>
<td>8 (m), 1 (f)</td>
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<td>294</td>
<td>882</td>
<td>Cycling</td>
<td>Well-trained</td>
<td>12 (m)</td>
<td>9</td>
</tr>
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<td>3000</td>
<td>364</td>
<td>1271</td>
<td>Cycling</td>
<td>Sub-elite</td>
<td>5 (f)</td>
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<td>504</td>
<td>1411</td>
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<td>8 (m)</td>
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<td>504</td>
<td>907</td>
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<td>Well-trained</td>
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<td>294</td>
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<td>Endurance</td>
<td>Well-trained</td>
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<td>536</td>
<td>Triathlon</td>
<td>Well-trained</td>
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<td>712</td>
<td>Triathlon</td>
<td>Well-trained</td>
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<td>Football (AFL)</td>
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<td>Metres</td>
<td>Activity</td>
<td>Level</td>
<td>Hours</td>
<td>Gender</td>
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<td>594</td>
<td>Swimming</td>
<td>Elite</td>
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<td></td>
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<tr>
<td>Robach 2006</td>
<td>2500-3000</td>
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<td>Swimming</td>
<td>Elite</td>
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<td>Elite</td>
<td>4 (m), 2 (f)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Robertson et al (2010) protocol consisted of LHTL protocol with an additional 4 to 5 hr of hypoxia training per week. (1) natural, (2) simulated, (m) male, (f) female. Measures per participant includes baseline, during- and post- altitude measures. Km.hr measure is calculated as the accumulated altitude duration and altitude.

For example, if an athlete stays at 2000 m for 24 hr then stays at 3000 m for 336 hr km.hr is: \( ((2000 \text{ m}/1000) \times 24 \text{ hr}) + ((3000/1000) \times 336 \text{ hr}) = 1056 \text{ km}. \)
Chapter 3: The athlete’s response to hypoxia

3.3 Results

3.3.1 First two days at natural LHTH altitude (haemoglobin concentration only)

These data (n = 52) were analysed separately from the remaining during-altitude [Hb] analysis, as the observed changes were considerably larger than would be expected if there were no change in [Hb] with zero time at altitude. This is in contrast to the changes at subsequent times at natural altitude, and all times at simulated altitude, which were consistent with there being no change in [Hb] with zero time at altitude. The final model established baseline [Hb] values (p = 0.002) and sex (p = 0.000) to have a significant effect. The fixed effects in the final model explain 26% of the total observed variation.

3.3.2 During altitude: Haemoglobin concentration

Three models were considered with exponential functions (chosen in response to physiological considerations) of days at altitude, altitude hours or km.hr, used to represent altitude dose (n = 354). The difference between these three models was minimal (AIC = 652.1, 652.9 and 637.2, respectively) (Appendix A, Figure II), with a slight preference for km.hr. The final km.hr model included baseline [Hb] as a fixed effect, and a continuous AR(1) correlation structure within subjects.

The observed deviations in [Hb] from baseline values tend to increase with increasing altitude dose (Figure 3.1). After approximately 1000 km.hr at altitude, the observed [Hb] increase plateaus at 0.94 g/dL [95% CI (0.69, 1.20)]. Numerous factors potentially influencing [Hb] differentiation during altitude were investigated, yet only baseline [Hb] levels (p = 0.004) presented with statistical significance. Baseline [Hb] > 14.4 g/dL (females) and > 16.0 g/dL (males) presented with a negative correlation to the subsequent change value (p = 0.005). That is, large baseline [Hb] values are more likely to result in a smaller subsequent change value at altitude (Appendix A, Figure III). Age and sex were not found to be significant. This, however, is most likely a reflection of the restricted data set, with minimal female data and a homogeneous age range. The fixed effects in the final model explain 18% of the total observed variation in [Hb].
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3.3.3 During altitude: Reticulocyte percentage (square-root scale)

The final model for sqrt(RET%) (n = 295) included a cubic expression in log(km.hr). The model included sex (p = 0.005) and the baseline values of sqrt(RET%) as additional fixed effects. An allowance for different residual standard deviations for males and females and autocorrelation within subjects was also made. The form of autocorrelation used enabled an evaluation of the analytical SD, which was estimated to be 0.068. Baseline values again presented with a negative correlation (p = 0.000). An increase in delta RET% is observed within the first 300 km.hr before returning to basal levels as the altitude duration continues (Figure 3.1). Interestingly, when baseline samples were collected at 1200 m [11,13,30] the expected increase in RET% at the subsequent altitude of interest (2500 to 3000 m) was less substantial in comparison to changes observed when baseline samples were collected at sea level or close to. The fixed effects of the final model explain 10% of the total observed variation.

3.3.4 During altitude: OFF-score

The final model (n = 277) included a quadratic expression in log(km.hr). Additional fixed effects were sex (p = 0.001) and baseline values (p = 0.000), with an allowance for autocorrelation within subjects. OFF-score values appear to drop below baseline within the first 200 km.hr before increasing above baseline levels for values collected > 1000 km.hr, however the range of individual variation is wide (Figure 3.1). The fixed effects in the final model explained (only) 7% of the total observed variation.

3.3.5 Post altitude: Haemoglobin concentration

The final model consisted of an exponential function of the number of days post-altitude (again chosen in response to physiological considerations) with baseline [Hb] (p = 0.000), age (p = 0.043) and sex (p = 0.000) as additional fixed effects, and a continuous AR(1) correlation structure within subjects (n = 275). Post-altitude [Hb] values return to baseline levels within two weeks (Figure 3.2). However, individual responses vary, with some delta values falling below baseline within three days post altitude. The fixed effects in the final model explained 24% of the total observed variation.
Figure 3.1. Observed change values in [Hb], sqrt(RET%) and OFF-score during altitude. This data excludes [Hb] values collected on days 1 and 2 at LHTH altitude. The curve in each panel refers only to the km.hr (fixed effect) component of the final model.
3.3.6 Post altitude: Reticulocyte percentage (square-root scale)

The final model was fitted with quadratic terms in days post-altitude combined with an interaction with log(km.hr) and a continuous AR(1) correlation structure within subjects (n = 258). Baseline sqrt(RET%) (p = 0.000), age (grouped as < or ≥ 20 years) (p = 0.000) and sex (p = 0.014) were included as additional fixed effects. An allowance for autocorrelation within subjects, and different residual standard deviations for males and females was made. Values appear to decline below baseline levels post-altitude (Figure 3.2), and the magnitude of the decrease appears to be determined by the altitude dose (as km.hr). The fixed effects in the final model explained 21% of the total observed variation.

3.3.7 Post altitude: OFF-score

Post-altitude (n = 257) the model of best fit was one with a simple linear term in days post altitude, though the term did not reach statistical significance (p = 0.083). Also included in the model were sex (p = 0.000) and baseline values (p = 0.000). However, the majority (80%) of the post-altitude data for OFF-score values were obtained during the first seven days post-altitude. No clear trend was observed from the post-altitude model (Figure 3.2). The fixed effects in the final model explained 16% of the total observed variation.

3.3.8 Extreme change values in haemoglobin concentration

The extreme delta values for [Hb], sqrt(RET%) and OFF-score for both during- and post-altitude phases are described in Table 3.2. These values represent 1% of the data (refer to Appendix A, Figures III to VIII for dot plots). The data was limited for delta [Hb] during the first two days at altitude (natural, LHTH only) and not included in Table 3.2 (44 M, 8 F). For these data the largest, negative, change values for males and females were -0.3 and -0.4 (g/dL), respectively, and the maximum, positive, change values for males and females were 2.3 and 0.9 (g/dL), respectively.
Figure 3.2. Observed change values in $[\text{Hb}]$, $\sqrt{\text{RET}}\%$ and OFF-score post altitude. The curve(s) in each panel refers only to the days post-altitude component of the fitted model. For $\sqrt{\text{RET}}\%$ three curves are presented in response to altitude dose (as km.hr).
Table 3.2. Extreme change values (high and low) observed during- and post-altitude for delta [Hb] (g/dL), delta sqrt(RET\%) and delta OFF-score. Values represent 1% of each respective variable for the two phases of altitude (number of observations).

<table>
<thead>
<tr>
<th>DURING</th>
<th>Males</th>
<th>Min, Max</th>
<th>Females</th>
<th>Min, Max</th>
<th>All</th>
<th>Min, Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb] *</td>
<td>≤ -1.4 (2)</td>
<td>-1.5, 3.0</td>
<td>≤ -1.0 (1)</td>
<td>-1.0, 2.4</td>
<td>≤ -1.2 (4)</td>
<td>-1.5, 3.0</td>
</tr>
<tr>
<td>(n = 258 M, 87 F)</td>
<td>≥ 2.6 (3)</td>
<td></td>
<td>≥ 2.4 (1)</td>
<td></td>
<td>≥ 2.6 (3)</td>
<td></td>
</tr>
<tr>
<td>sqrt(RET%)</td>
<td>≤ -0.322 (2)</td>
<td>-0.343, 0.433</td>
<td>≤ -0.315 (1)</td>
<td>-0.315, 0.508</td>
<td>≤ -0.315 (3)</td>
<td>-0.343, 0.508</td>
</tr>
<tr>
<td>(n = 222 M, 73 F)</td>
<td>≥ 0.388 (3)</td>
<td></td>
<td>≥ 0.502 (2)</td>
<td></td>
<td>≥ 0.472 (3)</td>
<td></td>
</tr>
<tr>
<td>OFF-score</td>
<td>≤ -23.6 (2)</td>
<td>-24.3, 35.4</td>
<td>≤ -31.4 (1)</td>
<td>-31.4, 28.3</td>
<td>≤ -26.8 (3)</td>
<td>-31.4, 35.4</td>
</tr>
<tr>
<td>(n = 204 M, 73 F)</td>
<td>≥ 25.5 (2)</td>
<td></td>
<td>≥ 28.2 (1)</td>
<td></td>
<td>≥ 27.1 (3)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>POST</th>
<th>Males</th>
<th>Min, Max</th>
<th>Females</th>
<th>Min, Max</th>
<th>All</th>
<th>Min, Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb]</td>
<td>≤ -1.3 (2)</td>
<td>-1.4, 2.7</td>
<td>≤ -1.1 (1)</td>
<td>-1.1, 3.1</td>
<td>≤ -1.3 (2)</td>
<td>-1.4, 3.1</td>
</tr>
<tr>
<td>(n = 217 M, 67 F)</td>
<td>≥ 2.5 (2)</td>
<td></td>
<td>≥ 3.1 (1)</td>
<td></td>
<td>≥ 2.5 (3)</td>
<td></td>
</tr>
<tr>
<td>sqrt(RET%)</td>
<td>≤ -0.409 (2)</td>
<td>-0.415, 0.370</td>
<td>≤ -0.437 (1)</td>
<td>-0.438, 0.343</td>
<td>≤ -0.409 (3)</td>
<td>-0.438, 0.370</td>
</tr>
<tr>
<td>(n = 195 M, 63 F)</td>
<td>≥ 0.364 (2)</td>
<td></td>
<td>≥ 0.343 (1)</td>
<td></td>
<td>≥ 0.343 (3)</td>
<td></td>
</tr>
<tr>
<td>OFF-score</td>
<td>≤ -20.9 (2)</td>
<td>-23.2, 37.0</td>
<td>≤ -20.6 (1)</td>
<td>-20.7, 53.0</td>
<td>≤ -20.6 (3)</td>
<td>-23.2, 53.0</td>
</tr>
<tr>
<td>(n = 194 M, 63 F)</td>
<td>≥ 33.8 (2)</td>
<td></td>
<td>≥ 52.9 (1)</td>
<td></td>
<td>≥ 33.8 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* Delta [Hb] during altitude only includes data where LHTH > 2 days.

a Two of these values were from the same subject (but different subjects for [Hb], RET\% and the OFF-score).

b All three values were from the same subject.
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3.3.9 Within-subject variance calculations

Estimates of the within-subject standard deviation estimates for delta [Hb], sqrt(RET%) and OFF-score are given in Appendix A, Table II (estimates of the between-subject standard deviations are also given). The delta sqrt(RET%) analysis found evidence of different within-subject variability for males and females, with an estimated ratio of standard deviations (F/M) of 1.30 [95% CI (1.08, 1.60), p = 0.005] and 1.29 [95% CI (1.04 to 1.60), p = 0.018] for both during- and post-altitude data, respectively. This was also reflected in the OFF-score analysis with the F/M ratio estimated to be 1.22 [95% CI (0.95 to 1.55), p = 0.099] and 1.30 [95% CI (0.99 to 1.71), p = 0.050] during- and post-altitude, respectively.

3.4 Discussion

This meta-analysis describes the time course of the expected fluctuations in [Hb] and RET% and the resulting OFF-score variations both during- and post-altitude. A comprehensive review of the haematological response to hypoxia will allow both the ABP expert and sports physician to make a more informed decision on an athlete profile associated with altitude.

3.4.1 First two days at natural altitude (LHTH)

During the first two days of natural altitude an increase in [Hb] was observed, most likely the result of a plasma volume contraction and haemoconcentration.\textsuperscript{[40]} The known decrease in plasma volume during an ascent to altitude has been attributed (in part) to insufficient fluid replacement to offset a suppression of the renin-angiotension axis and increases in urinary, respiratory and transcutaneous fluid loss.\textsuperscript{[41]} Previously, plasma volume has been found to decrease within two hours of altitude exposure (at 3600 m), and is regarded as a short term acclimation response designed to stabilise the arterial oxygen content.\textsuperscript{[8]} The magnitude of the plasma volume contraction is proportional to hypoxic stress, where 3000 m ascents have been observed to result in a volume decrease of approximately -15\%.\textsuperscript{[40,42]} Interestingly, in this meta-analysis, no such volume-induced shift in [Hb] was observed in the initial days of simulated LHTL altitude studies. This may be due to the oscillating nature of the LHTL protocol, where an insufficient duration at altitude resulted in no dehydrating effects. Differences in the time of the blood withdrawal may also influence these results where
morning bloods (collected directly after a night at altitude) may be more concentrated in comparison to a later blood withdrawal (where time spent at sea-level may have allowed for the body to re-hydrate). It is therefore recommended that samples collected within the first week at altitude consider the impact of diuretic fluid loss and the resulting plasma volume contraction on concentration-based ABP markers, primarily [Hb] and the OFF-score.

3.4.2 During altitude

The observed delta [Hb] values during altitude exposure were indeed influenced by altitude dose. Within the initial 1000 km.hr [Hb] increased until reaching a plateau of 0.94 g/dL [95% CI, (0.69, 1.20)] (Figure 3.1). The predicted gradual increase in [Hb] supports previous findings that estimate an increase in HbM of 1% for every 100 hr spent at altitude, given a sufficient elevation.\(^{[9,26]}\) The observed increases from baseline [Hb] values (omitting the spike observed during the first two days of natural altitude) indicate a true increase in HbM, rather than a change influenced by plasma volume. Altitude protocol (LHTH vs LHTL) did not influence delta [Hb] (p > 0.05). However, it must be highlighted that the majority of LHTH studies were performed around 2500 m, while the majority of the LHTL studies were performed at 3000 m. The response of RET\% to hypoxic stress was immediate, with an increase from baseline levels observed within the first 300 km.hr (Figure 3.1), reflecting an increased rate of red cell turnover. The RET\% levels then returned to baseline, as supposedly the red cell mass had increased sufficiently to support optimal oxygen transport in the hypoxic environment.\(^{[43]}\) Inversely, the delta OFF-score values declined within the first 200 km.hr before returning to near baseline levels. For an altitude dose exceeding 1000 km.hr an increase in OFF-score values was observed. This is a reflection of the decrease in RET\% and increase in [Hb], which is predicted to reach a maximum increase by approximately 1000 km.hr (Figure 3.1).

Currently, the effect of altitude is interpreted within the ABP on an individual basis in accordance with the World Health Organisation (WHO) recommendations.\(^{[44]}\) These recommendations are based on a 1945 report of the [Hb] fluctuations associated with long-term altitude exposure, from a non-exercising population (the impact of long-term exposure on peripheral blood markers is beyond the scope of this meta-analysis). At altitudes of 3000 m, an increase of +1.9 g/dL in [Hb] is predicted by WHO, nearly twofold larger than the increase predicted within this current meta-analysis (Figure 3.1),\(^{[44]}\) suggesting the WHO
values may be too conservative in an anti-doping context (albeit other factors are taken into account when interpreting an ABP profile). Although elevation is a determining factor of the magnitude of change, duration at altitude, altitude protocol, and also the population of interest must be specified when considering the haematological fluctuations at altitude. It thus remains essential that altitude protocol, elevation and duration be recorded when collecting an anti-doping sample associated with a sojourn at altitude in order to gain understanding of the altitude dose that the athlete had been exposed to. These factors must also be considered when planning an athlete’s altitude training regime in order for the desired effects to be achieved.

3.4.3 Post-altitude

Currently, an athlete undergoing anti-doping testing is required to answer a number of questions specifying whether or not they have been at altitude at any time in the two-weeks prior to providing the sample. Yet, the time course to normalisation post-altitude is currently not well defined within the altitude literature. Here, normalisation of [Hb] baseline values occurred within two weeks post-altitude. However, Figure 3.2 shows some individuals with delta [Hb] values > 1 g/dL at 15 days post-altitude, while some values fell below baseline levels. Previous studies have demonstrated HbM to be on average 3.4% higher than pre-altitude values up to 20 days post-altitude,[45] while others have shown HbM to return to baseline from approximately one to two weeks post-altitude.[11,21] Decreases in HbM have also been observed as soon as three days [9] and nine days [15] post-altitude (-1.5% and -3%, respectively). In terms of [Hb] (as a concentration-based measure) the influence of altitude becomes increasingly ambiguous as the number of days post-altitude increases. Altitude is commonly used in preparation for competition and thus a tapering period may occur, which in turn may influence plasma volume levels and consequently [Hb].[46] Additionally, a change in climate when returning to sea level (natural altitude environments are often cooler) may also influence plasma volume and thus [Hb].[7,8] This highlights the multifaceted physiological response to altitude beyond that directly influenced by changes in oxygen availability.

Altitude dose (as km.hr) appears to influence the post-altitude RET% values. Post-altitude RET% data presents a decline from baseline levels. However, athletes who spent 1500 km.hr at altitude, as opposed to 500 km.hr at altitude, presented with a larger decrease (Figure 3.2).
This is most likely a reflection of a larger increase in red cell mass expected to result from a higher altitude dose. The mechanism behind post-altitude haematological fluctuations is still debated. However, decreases in [Hb] along with the more dramatic reduction in RET% (Figure 3.2), suggest EPO suppression on return to sea-level and a decline in erythroid progenitors. Decreases in EPO two days post-altitude, and subsequent declines in RET% at five to nine days post-altitude have been previously observed,[15] possibly the result of decreased red cell production in the bone marrow and/or increased peripheral destruction of red blood cells. Neocytolysis has also been suggested as a regulating mechanism of HbM when it is mal-adapted to its environment (i.e. in excess due to hypoxia). The sudden decreased in EPO on return to sea-level may result in the destruction of neocytes by reticuloendothelial phagocytes.[47] Although the exact mechanism of the decrease in RET% post altitude remains equivocal it is of importance to note that the decline is indeed influenced by altitude dose, and the level of the red cell production at altitude is therefore most likely to be a key influencing factor.

Despite the post altitude trends observed in both [Hb] and RET% the post-altitude OFF-score values were ambiguous (Figure 3.2). This is most likely a reflection of the data set where 80% was collected within the first seven days upon return to sea-level. Further research into the haematological response post-altitude is therefore recommended. However, the minimum two-week threshold for reporting altitude should be maintained for any anti-doping sample.

3.4.4 Baseline values

Baseline values were found to be a highly significant covariate in the analysis of all three variables both during- and post-altitude, with higher baseline values more likely to be associated with a smaller change value at altitude (Appendix A, Figure III). High baseline [Hb] values may be more adaptable to the low oxygen environment, thus dampening the erythropoiesis response and resulting in lower change values in both RET% and [Hb] at altitude. Rates of erythropoiesis at altitude may also be altered by functional iron deficiency (and low [Hb] values).[43] For example, four individuals were known to be iron deficient at baseline and removed from this meta-analysis due to irregular response patterns. Recording accurate baseline samples is thus integral to ensure the true haematological response to altitude is recorded so a precise assessment can be made by the sports physician. The altitude at which the baseline measure was collected should also be considered when analysing the
influence of altitude. Three studies collected pre-values at 1200 m (involving 4 days of initial testing), which appeared to influence the RET% response, resulting in smaller change values.\textsuperscript{[11,13,30]} It may be that this low altitude stimulated erythropoiesis prematurely, resulting in higher RET% baseline values and thus smaller than expected delta values during- and post-altitude. Although the data are limited, this highlights the importance of recording the location of the baseline sample collection.

### 3.4.5 Maximum changes and within-subject variability

The ABP is an individual-based anti-doping approach, a superior method because it caters for genetically unique individuals. This individualised approach also has applications when monitoring an athletes’ health status, where unique physiologies may be observed. This is demonstrated in the failure of the “no-start” rule\textsuperscript{[48]} whereby upper limits for [Hb] did not allow for athletes who have a genetic pre-disposition for higher values. Additionally, athletes who live and train at altitude must not be unfairly penalised or unjustly sanctioned, and the rate of false-positive results must be kept at an absolute minimum. It is thus important to acknowledge the extreme haematological fluctuations observed at altitude in order to comprehend the plausible limits of natural variations.

Individual studies support the notion that altitude improves performance, but not necessarily for all athletes\textsuperscript{[49-51]} and the response within an individual can also vary.\textsuperscript{[52]} Here, a small number of extreme individual delta values for [Hb], sqrt(RET%) and OFF-score were observed (representing 1% of the data) which fell both well above and below baseline levels (Table 3.2). A number of possible factors may contribute to these extreme delta values, beyond the influence of altitude. First, training load or fatigue may be a factor; although this was controlled in most studies, athletes may still be tempted to train beyond their normal volumes in the performance testing setting.\textsuperscript{[7,53]} Further, haematological assessments performed on a rest-day may influence the result.\textsuperscript{[54]} Timing of the blood withdrawal will also be influenced by the circadian rhythm of [Hb] and RET%,\textsuperscript{[55]} however, within this meta-analysis, the timing of the blood withdrawals varied between studies so no definitive conclusions can be made. Differences between studies in temperature and seasonal variation are not considered here but can influence the results.\textsuperscript{[7]} Finally, hydration status of the athlete was not controlled and is also a potential confounding factor.\textsuperscript{[56]} Although most of these factors are controlled for within the ABP paradigm, with strict sample collection protocols
(for example, a controlled length time for tourniquet use) and requirements for athletes to refrain from exercise two hours prior to testing, the altitude studies contributing data to this meta-analysis were not strictly anti-doping related, and therefore, the majority did not follow WADA protocol. The low proportion of the overall variation described by fixed effects components of the fitted models highlights the multifaceted response triggered by altitude and the associated training adaptations. Therefore, the haematological fluctuations presented here must be considered with caution.

It must also be highlighted that although a number of altitude studies have demonstrated false-positive ABP scores, altitude has not been found to systematically alter ABP variables. This may be due to the focus of the ABP algorithm on within-subject variability (after a number of individual values have been collected), as opposed to between-subject variation. The within-subject standard deviations both during- and post-altitude was relatively low for [Hb], sqrt(RET%) and the OFF-score (Appendix A, Table II) despite the range of individual responses observed (Figures 1 and 2). Interestingly, the within-subject SD for RET% (and to an extent the OFF-score within-subject SD) was greater in females compared to males. The female menstrual cycle may in part influence this; however, female data were limited within this meta-analysis, and further research into the difference in response to hypoxia by sex may be of value. The range of individual variation demonstrated within this meta-analysis highlights the complexity of the haematological response associated with altitude. Previous attempts to introduce altitude into the ABP algorithms were abandoned due to this issue of complexity. The multifaceted components associated with altitude (resulting in various haematological shifts) confirm that altitude, as a single factor, cannot effectively be entered within the ABP Bayesian network due to this issue of complexity. However, a combination of the current ABP paradigm and reviews of the altitude literature, as presented here, will allow experts to make more informed individual decisions.

3.4.6 Limitations

A wide-range of haematology analysers were used across the 17 altitude studies (ADVIA 120, Sysmex XT 2000i, Sysmex SE9000 and Sysmex XE2100). Studies performed post 2000 were chosen to limit the influence of analytical variability stemming from analyser technology. In addition, sample handling was not standardised across the studies and the number of times a single sample was analysed was often not specified. Data on training load
were not collected for this meta-analysis, although most studies controlled load. Furthermore, it cannot be confirmed that all participants were clean of performance-enhancing drugs, although all studies assumed their participants to be clean. The results presented here must therefore be considered with caution as other factors, besides an altitude effect, may contribute to the observed changes in peripheral blood markers.

3.4.7 Conclusion

The degree of the haematological fluctuation is dependent on various components of altitude training, for example the initial volume response vs. the subsequent erythrocye response, LHTH vs. LHTL, altitude dose and the baseline level of the marker of interest. Altitude therefore remains a complex factor that may not be reasonably introduced directly into the ABP algorithm. However, improved knowledge of the effect of altitude on peripheral blood markers is key to providing the expert with fundamental information required to make an informed judgement on a suspicious ABP profile involving altitude. This knowledge of the unique haematological fluctuations observed in an athlete population also provides invaluable information to the sports physician monitoring the health status of an athlete.

3.4.8 Summary and recommendations

- Delta [Hb] depends on shifts in plasma volume as well as the erythrocyte response to hypoxia. Plasma volume influences the volumetric marker, [Hb], during the first two days of LHTH (this was not observed in LHTL).
- RET% and the OFF-score were the first markers to describe the erythrocyte response to altitude.
- Altitude protocol (LHTH vs. LHTL), elevation and duration must be specified.
- Altitude remains an influencing factor to haematological variation up to two weeks post-altitude.
- Altitude dose (as km.hr) is proportional to the observed decline in post-altitude RET% values.
- Baseline levels influence subsequent delta values in [Hb], RET% and the OFF-score.
- A wide range of individual responses to altitude was observed.

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3.5 References


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The use of biomarkers to describe plasma-, red cell- and blood volume from a simple blood test

This chapter is based on a peer-reviewed paper accepted and published in the American Journal of Haematology:

4.0 Abstract

Plasma volume and red cell mass are key health markers used to monitor numerous disease states, such as heart failure, kidney disease or sepsis. Nevertheless, there is currently no practically applicable method to easily measure absolute plasma or red cell volumes in a clinical setting. Here, a novel marker for plasma volume and red cell mass was developed through analysis of the observed variability caused by plasma volume shifts in common biochemical measures, selected based on their propensity to present with low variations over time. Once a month for 6 months, serum and whole blood samples were collected from 33 active males. Concurrently, the CO-rebreathing method was applied to determine target levels of Haemoglobin Mass (HbM) and blood volumes. The variability of 18 common chemistry markers and 27 Full Blood Count variables was investigated and matched to the observed plasma volume variation. After the removal of between-subject variations using a Bayesian model, multivariate analysis identified two sets of 8 and 15 biomarkers explaining 68% and 69% of plasma volume variance, respectively. The final multi-parametric model contains a weighting function to allow for isolated abnormalities in single biomarkers. This proof-of-concept investigation describes a novel approach to estimate absolute vascular volumes, with a simple blood test. Despite the physiological instability of critically ill patients, it is hypothesised the model, with its multi-parametric approach and weighting function, maintains the capacity to describe vascular volumes. This model has potential to transform volume management in clinical settings.

**Key words:** Plasma volume, red cell volume, blood volume, biological variation, Bayesian inference.
Chapter 4: The use of biomarkers to describe plasma-, red cell- and blood volume

4.1 Introduction

Plasma volume, the liquid component of the blood, plays a crucial role in countless physiological processes and is a key variable in many clinical decisions.\textsuperscript{[1]} For example, dialysis treatment of patients with Chronic Kidney Disease (CKD) is based on the removal of a defined amount of intravascular fluid, while intensive care or heart failure patients require strict fluid monitoring to improve their health outcome. Furthermore, many variables measured in clinical chemistry are defined as concentrations in a given amount of blood, and thus are highly dependent on plasma volume. Nevertheless, there is currently no easily applicable method to accurately measure plasma volumes in a clinical setting.\textsuperscript{[2-5]} Most direct tests of plasma volume rely on indicator dilution methods and are time consuming, expensive and cumbersome in methodology. For these reasons, plasma volume is mostly indirectly estimated using concentration-based blood measures such as Haematocrit (HCT) and Haemoglobin Concentration ([Hb]), which are evaluated longitudinally, and changes in these variables are attributed to shifts in plasma volume.\textsuperscript{[6]} Such assessment methods are of limited utility as red cell mass stability and uniform vascular mixing is assumed; yet is not guaranteed, especially in clinical conditions where red cell mass is affected, such as CKD.

Red blood cells represent the main cellular constituent of blood and are the principal means of delivering oxygen to the body tissues. Measures of red cell volume are indicative of oxygen-carrying capacity, and traditionally involve concentration-based blood measures obtained as part of a full blood count. For example, the World Health Organisation (WHO) has specified some thresholds on [Hb] to define anaemia.\textsuperscript{[7]} However, this neglects the fact that [Hb] can be low because of a high plasma volume rather than a true decrease in red cell volume. This pseudo-anaemia may be observed in instances such as patients with kidney disease, deliberate hypotensive anaesthesia in intensive care, and as an athletic adaptation to aerobic exercise. Red cell volume can be directly measured after the injection of tracers in the body, such as radioactive markers \textsuperscript{[2-5]} or Carbon Monoxide (CO)\textsuperscript{[8-10]} but these methods are impractical in daily clinical practice. A viable method that provides a robust estimation of red cell mass or volume, or at least a substantive target, that can be used to titrate the dose of an erythropoiesis stimulating agent, for example, is currently still lacking in clinical practice.

Previous investigations have attempted to identify (novel) biomarkers capable of describing vascular volumes. Alis et al \textsuperscript{[11]} recently attempted to identify common serum biomarkers as markers for plasma volume; here, Total Protein (TP), Albumin (ALB), Gamma-Glutamyl
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Transferase (GGT) and Calcium (Ca) were found to correlate significantly with changes in plasma volume following high intensity exercise, and it was concluded that Ca concentration had potential as a biomarker of haemoconcentration in high-intensity exercise. The study however, focused on plasma volume changes using the Dill & Costill equation,\[6\] rather than calculating absolute volumes. Nevertheless, the presence of ‘plasma descriptive’ biomarkers was recognised. Subsequently, TP has been regarded as a potential marker of vascular volumes,\[11\] however, the protein is vulnerable to changes in the reflection coefficient of the vasculature; the so-called “capillary leak”, a common yet unpredictable condition observed in the critically ill. It is unlikely that a single biomarker will hold the capacity to describe absolute vascular volumes with precision, and, it is even less likely that a single biomarker may be applied to hospital patients where uniform physiology is uncertain. However, the ability to measure absolute blood volumes from a simple blood test presents a possible solution to the age-old problem of volume management. Further, the ability to calculate red cell mass has the potential to greatly enhance our capacity to monitor erythropoiesis.

Therefore, the aim of the present study is to develop and validate markers of red cell, plasma and blood volumes based on the consistency of the changes over time in a set of concentration-based variables after the removal of undesired between-subject biological variations. Most common biomarkers are known to present with significantly lower within-than between-subject variations.\[12\] Since all concentration-based analytes are affected the same way by plasma volume shifts, as opposed to a more specific effect caused by a pathology in a single or at least limited number of biomarkers, it is hypothesised that a combination of commonly measured biomarkers (associated to different, therefore somehow independent, physiological and/or pathological processes) can lead to a marker of plasma volume from a series of simple blood tests.

4.2 Methods

Thirty-three healthy, male, endurance athletes aged 25-53 years were monitored over six months (one test per subject per month). Subjects were withheld from the study if they presented with anaemia, polycythaemia, haemophilia, low blood pressure, or an acute pathology. All subjects provided written consent, which was approved by the Shafallah Medical Genetics Centre, Doha, Qatar (Project Nr. 2013-002). First, a serum sample (10 mL BD vacutainer serum tube, ref 367820, Plymouth, United Kingdom) and a whole blood
sample (4 mL BD vacutainer K2 EDTA tube, ref 367861, Plymouth, United Kingdom) were collected. Blood collection and analysis followed strict protocols recommended by the World Anti-Doping Agency (WADA) to minimise analytical variation.\[13\] Specifically, subjects were asked to refrain from physical exercise for 2 hr prior to blood collection (unless specified otherwise) and 10 min prior to blood collection, subjects were seated with their feet placed on the ground, allowing for equilibrium of vascular volumes. Second, the Haemoglobin Mass (HbM) of all subjects was determined using a Carbon Monoxide (CO) rebreathing method in order to calculate the control measure of plasma volume (see below). The subjects' body mass was also monitored. Elapsed times between the 6 monthly measures varied (minimum 10 days, maximum 76 days between measures) with a mean of 32 days between measures. At month 6, after completion of the final venous blood collection and CO-rebreathing manoeuvre (performed as per the prior 5 months), the 33 subjects performed an exercise challenge designed to promote an acute, maximal shift in plasma volume.\[14\] The exercise challenge involved a 30 min maximal step-test on a cycle ergometer (5 min steps of 25-50 W increments were completed until the subject reached volitional exhaustion) under standard laboratory conditions. Blood lactate, heart rate and Rating of Perceived Exertion (RPE, Borg Category-Ratio 10 scale)\[15\] were collected to quantify if the subject had reached a maximal effort. Subjects were asked to refrain from drinking during the effort to ensure a maximal plasma volume shift, however, on completion of the exercise subjects could drink water *ad libitum*. Immediately post- and 1 hr post-exercise, venous blood (serum and whole blood) was collected. The study design is depicted in Figure 4.1.

**4.2.1 Blood analysis**

All blood samples were analysed or aliquoted within 1 hr of collection under standard laboratory conditions. A complete blood count analysis was performed using a Sysmex XT 2000i analyser (Kobe, Japan). The following 27 measures reported by the instrument were used for further analysis: Haematocrit (HCT), Haemoglobin concentration [Hb], Platelets (PLT), White Blood Cells (WBC), Red Blood Cells (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Cell Distribution Width Standard Deviation (RDW_SD), Red Cell Distribution Width Coefficient of Variation (RDW_CV), Platelet Distribution Width (PDW), Mean Platelet Volume (MPV), Platelets Large Cell Ratio (P_LCR), Plateletcrit
(PCT), Neutrophils (NEUT), Monocytes (MONO), Eosinophils (EO), Basophils (BASO), Lymphocytes (LYMPH), Reticulocytes Number (RET#), Reticulocytes Percentage (RET%), Low Fluorescence Reticulocytes (LFR), Medium Fluorescence Reticulocytes (MFR), High Fluorescence Reticulocytes (HFR), Immature Reticulocyte Fraction (IRF), Reticulocytes Haemoglobin Content (RET_Hb) and Red Blood Cells Haemoglobin Content (RBC_Hb). All sample analyses were performed in accordance with the manufacturer’s specifications using routine laboratory procedures.

Serum samples were spun down at 4°C and 1500 rpm for 10 min, and aliquots immediately stored at -80°C. All serum samples were batch analysed 2 months after month 6 of sample collection in a random order with a Dimension Integrated Chemistry System (Siemens, Germany). The following 18 chemistry variables were analysed: Transferrin (Tfn), Alanine Aminotransferase (ALT), Albumin (ALB), Alkaline Phosphatase (ALP), Calcium (Ca), Chloride (Cl), Sodium (Na), Creatinine (CRE), Free Thyroxine (FT4), Triiodothyronine (T3), Thyroxine (T4), Total Protein (TP), Magnesium (Mg), Potassium (K), Cholesterol (CHOL), High-density Lipoprotein (HDL), Low-density Lipoprotein (LDL) and Triglyceride (TG). The 18 serum-based biomarkers described above were chosen as they are known to present with low within-subject variations. Exclusive of TG, FT4 and ALT, all 15 other biomarkers presented with lower within- than between-subject variations, and all within-subject coefficient of variations were lower than 10%, at the additional exception of ALP.

In total, 8 blood collections of these 45 blood variables on 33 subjects led to 11,880 readings.

4.2.2 Haemoglobin mass determination

The optimised CO-rebreathing method for measurement of HbM and blood volume was performed as previously described by Schmidt and Prommer,[8] with slight modifications (typical error given as a coefficient of variation is 1.3%). The method has been evaluated to have superior accuracy compared to other indicator dilution-based methods.[16] Capillary fingertip samples (200 uL) were collected pre- and 7 min post-CO administration; these were immediately analysed in quintuplet for percent Carboxyhaemoglobin (HbCO%) using an ABL80 flex blood gas analyser (Radiometer, Denmark). Lung CO levels were determined pre- and 4 min post-CO administration using a portable gas monitor (Dräger Pac 7000, Germany). At minute 0, a body mass adapted dose of CO (1.2 mL/kg) was applied as a tracer
through a closed rebreathing apparatus (glass spirometer) and rebreathed, together with pure oxygen, for 2 min. Total HbM was calculated through the difference of CO levels pre- and post-CO rebreathing, in consideration to the applied CO bolus, level of CO remaining in the lungs and environmental factors (temperature and barometric pressure). Equipment used within this procedure was subject to frequent internal and external quality controls and double baseline measures were collected in the first month (Figure 4.1).

Red cell, plasma and blood volumes were calculated as follows:

1) **Red Cell Volume (mL)** = HbM(g) ÷ MCHC (g/L) x 100

2) **Blood Volume (mL)** = HbM(g) x 100 ÷ [Hb] (g/dL) ÷ 0.91

3) **Plasma Volume (mL)** = Blood volume (mL) – Red cell volume (mL)

The scaling coefficient (0.91) in equation (2) refers to the F-cell ratio.\[17\]
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Figure 4.1 Study timeline. Seated model represents the pre-testing preparation i.e. no exercise 2 hr prior and subjects initially remained seated for 10 min. Droplets represent blood collection. CO symbol represents the CO-rebreathing procedure (collected immediately post blood withdrawal). 6a represents the 6th blood collection and HbM measure; subjects then immediately completed the maximal exercise effort (6b), blood samples were collected immediately (6c) and 1 hr (6d) post-exercise. One blood collection and HbM measure was performed per subject per month, with the exception of month 6 where two additional blood samples were collected post exercise challenge. The duplicate symbols under month 1 indicate the collection of a double baseline measure.
4.2.3 Statistical analysis and development of the plasma volume model

All data were analysed with Matlab version 8.3. First, a target for HbM was defined with the CO-rebreathing method. Analyses of variance with subject as the group variable were performed on all 45 biomarkers to derive within- and between-subject components of variance. An adaptive Bayesian model was applied to remove undesired between-subject components on all variables.\[^{18}\] The adaptive Bayesian model consists of a hierarchical Bayesian network that allows the derivation of individual distributions of any marker measured in biological fluid.\[^{19}\] Within- and between-subject components of variance are added as hard evidence in the Bayesian network so that a prior distribution can be generated. The Bayesian network allows then to move from prior distributions of biomarker values to posterior distributions when individual test results are further added as hard evidence.\[^{18,19}\] The use of two baseline values already allows for the removal of 80% of between-subject biological variations and is used to define individual reference ranges. The use of individual reference ranges is key to finding the biomarkers presenting with variations that are correlated to plasma volume shifts, as well as putting the effect of plasma volume shifts on the same scale for each biomarker. A Z-score formalism was chosen, with each Z-score value corresponding to the number of standard deviations from the expected mean computed by the adaptive model. Linear regression analysis was performed between the Z-score values found after the application of the adaptive model on plasma volume and the Z-scores computed for all serum-based biomarkers. The regression analysis was performed with and without the values obtained following the acute exercise challenge. A correction factor was included in the adaptive model to derive individualised values of biomarkers corrected for plasma volume variations. A leave-one-subject-out cross-validation procedure was used to limit over-fitting and prevent the use of parameters derived from data coming from the same subject, in order to guarantee a good generalisation of the model on new subjects.

In order to select the variables of interest, a Principal Component Analysis (PCA) was performed on various combinations of the 45 variables with the aim to explain the largest amount of the variance in plasma volume while finding a marker that is robust to changes in a single variable, or in a subset of variables that are correlated with each other. For example, HCT is strongly correlated to [Hb] and RBC, and all three variables are associated with red cell mass. If all three markers were chosen within the model, excessive weighting would be given to red cell mass as compared to plasma volume, making this particular biomarker set undesirable. Formally, if $N-I$ out of $N$ biomarkers present an increase (represented by $N-I$
positive Z-score values) and one biomarker presents a strong decrease (given by a Z-score significantly lower than zero), such as low platelets caused by an immune system problem, this inconsistency can be taken into account and a low weight attributed to the contribution of the platelet count in the computation of the marker of plasma volume. A weighting function has been determined as a normal probability density function applied on the residual of a first estimate of plasma volume shifts as determined from the PCA. A level of confidence can then be defined as the exponential of the sum of this weighting function normalised between 0 and 1. More explicitly, if $Z_i$ is a vector of size $N$ that represents the variations in each of the $i=1,...,N$ biomarkers ($Z_i$ represented as a Z-score obtained by the adaptive model), and $F_i$ a vector of size $N$ that represents the contribution of each biomarker $i$ as determined from the PCA (the higher the factor $F_i$, the higher the effect of marker $i$ in plasma volume shifts), a first estimate in plasma volume $\Delta PV_1$ is calculated as $\Delta PV_1 = \sum_{i=1,...,N} (Z_i \cdot F_i)$, where the subscript indicates that the sum applies for $i=1,...,N$. The residual $R_i$ becomes a vector of size $N$ given by $R_i = Z_i - \Delta PV_1 \cdot F_i$ and the weighting function $W_i$ another vector of size $N$ computed as the normal probability function of $R_i$. The final estimate in plasma volume shift $\Delta PV_2$ becomes $\Delta PV_2 = \sum_{i=1,...,N} (Z_i \cdot F_i \cdot W_i)$, i.e. the contribution of each marker $i$ is further weighted by $W_i$ and the confidence in this estimate is the exponential of the sum of the $W_i$ normalised between 0 and 1. For example, if all $N$ marker values show a relative increase over the individual baseline given by a $Z_i \approx 1.0$ for $i=1,...,N$, i.e. with small variations around 1.0 for all $N$ biomarkers, $\Delta PV_1$ will be close to 1.0 and the confidence level very high. On the other hand if one of the $N$ marker values show an important decrease, say $Z_j = -2.0$, this value is inconsistent with the other $N-1$ increases and in turn $W_j$ would be low for the one inconsistent $N$ marker value and $\Delta PV_2$ remains close to 1.0. If important deviations are observed between all $Z_i$ the level of confidence will be low. The higher the value of $N$ and the higher the independence of the $N$ biomarkers, the higher the chance of the model to catch variations associated to plasma volume shifts and not to other physiological and/pathological effect(s). Refer to Appendix B for detailed instructions of the final model and its practical application.

4.3 Results

The HbM correlated with body mass for all 33 subjects ($R^2=0.61$), allowing body mass to be used as a reference tool to estimate HbM (see Appendix B, Figure I). The mean, between- and within-subject components of variance are shown in Table 4.1 for all 45 variables. The data obtained after the acute exercise challenge were excluded from the analysis of variance.
as the data is not representative of normal variations of the biomarkers. The outputs of the adaptive model were given as a Z-score to represent the variations over an individual expected mean. A universal within-subject variance was assumed here.

Salts (Na, Cl, Ca, Mg) presented the most stable profiles, together with TP and ALB. However, good stability is not the only criterion that a biomarker should fulfil to exhibit a correlation with plasma volume. Table 4.1 shows the resulting p-values (significance set at p < 0.05) from the linear regression analysis, performed between the Z-score values found after the application of the adaptive model on plasma volume and the Z-scores computed for all serum-based biomarkers. When the exercise challenge data were taken into account, all 18 serum-based biomarkers showed a significant correlation with plasma volume at the notable exception of Cl and T3. When these data were excluded, HDL, ALT, ALP, K, Mg and Na no longer showed any association to plasma volume (Table 4.1). The same procedure was applied to all biomarkers obtained from the full blood count. The [Hb] showed low within-subject variations, as well as a strong correlation to plasma volume, as did WBC, PCT, NEUT and MONO (Table 4.1).

Two sets of biomarkers were found based on the criteria of the PCA a set of 8 biomarkers that included [Hb], Tfn, CRE, Ca, PLT, LDL, ALB and TP (Set 1), and a set of 15 biomarkers that included, in addition to the 8 above, CHOL, TG, T4, WBC, PCT, NEUT and MONO (Set 2). The first component of variance was able to explain 68% and 69% of the variations in plasma volume for the first and second set of biomarkers, respectively. In both cases, the marker with the highest contribution in the first component of variance was TP.

Figure 4.2 shows the relation between the measured changes in plasma volume (x-axis) and the relative changes in the marker of plasma volume (y-axis) (using the panel of 8 biomarkers, Set 1). The values found after the acute exercise challenge can be seen at the bottom left, with a diminution in plasma volume of up to 5-7 standard deviations from normal day-to-day variations.

Figure 4.3 represents the data from two randomly selected subjects (the same graphs for the remaining 31 subjects are provided in Appendix B, Figures II-XVII). The top panel compares the longitudinal profile of the variations in plasma volume, calculated with 1) the gold-standard CO-rebreathing method, and 2) the marker of plasma volume (using the panel of 8 biomarkers, Set 1). The bottom panel shows the confidence in the marker of plasma volume ranges from 25 to 100%.

Figure 4.4 provides a simplified step-by-step summary of the final plasma volume model.
Table 4.1. Mean, within-subject variation (WS\textsubscript{var}), between-subject variation (BS\textsubscript{var}), individual (CV\textsubscript{i}) and group (CV\textsubscript{g}) coefficients of variation of whole blood, serum and volume variables.

<table>
<thead>
<tr>
<th>Whole Blood Variables</th>
<th>Mean</th>
<th>WS\textsubscript{var}</th>
<th>BS\textsubscript{var}</th>
<th>CV\textsubscript{i}</th>
<th>CV\textsubscript{g}</th>
<th>(P_{\text{with exercise}})</th>
<th>(P_{\text{without exercise}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb] (g/dL) (^1)</td>
<td>14.8</td>
<td>0.186</td>
<td>0.449</td>
<td>2.91</td>
<td>4.53</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>42.29</td>
<td>1.2860</td>
<td>2.7548</td>
<td>2.68</td>
<td>3.92</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>PLT (x10(^3)/\mu L) (^1)</td>
<td>241</td>
<td>362</td>
<td>2870</td>
<td>7.89</td>
<td>22.23</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>WBC (x10(^3)/\mu L) (^2)</td>
<td>701</td>
<td>5895</td>
<td>17500</td>
<td>10.96</td>
<td>18.88</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>RBC (x10(^6)/\mu L)</td>
<td>485</td>
<td>213</td>
<td>1014</td>
<td>3.01</td>
<td>6.57</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>87.4</td>
<td>0.569</td>
<td>13.1</td>
<td>0.86</td>
<td>4.14</td>
<td>0.125</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.6</td>
<td>0.0805</td>
<td>1.41</td>
<td>0.93</td>
<td>3.88</td>
<td>&lt; 0.005</td>
<td>0.989</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>35.0</td>
<td>0.142</td>
<td>0.27</td>
<td>1.07</td>
<td>1.48</td>
<td>0.122</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>RDW_SD (fL)</td>
<td>39.8</td>
<td>0.691</td>
<td>3.60</td>
<td>2.09</td>
<td>4.77</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>RDW_CV (%)</td>
<td>12.6</td>
<td>0.0511</td>
<td>0.182</td>
<td>1.79</td>
<td>3.38</td>
<td>&lt; 0.005</td>
<td>0.726</td>
</tr>
<tr>
<td>PDW (fL)</td>
<td>114</td>
<td>40.6</td>
<td>155</td>
<td>5.60</td>
<td>10.95</td>
<td>&lt; 0.005</td>
<td>0.508</td>
</tr>
<tr>
<td>MPV (x10(^2)/fL)</td>
<td>98.2</td>
<td>9.71</td>
<td>40.9</td>
<td>3.17</td>
<td>6.51</td>
<td>&lt; 0.005</td>
<td>0.333</td>
</tr>
<tr>
<td>P_LCR (%)</td>
<td>241</td>
<td>520</td>
<td>2272</td>
<td>9.46</td>
<td>19.76</td>
<td>&lt; 0.005</td>
<td>0.370</td>
</tr>
<tr>
<td>PCT (x10(^3)/%)</td>
<td>23.4</td>
<td>3.45</td>
<td>19.8</td>
<td>7.94</td>
<td>19.03</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>NEUT (x10(^3)/\mu L) (^2)</td>
<td>362</td>
<td>5551</td>
<td>5824</td>
<td>20.59</td>
<td>21.09</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>MONO (x10(^3)/\mu L) (^2)</td>
<td>63.2</td>
<td>84.4</td>
<td>173</td>
<td>14.54</td>
<td>20.87</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>EO (x10(^3)/\mu L)</td>
<td>20.9</td>
<td>37.1</td>
<td>162</td>
<td>29.08</td>
<td>60.79</td>
<td>&lt; 0.005</td>
<td>0.767</td>
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<tr>
<td>BASO (x10(^3)/\mu L)</td>
<td>3.52</td>
<td>0.84</td>
<td>2.54</td>
<td>26.10</td>
<td>45.26</td>
<td>&lt; 0.005</td>
<td>0.135</td>
</tr>
<tr>
<td>LYMPH (x10(^3)/\mu L)</td>
<td>353</td>
<td>2415.10</td>
<td>2909.80</td>
<td>13.91</td>
<td>15.27</td>
<td>&lt; 0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>RET (x10(^3)/\mu L)</td>
<td>573</td>
<td>8936.40</td>
<td>15894.00</td>
<td>16.48</td>
<td>21.98</td>
<td>&lt; 0.005</td>
<td>0.049</td>
</tr>
<tr>
<td>%RET (%)</td>
<td>1.18</td>
<td>3.86</td>
<td>5.13</td>
<td>16.69</td>
<td>19.23</td>
<td>0.143</td>
<td>0.483</td>
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<tr>
<td>LFR (x10(^3)/%)</td>
<td>924</td>
<td>611</td>
<td>485</td>
<td>2.67</td>
<td>2.38</td>
<td>0.233</td>
<td>0.047</td>
</tr>
<tr>
<td>MFR (x10(^3)/%)</td>
<td>67.5</td>
<td>454</td>
<td>359</td>
<td>31.58</td>
<td>28.07</td>
<td>0.469</td>
<td>0.133</td>
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<tr>
<td>HFR (x10(^3)/%)</td>
<td>9.87</td>
<td>41.2</td>
<td>6.16</td>
<td>65.10</td>
<td>25.15</td>
<td>0.402</td>
<td>0.014</td>
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<tr>
<td>IRF (x10(^3)/%)</td>
<td>75.5</td>
<td>611</td>
<td>485</td>
<td>32.74</td>
<td>29.17</td>
<td>0.234</td>
<td>0.047</td>
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<table>
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<tr>
<th>Chemistry Variables</th>
<th>Mean</th>
<th>$WS_{var}$</th>
<th>$BS_{var}$</th>
<th>$CV_i$</th>
<th>$CV_g$</th>
<th>$P_{exercise}$ with</th>
<th>$P_{without}$ exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfn (mg)$^1$</td>
<td>2.56</td>
<td>0.03</td>
<td>0.06</td>
<td>6.42</td>
<td>9.16</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>37.7</td>
<td>64.5</td>
<td>57.4</td>
<td>21.30</td>
<td>20.10</td>
<td>0.007</td>
<td>0.752</td>
</tr>
<tr>
<td>ALB (g/L)$^1$</td>
<td>41.9</td>
<td>3.85</td>
<td>4.65</td>
<td>4.65</td>
<td>5.15</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
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<tr>
<td>ALP (U/L)</td>
<td>69.0</td>
<td>102</td>
<td>155</td>
<td>14.64</td>
<td>18.04</td>
<td>&lt; 0.005</td>
<td>0.443</td>
</tr>
<tr>
<td>Ca (mmol/L)$^1$</td>
<td>2.30</td>
<td>0.01</td>
<td>0.004</td>
<td>3.82</td>
<td>2.85</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Cl (mmol/L)</td>
<td>100</td>
<td>4.32</td>
<td>0.67</td>
<td>2.08</td>
<td>0.82</td>
<td>0.444</td>
<td>0.005</td>
</tr>
<tr>
<td>Na (mmol/L)</td>
<td>138</td>
<td>6.01</td>
<td>0.21</td>
<td>1.78</td>
<td>0.33</td>
<td>&lt; 0.005</td>
<td>0.082</td>
</tr>
<tr>
<td>CRE (U/L)$^1$</td>
<td>91.0</td>
<td>63.7</td>
<td>75.5</td>
<td>8.77</td>
<td>9.55</td>
<td>&lt; 0.005</td>
<td>0.024</td>
</tr>
<tr>
<td>FT4 (mIU/L)</td>
<td>0.97</td>
<td>0.003</td>
<td>0.01</td>
<td>5.9</td>
<td>11.8</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>T3 (nmol/L)</td>
<td>1.02</td>
<td>0.29</td>
<td>0.02</td>
<td>52.43</td>
<td>13.04</td>
<td>0.221</td>
<td>0.525</td>
</tr>
<tr>
<td>T4 (µg/dL)$^2$</td>
<td>5.43</td>
<td>0.18</td>
<td>0.66</td>
<td>7.81</td>
<td>14.96</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>TP (g/L)$^1$</td>
<td>74.7</td>
<td>8.66</td>
<td>8.54</td>
<td>3.94</td>
<td>3.91</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>0.81</td>
<td>0.001</td>
<td>0.004</td>
<td>4.62</td>
<td>7.41</td>
<td>&lt; 0.005</td>
<td>0.593</td>
</tr>
<tr>
<td>K (mmol/L)</td>
<td>4.26</td>
<td>0.08</td>
<td>0.02</td>
<td>6.54</td>
<td>3.43</td>
<td>&lt; 0.005</td>
<td>0.313</td>
</tr>
<tr>
<td>CHOL (mmol/L)$^2$</td>
<td>4.76</td>
<td>0.21</td>
<td>0.38</td>
<td>9.63</td>
<td>12.95</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.39</td>
<td>0.03</td>
<td>0.09</td>
<td>11.82</td>
<td>21.58</td>
<td>&lt; 0.005</td>
<td>0.163</td>
</tr>
<tr>
<td>LDL (mmol/L)$^1$</td>
<td>2.89</td>
<td>0.09</td>
<td>0.37</td>
<td>10.38</td>
<td>21.05</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>TG (mmol/L)$^2$</td>
<td>1.27</td>
<td>0.26</td>
<td>0.16</td>
<td>40.15</td>
<td>31.50</td>
<td>&lt; 0.005</td>
<td>0.025</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume Measures</th>
<th>Mean</th>
<th>$WS_{var}$</th>
<th>$BS_{var}$</th>
<th>$CV_i$</th>
<th>$CV_g$</th>
<th>$P_{exercise}$ with</th>
<th>$P_{without}$ exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbM (g)</td>
<td>885</td>
<td>385</td>
<td>23000</td>
<td>2.22</td>
<td>17.14</td>
<td>0.48</td>
<td>0.71</td>
</tr>
<tr>
<td>Plasma Volume (L)</td>
<td>3.47</td>
<td>0.03</td>
<td>0.40</td>
<td>4.74</td>
<td>18.23</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

In the ‘variables’ column (1) indicates biomarkers of sets 1 and 2 of the plasma volume model; (2) indicates the additional markers involved in set 2 of the plasma volume model. $P_{without~exercise}$ indicates the p-value obtained with and without the exercise data included. *Data obtained after exercise were excluded, as these are representative of extreme biomarker variations associated with an atypical biological state.*
Figure 4.2. Measured changes in plasma volume (CO rebreathing method) versus the changes in the newly developed marker of plasma volume. Changes are given in number of deviations from an individual mean as computed by an adaptive Bayesian model. Plus marker represents measures taken post exercise; Circle marker represents no exercise; $R^2=0.72$. The panel of 8 biomarkers, Set 1, is applied here.
**Figure 4.3. Application of the plasma volume marker in two subjects.** *Top:* measured variations (given as a Z-score) in plasma volume calculated with the gold-standard CO-rebreathing method (black) and variations in plasma volume estimated using the panel of 8 biomarkers, Set 1 (grey). *Bottom:* confidence in plasma volume marker. Test number indicates months 1 to 6 sample collections; test 7 represents post-exercise collection; test 8 represents 1hr post-exercise collection.
Figure 4.4. Summary of the plasma volume model. " Target values can be calculated from anthropometric characteristics, such as body mass, or estimated based on published population references. * Method remains applicable when only partial information is available.
4.4 Discussion

In this proof of concept investigation, a marker of plasma volume is described, developed from the within-subject variations of a set of concentration-based variables, measured in whole blood and serum. This novel marker explains ~67% of the variation in plasma volume. Additionally, the derivation of the marker of plasma volume, together with a classic measurement of [Hb], allows for the estimation of HbM.

At present, clinical volume management is focused on indirect measures, encompassing dialysis machines or devices, which monitor, in real-time, HCT and or [Hb] and report percentage changes in intravascular volumes. Although this approach provides an instantaneous, non-invasive measure of relative blood volumes, a number of confounding factors influence the accuracy. First, concentration changes of blood constituents assumes the total amount of constituent in circulation is constant and mixing is uniform, which is often not the case in hospital patients. Second, the approach is dependent on hydration status, where an identical decrease in relative blood volume can result from very different absolute blood volume values, particularly during haemodialysis. Finally, changes in posture, food-intake, exercise and the administration of intravenous fluids have the potential to influence the method’s validity. Clinicians must therefore currently interpret relative blood volume measures with caution. The plasma volume model, described here, circumvents a number of the confounding factors described above. First, the application of PCA allows for the identification of an optimal combination of biomarkers, with low within-subject variation, which, correlate with plasma volume variations, yet when combined, avoids excessive weighting to a single factor (Table 4.1). This multi-parametric approach ensures good generalisation of the model, allowing for its application to individual scenarios such as an abnormal reading in a single marker, or non-uniform mixing of a single constituent. Hydration status of the patient is irrelevant, as the plasma volume model estimates absolute volumes, as opposed to relative values. The model presented here does not require full knowledge of heterogeneous factors (for example, age, sex, ethnicity), biological pathways, and or previous individual biomarker values. It is able to make the best description of vascular volumes with partial information. This is made possible with the application of serial observations. Individual vascular volumes can be inferred from biomarker values following the effect to cause (and not the usual cause to effect) relationship using Bayesian inference.
By applying a multi-parametric approach the likelihood of correctly explaining vascular volumes is improved. This has been demonstrated here with two sets of biomarkers identified to correlate highly with plasma volume change. The first set incorporates [Hb], Tfn, CRE, Ca, PLT, LDL, ALB and TP and explains 68% of plasma volume variance, while the second set includes all the latter biomarkers with the addition of CHOL, T4, WBC, PCT, NEUT and MONO, explaining 69% of plasma volume variance. Although the second set increases the percentage of explained variance by 1% only, it includes some markers associated to other biological processes, and therefore is believed to be more robust in situations when multiple biomarkers can be affected by multiple factors other than plasma volume. It is possible that the two sets of markers are pathology-specific, where one set is more appropriate for an individual scenario. Further, TP has been identified as the highest contributor to the first component of variance in both Set 1 and Set 2.

The proposed multi-parametric approach has the advantage of improved specificity with an increased number of biomarkers. In addition to the multi-parametric approach, a weighting function has been applied to the model described to allow for isolated abnormalities in a single biomarker. For example, ALB is an inverse acute phase reactant that is actively metabolised, and also equilibrates with the interstitial space. Approximately 5% of plasma ALB crosses into the extravascular space every hour, increasing to 15% per hour in patients with septic shock.\[20\] Thus, ALB levels can decline significantly in the critically ill. It is generally accepted that the measured volume of ALB distribution exceeds plasma volume, yielding exaggerated results of vascular volumes (false high values); this is true in all patients with impaired capillary integrity. The addition of a weighting function allows for a significant decline in ALB by compensating the abnormal value with the results from the additional plasma volume descriptive biomarkers. Therefore, despite the physiological instability of critically ill patients, the model described here, with its multi-parametric approach and additional weighting function, maintains the capacity to describe absolute vascular volumes. The weighting function thus allows for one abnormal biomarker reading; however, in a case where more than three of the plasma volume markers are abnormal, the confidence of the model would be low. Further validation studies are therefore required to assess the behaviour of the model in non-healthy patients.

Furthermore, the novel marker of plasma volume, described here, together with a classic measurement of [Hb], allows for the estimation of HbM using the formulae (1) to (3). As [Hb] is dependent on both the total mass of circulating haemoglobin (HbM) and the plasma...
volume in which it is suspended, it is possible to infer HbM with knowledge of [Hb] and plasma volume. HbM has been identified as a potential marker to monitor the clinical status of patients as it is the primary determinant of the total oxygen-carrying capacity of blood and physical fitness, both of which have a positive association with reduced patient mortality and morbidity.\textsuperscript{21,22} Additionally, HbM may provide an insight into the level of anaemia in perioperative or critical care patients. Anaemia is associated with adverse health outcomes, however, it is often not well defined by [Hb] \textsuperscript{7} as this biomarker is influenced by shifts in plasma volume. A clinically available estimation of HbM, by application of the proposed model for plasma volume, may thus provide further insight into the link between anaemia and adverse health outcomes, thereby optimising patient monitoring and intervention strategies.

An innovative approach to assess blood volumes has been presented here, where a panel of biomarkers (easily obtainable through a simple blood test and standard chemistry and whole blood analysis) hold the capacity to define a patient’s plasma volume, red cell volume, blood volume and red cell mass, enabling physicians to monitor a patient’s fluid balance and erythropoiesis. The method can be applied to all fields in which a strict balance between red cell and plasma volumes is required. Nevertheless, further studies to validate the model in unhealthy patients are still necessary.
Chapter 4: The use of biomarkers to describe plasma-, red cell- and blood volume

4.5 References


Chapter 4: The use of biomarkers to describe plasma-, red cell- and blood volume


A step towards removing plasma volume variance from the Athlete’s Biological Passport: The use of biomarkers to describe vascular volumes from a simple blood test

This chapter is based on a peer-reviewed paper *accepted and published* in Drug Testing and Analysis:

5.0 Abstract

The haematological module of the Athlete’s Biological Passport (ABP) has significantly impacted the prevalence of blood manipulations in elite sports. However, the ABP relies on a number of concentration-based markers of erythropoiesis, such as Haemoglobin Concentration ([Hb]), which are influenced by shifts in plasma volume. Fluctuations in plasma volume contribute to the majority of biological variance associated with volumetric ABP markers. Our laboratory recently identified a panel of common chemistry markers (from a simple blood test) capable of describing ~67% of plasma volume variance, presenting an applicable method to account for volume shifts within anti-doping practices. Here, this novel plasma volume marker was included into the ABP adaptive model. Over a 6-month period (one test per month), 33 healthy, active males provided blood samples and performed the CO-rebreathing method to record plasma volume (control). In the final month participants performed a single maximal exercise effort to promote a plasma volume shift (mean plasma volume decrease -17%, 95% CI -9.75 to -18.13%). Applying the ABP adaptive model, individualised reference limits for [Hb] and the OFF-score were created, with and without the plasma volume correction. With the plasma volume correction, an average of 66% of [Hb] within-subject variance is explained, narrowing the predicted reference limits, and reducing the number of atypical ABP findings post-exercise. Despite an increase in sensitivity there was no observed loss of specificity with the addition of the plasma volume correction. The novel plasma volume marker presented here has the potential to improve the ABP’s rate of correct doping detection by removing the confounding effects of plasma volume variance.

Key words: Plasma volume, biological variation, Bayesian inference, blood doping, Athlete’s Biological Passport
5.1 Introduction

An enhanced understanding of biological variability and the confounding factors influencing haematological fluctuations form the foundations of the anti-doping tool, the Athlete’s Biological Passport (ABP). Since its introduction in 2008, the haematological module of the ABP has contributed to a significant decline in blood manipulations in elite sports,[1] providing a timeless anti-doping tool, focused on irregular physiological changes, independent of the development of new illicit blood manipulation techniques.[2] Based on Bayesian principles, the ABP identifies abnormal patterns in an individual’s blood profile through the development of individualised reference limits for key biomarkers of erythropoiesis.[2,3] However, a number of key haematological markers of the ABP are measured as a concentration in plasma (for example Haemoglobin Concentration, \([\text{Hb}]\)), and can thus be influenced by shifts in plasma volume. In an average male, plasma makes up 55% of the body’s total blood volume and can rapidly change within hours or days. The variability associated with plasma volume becomes more problematic within an athlete cohort undergoing haematological testing, since exercise,[4-6] altitude,[7,8] time of competitive season,[6] heat,[9,10] posture[11] and hydration status[12,13] are all major external dictators, with the ability to shift vascular volumes by more than 10%. [14,15] Such extreme plasma volume changes consequently influence the concentration-based haematological variables of the ABP, which may therefore confound ABP results.[16] For example, an unscrupulous athlete can mask an abnormally high \([\text{Hb}]\) by simple haemodilution, and alternatively, a clean athlete suffering from severe dehydration (haemoconcentration, and increased \([\text{Hb}]) may be flagged with a suspicious ABP profile. Currently, in an anti-doping case where plasma volume shifts are claimed to be the cause for an irregular profile, it is the role of the expert evaluating the profile to make a judgment based on the available information, as to whether or not the abnormality is truly the result of a natural volume shift or if a blood manipulation is the most probable cause.[17]

Originally, Haemoglobin Mass (HbM), estimated via the Carbon Monoxide (CO) rebreathing procedure,[18] had been suggested as a method to circumvent the confounding effects of plasma volume variability.[19-22] A measure of HbM is independent of shifts in plasma volume as the technique calculates the absolute mass of haemoglobin in an individual, rather than the \([\text{Hb}]\). However, the application of the CO-rebreathing method into anti-doping practices was abandoned due to a number of practical factors. First, the administration of CO to elite athletes involves complex ethical issues; second, the method requires the test-subject to be
fully compliant (providing opportunities for dishonest athletes to “cheat” the test); third, a potentially toxic gas cannot be administered to healthy competing athletes; and finally, the test cannot be standardised between laboratories, as it requires a human, not a machine, to perform the protocol. Alternative methods to directly measure plasma volume include the injection of radioactive tracers, such as $^{51}$Chromium ($^{51}$Cr), or the administration of dyes, such as indocyanine green, which bind to plasma proteins to provide volume estimates.$^{[23,24]}$ However, these techniques are often time-consuming and cumbersome, and the inherent dangers of radioactive isotopes render such methods unsuitable for anti-doping testing. Recently, a promising method to estimate absolute plasma volume from a simple blood test was demonstrated in our laboratory.$^{[25]}$ The association between normal biological variations observed in common serum biomarkers and plasma volume (calculated using the gold-standard CO-rebreathing method)$^{[26]}$ identified a panel of biomarkers capable of describing ~67% of plasma volume variance.$^{[25]}$ This novel approach (requiring only a blood test) has potential applications in anti-doping, where the variance associated with plasma volume can be quantified in a way which cannot be easily manipulated by an unscrupulous athlete, and which does not compromise the health of an athlete undergoing routine anti-doping testing.

In this investigation, the application of the novel marker for plasma volume will be applied to the haematological module of the ABP. It is hypothesised that by removing the variance component associated with plasma volume fluctuations, the sensitivity of the ABP reference calculations will be enhanced. The capacity to estimate an individual’s plasma volume will reduce the possibility for dishonest athletes to mask a blood manipulation by expanding their plasma volume, or to fraudulently claim plasma volume fluctuations to be the cause of an abnormal ABP profile.

5.2 Methods

The experimental methods used by our laboratory are described in detail elsewhere.$^{[25]}$ In summary, 33 healthy, male, endurance-trained athletes aged 25-53 years were monitored periodically over six months (one test per subject per month). All subjects provided written consent, approved by the Shafallah Medical Genetics Center, Doha, Qatar (Project Nr. 2013-002). The subject cohort was assumed to be clean of any banned substance, but this cannot be proven with certainty.
Chapter 5: Removing plasma volume variance from the Athlete’s Biological Passport

The monthly testing followed the protocol subsequently described. First, a serum sample (10 mL BD vacutainer serum tube, ref 367820, Plymouth, United Kingdom) and a whole blood sample (4 mL BD vacutainer K2 EDTA tube, ref 367861, Plymouth, United Kingdom) were collected. Blood collection and analysis followed strict protocols, recommended by the World Anti-Doping Agency (WADA), in order to minimise pre analytical and analytical variability.[27] Last, the HbM of all subjects was determined using the optimised CO-rebreathing method in order to calculate the control measure of plasma volume.[18] Double baseline measures were performed in month 1.

Red cell, plasma and blood volumes were calculated as follows:

1. \( \text{Red Cell Volume (mL)} = \frac{\text{HbM(g)}}{\text{MCHC (g/dL)}} \times 100 \)

2. \( \text{Blood Volume (mL)} = \frac{\text{HbM(g)} \times 100}{[\text{Hb}] (\text{g/dL})} \div 0.91 \)

3. \( \text{Plasma Volume (mL)} = \text{Blood volume (mL)} - \text{Red cell volume (mL)} \)

The scaling coefficient (0.91) in equation (2) refers to the F-cell ratio.[28]

At month 6, whole blood and serum samples were collected, followed by the CO-rebreathing manoeuvre (performed as per the prior 5 months). Subjects were then transferred to a cycle ergometer where they performed an exercise challenge designed to promote an acute, maximal shift in plasma volume.[29] The exercise challenge involved a 30 min maximal step-test on a cycle ergometer (5 min steps of 25-50 W increments were completed until the subject reached volitional exhaustion) under standard laboratory conditions. Blood lactate, heart rate and Rating of Perceived Exertion (RPE, Borg Category-Ratio 10 scale) [30] were collected to quantify the attainment of a maximal effort (blood lactate > 6mmol.L\(^{-1}\), max heart rate > 90%, RPE score ≥ 9, cycling cadence ≥ 80rpm).[31] Subjects were asked to refrain from drinking during the effort to ensure a maximal plasma volume shift, however, on completion of the exercise, subjects could drink water \textit{ad libitum}. Immediately post- and 1 hr post-exercise, venous blood (serum and whole blood) was collected (Figure 5.1).
Figure 5.1. Summary of Month 6 testing procedure. The testing procedures represented by the first 3 boxes (seated 10 min, blood collection and CO-rebreathing) were performed exactly as per the previous 5 months of testing. However, at month 6, immediately after completion of the CO-rebreathing procedure the subject was moved to a cycle ergometer where he completed a 10 min warm up at a self-selected power (<100 W). The subject then began the step-test (grey columns). Each grey column represents 5 min steps at a set power output, with the workload increasing by 25 or 50 W (dependent on the subject's fitness level) every 5 min. The test involved ~6 steps (30 min of cycling) with the final step being a maximal effort. The starting threshold was selected based on the individual’s fitness and training history, here a starting threshold of 150 W is given as an example. Down arrows represent an assessment of the subject’s effort (via lactate, heart rate and RPE data collection). Immediately upon completion of the maximal cycling effort the subjects were seated and blood was collected. Subjects remained seated for a further 1 hr before the final blood collection.
A complete blood count analysis, including Reticulocytes, was performed within 1 hr of blood collection using a Sysmex XT 2000i analyser (Kobe, Japan). Serum samples were batch analysed (on completion of the 6 months of data collection) in a random order with a Dimension Integrated Chemistry System (Siemens, Germany) for the following 18 chemistry variables (approximately 1.5 mL serum per subject was required): Transferrin (Tfn), Alanine Aminotransferase (ALT), Albumin (ALB), Alkaline Phosphatase (ALP), Calcium (Ca), Chloride (Cl), Sodium (Na), Creatinine (CRE), Free Thyroxine (FT4), Triiodothyronine (T3), Thyroxine (T4), Total Protein (TP), Magnesium (Mg), Potassium (K), Cholesterol (CHOL), High-density Lipoprotein (HDL), Low-density Lipoprotein (LDL) and Triglyceride (TG). The above 18 serum-based biomarkers were chosen due to their propensity to present with low within-subject variations over time. All sample analyses were performed in accordance with the manufacturer’s specifications using routine laboratory procedures.

An in-depth description of the statistical methods used to develop the final plasma volume model can be found elsewhere. Our previous work identified two optimal sets of ‘volume descriptive’ biomarkers, including Set 1 using 8 biomarkers ([Hb], Tfn, CRE, Ca, PLT, LDL, ALB and TP), and a Set 2 using 15 biomarkers (encompassing the 8 biomarkers of Set 1 in addition to CHOL, TG, T4, WBC, PCT, NEUT and MONO) explaining 68 and 69% of plasma volume variations. In the current investigation, the set of 8 biomarkers (Set 1) was applied to the ABP paradigm.

A detailed step-by-step application of the plasma volume model can be found in the supplementary material of our previous work. In brief, target values for plasma volume are first defined (here, the CO-rebreathing method was used to estimate plasma volume priors, however, anthropometric characteristics, such as body mass, can also be used to establish priors) and an initial individual distribution for the 8 plasma volume biomarkers was calculated with an adaptive model. After the establishment of target values (month 1), additional serum and whole blood samples were collected and analysed for the set of 8 plasma volume markers (months 2 to 6). An adaptive Bayesian model was applied to the plasma volume markers to derive individual Z-scores for each marker. A weighting function was then applied to combine the individual marker Z-scores to calculate the Z-score associated with plasma volume. A confidence level was associated to the plasma volume Z-score. Finally, an estimate of plasma volume was made from the expected mean, returned by the adaptive model, together with the estimated Z-score and confidence level.
Using an adaptive Bayesian model and the principles of the ABP, with a set specificity level of 99% and 99.9%, a longitudinal profile for each subject was created, where individual [Hb] and OFF-score values (OFF-score = [Hb] (g/dL) x 10 − 60 x \(\sqrt{\text{RET}\%}\)) \(^{[33]}\) were entered as hard evidence into the Bayesian model to compute individualised reference limits. Individual results of the panel of 8 plasma volume markers were then derived from the adaptive model (Figure 5.2) and the estimation of plasma volume (at the time of sample collection) was used to correct the individual ABP reference calculations.

Figure 5.3 provides an overview from sample collection and analysis to the development of individual reference limits within the ABP paradigm with the proposed addition of the plasma volume correction.
Figure 5.2. Graphical representation of the Bayesian model used to interpret a marker M. Here, M is a vector of size 8, representing the 8 markers of plasma volume shifts or the OFF-score or [Hb]. Sex (male, female) and age (year) are heterogenous factors entered as hard evidence, with a linear effect on the prior individual mean (µ) of size 8. When the 8 markers of plasma volume are measured in an individual, the values are entered as hard evidence and the plasma volume is estimated using Bayesian inference. A universal standard deviation (σ) is chosen for all markers. Then, knowledge of the plasma volume is used to correct the individual means, this time following the causal direction. Finally, individual limits of M are derived for a given specificity level.
Chapter 5: Removing plasma volume variance from the Athlete's Biological Passport

Collect Serum and EDTA samples from Athlete X and transport to an accredited laboratory

→

Analyse EDTA sample for ABP haematological variables and PV markers (PLT, WBC, PCT, NEUT and MONO)

Analyse Serum sample for PV markers (Tfn, CRE, Ca, LDL, ALB, TP, CHOL, TG and T4)

→

Apply adaptive model to all variables

→

Calculate the change in PV *

→

Apply the PV correction factor to concentration-based ABP variables ([Hb] and the OFF-score)

→

Haematological data is entered into Athlete X's ABP electronic file (with the PV correction applied to the [Hb] and the OFF-score values) and individual reference limits are updated

→

Abnormal values falling outside the calculated individual limits for Athlete X are electronically flagged and analysed by an expert panel

Figure 5.3. Applying the plasma volume (PV) correction to the ABP paradigm. First, an additional serum sample must be collected, prior to EDTA whole blood collection, to avoid sample contamination. All sample collection, transport, analysis and storage must follow WADA protocol to limit the influence of analytical variation. Refer to Lobigs et al. for a detailed description of the plasma volume correction calculations.
5.3 Results

Table 5.1 shows the frequency of atypical values (i.e. a value that falls outside the predicted reference range, which in this scenario would be regarded as false positives) recorded with and without the plasma volume correction (for 99% and 99.9% specificity levels). There was no change to the frequency of false positive [Hb] values with and without the plasma volume correction without exercise (99% set specificity, 3/228, p = 1). Samples collected immediately post-exercise presented with more false positive [Hb] values. However, the frequency of false positive [Hb] values post-exercise was significantly reduced when the plasma volume correction was applied (p < 0.0001). All [Hb] values recorded 1 hr post-exercise returned to (near) pre-exercise levels. A similar pattern was observed for OFF-score results (Table 5.1).

Figure 5.4 shows the results of the adaptive model for the 8 plasma volume markers (A to H). All 8 plasma volume markers show similar changes over time despite being associated with different biological pathways and biological functions. These similarities in changes between the 8 plasma volume markers are best represented by the correlation of the individual marker z-score sequences (Figure 5.4, J). The confidence in the plasma volume marker (Figure 5.4, K) is high when there is consistency in the plasma volume marker z-scores and low when there is a high dispersion. For example, for Test 8 (1 hr post-exercise) 5 of 8 plasma volume marker z-scores are close to the individual mean (z-scores close to zero), but LDL, TP and ALB are higher than the individual mean (z-scores ≥ 1) (Figure 5.4, J), this results in a low confidence level (~30%) (Figure 5.4, K). Nevertheless, much of the variance in plasma volume is explained by our model when comparing the plasma volume z-scores calculated with the gold-standard CO-rebreathing method and the plasma volume model (Figure 5.4, L). When applied to all subjects, an analysis of variance (with “subject” as random effect) of the [Hb] data resulted in the removal of an average of 66% of [Hb] within-subject variance after the application of the plasma volume correction. Despite this, the frequency of atypical values did not increase, that is, despite an improvement in the sensitivity of the reference limit calculations with the application of the plasma volume correction, no loss of specificity was observed in [Hb] or OFF-score calculations without exercise (Table 5.1, Figure 5.4, A and I).

A significant decline in plasma volume was observed during the exercise challenge, calculated using the CO-rebreathing method (minimum -8.2%, maximum -26.97%). Immediately post-exercise, plasma volume decreased by an average of -17.04% (95% CI - 9.75 to -18.13 %). At 1 hr post-exercise plasma volume was on average -2.11% (95% CI - 0.09 to -3.22 %) less than pre-exercise measures.
Table 5.1. Frequency of individual [Hb] and OFF-score values exceeding the predicted reference ranges calculated by the adaptive model with and without the plasma volume correction (for 99% and 99.9% set specificity levels).

<table>
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<tr>
<th>Haemoglobin Concentration</th>
<th>No Exercise</th>
<th>Post Exercise</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99%</td>
<td>3/228</td>
<td>23/31</td>
<td>26/259</td>
</tr>
<tr>
<td>99.9%</td>
<td>0/228</td>
<td>11/31</td>
<td>11/259</td>
</tr>
<tr>
<td><strong>With Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99%</td>
<td>3/228</td>
<td>1/31</td>
<td>4/259</td>
</tr>
<tr>
<td>99.9%</td>
<td>0/228</td>
<td>0/31</td>
<td>0/259</td>
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<table>
<thead>
<tr>
<th>OFF-Score</th>
<th>No Exercise</th>
<th>Post Exercise</th>
<th>All</th>
</tr>
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<tr>
<td><strong>Without Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99%</td>
<td>1/228</td>
<td>3/31</td>
<td>4/259</td>
</tr>
<tr>
<td>99.9%</td>
<td>0/228</td>
<td>1/31</td>
<td>1/259</td>
</tr>
<tr>
<td><strong>With Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99%</td>
<td>2/228</td>
<td>0/31</td>
<td>2/259</td>
</tr>
<tr>
<td>99.9%</td>
<td>0/228</td>
<td>0/31</td>
<td>0/259</td>
</tr>
</tbody>
</table>

*Note: 33 subjects were monitored over the course of this investigation, however, 5 samples were missing, resulting in n = 259.*
Figure 5.4. Observed stability of the plasma volume model and a comparison of the [Hb] and OFF-score reference limit calculations with and without the plasma volume correction. All graphs are derived from the data of Subject 9. Test number (x-axis) indicates the sample collections from months 1 to 6; test 7 represents post-exercise collection; test 8 represents 1 hr post-exercise collection. Graphs A to H show the results of the adaptive model for all 8 plasma volume markers ([Hb], Tfn, ALB, Ca, CRE, TP, PLT, LDL). Solid blue line represents Subject 9’s individual test result across the 6 months of testing. Red lines represent the reference limits calculated with the ABP adaptive model. For dashed red lines specificity is set at 99%, for solid red lines specificity is set at 99.9%. Graph A and I represent Subject 9’s [Hb] and OFF-score values (blue line), with calculated 99% (dashed red lines) and 99.9% (solid red lines) reference limits with (green lines) and without (red lines) the plasma volume correction. Graph J represents the individual z-scores for each plasma volume marker (blue = [Hb], red = Tfn, yellow = ALB, black = Ca, green = CRE, pink = TP, cyan = PLT, dashed blue = LDL). Graph K represents the calculated confidence level in the plasma volume model calculations. Graph L represents the change in plasma volume given as individual z-score calculations, calculated using a) the gold-standard CO-rebreathing method (blue) and b) the plasma volume model (red).
5.4 Discussion

By applying this novel plasma volume marker to the ABP paradigm, an average of 66% of [Hb] within-subject variance can be explained, differentiating between true changes in HbM and changes caused by shifts in plasma volume. Thus, the introduction of the plasma volume correction has the potential to refine the ABP’s ability to detect blood manipulations by improving the sensitivity of the calculated probability distributions of principal volumetric ABP markers, with no evidence that (with exercise cases excluded) the models specificity (rate of false positives) is different.

5.4.1 A multi-parametric approach to adjust for plasma volume variance

In the current ABP paradigm, plasma volume fluctuations represent a large portion of the biological variability associated with volumetric haematological parameters, once inter-individual variance components (heterogeneous factors such as age, sex and ethnicity) are removed with the development of individualised reference intervals.[34] Artificial plasma volume expanders can thus influence an athlete's ABP profile,[35] and several professional athletes have already been banned from competitive sport after the detection of a banned plasma volume expander, Hydroxyethyl Starch (HES), within their sample.[36] Such compounds are most likely administered to mask the effect of other prohibited substances,[37] such as an increased [Hb] resulting from recombinant erythropoietin (rhEPO) abuse. Additionally, studies have shown the simple acute intake of a high volume of water can expand plasma volume by around 4% within 40 to 60 min of ingestion, consequently decreasing volumetric ABP markers such as the OFF-score and [Hb].[15] The percentage of athletes found to have an atypical blood profile by the ABP (sensitivity) is thus challenged by plasma volume shifts.

To circumvent this plasma volume problem, the introduction of tracer techniques to directly estimate plasma volume have been suggested,[20,22] yet remains practically unsuitable for anti-doping assessments. Biomarkers to adjust for vascular shifts present an alternative approach. Previously, ALB was identified as a candidate plasma volume marker,[15,38] however, ALB is influenced by altitude, exercise[39] and nutritional status.[40,41] As such, it is unlikely that an individual marker will present with sufficient stability and low variability, which would be required to accurately and reliably identify plasma volume shifts in an anti-doping context. Consequently, a multi-parametric approach using common chemistry markers has been
suggested by our laboratory as a superior approach to estimate plasma volume, having the potential to be introduced into anti-doping practices.

5.4.2 A practical application of the plasma volume correction in anti-doping

The performance difference between Set 1 (8 biomarkers) and 2 (15 biomarkers) described previously is minimal, thus Set 1 was chosen here as a more cost effective approach. The analysis would require approximately 1.5 mL of serum (equating to a 3 mL serum vacutainer). Additionally, T4 (of Set 2) is likely to be unsuitable as an anti-doping biomarker due to the prevalence of thyroid medication in elite sports.

The initial step to developing individual plasma volume estimations using the plasma volume markers previously described is the establishment of target values (Figure 5.3). Body mass was found to correlate with plasma volume \( r^2 = 0.61 \), and is a non-invasive method to develop valid targets that may be applied in an anti-doping setting. Bayesian modeling was subsequently used to develop Z-scores for the 8 identified ‘volume descriptive’ biomarkers. This is a key step to removing the undesired inter-individual variance components of the selected biomarkers, and is key to the development of individualised results. From the calculated variance values of the set of 8 “plasma descriptive” biomarkers, plasma volume estimations can be inferred (Figure 5.3). The further addition of the weighting function within the plasma volume model allows for isolated abnormalities in a single “plasma descriptive” biomarker. This may occur, for example, in pathologies that affect mainly one variable of the panel: In the case of an athlete with congenital erythrocytosis, an inherited condition characterised by an increased number of erythrocytes and abnormally high [Hb], the plasma volume model would put less “weight” on [Hb] (one marker of the panel of 8, Set 1) so as to maintain an accurate estimation in this particular athlete. When more than one biomarker is abnormal, the confidence in the plasma volume model would be low (Figure 5.4, K). The addition of the weighting function is essential to reduce the probability of a false positive result. Once absolute plasma volume estimations for an individual athlete have been made, this information can be included into the adaptive model of the ABP to correct for changes in plasma volume (Figure 5.2). Both the novel plasma volume marker and the ABP follow the unique effect to cause direction of Bayesian inference; this allows for the calculation of individual plasma volume estimations and ABP reference ranges with only partial information.
Chapter 5: Removing plasma volume variance from the Athlete’s Biological Passport

5.4.3 Validation of the plasma volume marker during exercise

Currently, WADA protocols require athlete sample collections to occur 2 hr post-exercise or post-competition.[27] This protocol is designed, in part, to reduce the variability in the volumetric ABP parameters resulting from an exercise-induced haemoconcentration. Interestingly, in this investigation, no [Hb] value exceeded the predicted ABP reference intervals 1 hr post-exercise, verifying the minimal probability of a sample being affected by an exercise-induced plasma volume shift 2 hr post-physical activity. However, immediately post-exercise, subject 9 (Figure 5.4, A) recorded a [Hb] value outside of the predicted reference intervals (set specificity of 99%); in an anti-doping scenario this value would be flagged as atypical. This is true for 74% (99% specificity) of the post-exercise [Hb] values, highlighting the confounding results that a natural plasma volume shift can yield. When the plasma volume correction was applied one [Hb] value (3%) exceeded the 99% predicted reference limits post-exercise (where plasma volume decreased by approximately -17%). As expected, the OFF-score was more robust during the exercise-induced plasma volume shift with 3 of 31 samples recording atypical values (at 99% specificity level), due to the influence of Reticulocyte% (a non-volumetric marker) in the OFF-score algorithm.[33] It is assumed that the plasma volume marker would also describe shifts resulting from the use of illegal plasma volume expanders (given that the expanding agent influences no more than one of the plasma volume model biomarkers); however, further investigations are warranted in this context.

5.4.4 Further standardisation of the plasma volume marker

The success of the current ABP model has been in part due to the extremely stringent sample collection, transport, storage and analysis protocols, and it is essential that similarly strict protocols be established for the ‘volume descriptive’ biomarkers of the plasma volume model. The 8 biomarkers of the plasma volume marker were chosen based on their propensity to present with low variation over time. Nevertheless, additional research into the stability of the 8 proposed biomarkers during sample analysis between different labs is required to minimise the influence of analytical variability on the results. Frequent and standardised quality control assessments of the laboratory analysers must also be performed to minimise the within-laboratory variability. Given that most of these biomarkers are widely used in clinical practice, it can be assumed that adequate quality control schemes exist in order to minimise this risk. An assessment of the stability of the 8 plasma volume biomarkers, for
example, during heat-induced dehydration\textsuperscript{[13]} or an altitude-induced plasma volume shift\textsuperscript{[46]} would provide valuable information on the validity of the current plasma volume model. Further validation of the performance of the plasma volume model within an elite athlete cohort is also recommended. Finally, future research to apply the plasma volume markers in blood transfusion or Erythropoiesis Stimulating Agent (ESA) studies is recommended. It is hypothesised that blood manipulation techniques would only influence [Hb] and T\(\text{fn}\) and the remaining six plasma volume biomarkers (CRE, Ca, PLT, LDL, ALB and TP) would remain stable. With the addition of the weighting function (allowing for an abnormal marker value), the plasma volume correction would, in theory, remain valid following an illicit blood manipulation, however, the confidence level may be impacted if more than one biomarker is highly abnormal.

In summary, plasma volume shifts are a major confounder within the current ABP paradigm, allowing unscrupulous athletes to avoid detection by diluting their anti-doping test sample. The simplicity of the novel method used to estimate vascular volume from a routine blood test presented here, provides a desirable solution to the current confounding effects of plasma volume shifts within the ABP. With further research and validation studies, this plasma volume marker presents an encouraging step forward for anti-doping practices.
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5.5 References


Chapter 5: Removing plasma volume variance from the Athlete’s Biological Passport


Chapter 5: Removing plasma volume variance from the Athlete’s Biological Passport


Chapter 5: Removing plasma volume variance from the Athlete’s Biological Passport


Validation of a blood marker for plasma volume in endurance athletes during a live high: train low altitude training camp

This chapter is based on a peer-reviewed paper accepted and published in Drug Testing and Analysis:

Chapter 6: Validation of a blood marker for plasma volume

6.0 Abstract

Altitude is a confounding factor within the Athlete’s Biological Passport (ABP) due, in part, to the plasma volume response to hypoxia. Here, a newly developed plasma volume blood test is applied to assess the possible efficacy of reducing the influence of plasma volume on the volumetric ABP markers; Haemoglobin Concentration ([Hb]) and the OFF-score. Endurance athletes (n = 34) completed a 21 night simulated live high: train low protocol (14 hr/day at 3000m). Bloods were collected twice pre-altitude, at days 3, 8 and 15 at altitude and 1, 7, 21 and 42 days post-altitude. A full blood count was performed on the whole blood sample. Serum was analysed for Transferrin, Albumin, Calcium, Creatinine, Total Protein, and Low-Density Lipoprotein. The plasma volume blood test (consisting of the serum markers, [Hb] and platelets) was applied to the ABP adaptive model and new reference predictions were calculated for [Hb] and the OFF-score, thereby reducing the plasma volume variance component. The plasma volume correction refined the ABP reference predictions. The number of Atypical Passport Findings (ATPFs) for [Hb] was reduced from 7 of 5 subjects to 6 of 3 subjects. The OFF-score ATPFs increased with the plasma volume correction (from 9 to 13, 99% specificity); most likely the result of more specific reference limit predictions combined with the altitude-induced increase in red cell production. Importantly, all abnormal biomarker values were identified by a low confidence value. Although the multifaceted, individual physiological response to altitude confounded some results, the plasma volume model appears capable of reducing the impact of plasma volume fluctuations on [Hb].

Key words: Vascular volumes, biological variation, Bayesian inference, blood doping, Athlete’s Biological Passport
6.1 Introduction

Rather than the direct detection of a doping substance, the Athlete’s Biological Passport (ABP) is focused on the physiological changes resulting from doping practices (i.e., indirect detection). However, the ABP relies on a number of concentration-based markers, including Haemoglobin Concentration ([Hb]) and the OFF-score (OFF-score = [Hb] (g/dL) x 10 – 60 x √(RET%), and plasma volume fluctuations currently represent the majority of biological variation associated with these markers. Recently, plasma volume shifts resulting from acute hyper-hydration or an increase in training load have been found to influence an ABP profile. An alternative scenario where plasma volume shifts occur naturally is during exposure to altitude. It is well documented that altitude promotes a natural reduction in plasma volume in the first few days of ascent from sea level, reflected by an increase in [Hb]. As an athlete adapts to the low oxygen environment, red cell production is stimulated and a true increase in Haemoglobin Mass (HbM) is commonly observed. Of note, altitude is especially problematic to the ABP, as athletes commonly live and train in hypoxic environments to naturally improve their performance, often in the weeks preceding a major competition. Nevertheless, there is currently no method in place to correct volumetric ABP markers for such naturally occurring plasma volume shifts.

Recently, our laboratory developed a novel method to estimate absolute plasma volume through the observed variability of a panel of common biomarkers. This novel marker for plasma volume, requiring only a blood test, was suggested as a potential tool to adjust for volume fluctuations influencing the concentration-based markers of the ABP. By applying the plasma volume correction to the adaptive model of the ABP, the specificity of the resulting reference limit calculations were refined. However, before the plasma volume correction can be applied to anti-doping practices, validation of the model in a variety of relevant situations is necessary.

Within this study, an attempt is made to reduce the confounding effects of plasma volume shifts at altitude with the application of the plasma volume correction to the ABP adaptive model. It is hypothesised that altitude will stimulate an initial reduction in plasma volume; however, with the application of the plasma volume correction to the ABP adaptive model, this natural fluctuation of plasma volume will be accounted for.
6.2 Methods

34 sub-elite endurance athletes (19 m, 15 f) with at least 3 years of training history completed 21 days of simulated LHTL altitude training. Mean ± standard deviation for age and maximal oxygen uptake (VO\textsubscript{2max}) of males and females was 28.1 ± 9.2 years and 65.9 ± 6.6 (ml.kg\textsuperscript{-1}.min\textsuperscript{-1}) and 29.9 ± 6.3 years and 54.2 ± 5.6 (ml.kg\textsuperscript{-1}.min\textsuperscript{-1}), respectively. Athletes spent on average 14 hr/day at a simulated altitude (normobaric hypoxia) of 3000 m. All training was conducted at normoxia (~600m, Canberra, Australia). Participants were excluded if they showed signs of iron overload (haemochromatosis, haemosiderosis), polycythaemia, iron deficiency, or recently received a blood transfusion or donated blood. Baseline haematology and HbM measures\textsuperscript{[9]} were collected -14 and -1 day prior to altitude exposure (B1, B2) and then at days 3, 8 and 15 at altitude (A3, A8, A15) and +1, +7, +21 and +42 days post-altitude (P1, P7, P21, P42). Note that HbM was not measured at A3. A schematic diagram of the testing protocol is found in Figure 6.1. As part of a parallel investigation, subjects were split into three iron supplementation groups, placebo (no supplementation), oral supplementation or a course of intravenous (IV) injections. Details of the iron supplementation protocol\textsuperscript{[10]} and HbM protocol\textsuperscript{[7]} can be found elsewhere. Venous blood was sampled following strict World Anti-Doping Agency (WADA) blood collection and analysis protocol.\textsuperscript{[11]} A 3 mL whole blood K\textsubscript{2}EDTA sample (BD vacutainer, Ref 367856, Plymouth United Kingdom) was stored at room temperature (21 degrees) and analysed for Haemoglobin Concentration ([Hb]), Platelets (PLT), Mean Corpuscular Haemoglobin Concentration (MCHC), and Reticulocytes Percentage (RET%) on a Sysmex XT 2000i (Kobe, Japan) in automatic mode within 24 hours. An 8.5 mL serum sample (BD vacutainer, Ref 367988, Plymouth, United Kingdom) was collected, stored at room temperature for 15 min before centrifugation at 4°C and 1500 rpm for 10 min. Serum was aliquoted and stored at -80°C and batch analysed in random order with a COBAS Integra 400 (Roche Diagnostics, Switzerland) for the following 6 chemistry variables: Transferrin (Tfn), Albumin (ALB), Calcium (Ca), Creatinine (CRE), Total Protein (TP), and Low-Density Lipoprotein (LDL). The Australian Institute of Sport human ethics committee approved the study and all subjects provided written consent before participating.
Figure 6.1. Study design. Baseline HbM calculations (determined by the CO-rebreathing test) and blood collection were performed on day -14 and -1 day prior to altitude (B1, B2) (represented by the droplet). Subsequent measures occurred at days 3, 8 and 15 at altitude (A3, A8, A15) and +1, +7, +21 and +42 days post altitude (P1, P7, P21, P42). HbM was not collected at A3. Subjects received an oral supplement (iron or placebo glucose tablets) daily from day -14 to day 21 at altitude and three IV injections (iron or placebo), prescribed at day -14, day -1 prior to altitude and day 10 at altitude (represented by the needle).
Chapter 6: Validation of a blood marker for plasma volume

6.2.1 Statistics

A validation of the previously describe plasma volume model is performed here. Detailed descriptions of the statistical development of the plasma volume model and its application to the adaptive model of the ABP paradigm can be found elsewhere. Within the plasma volume model a confidence level is associated with the calculated plasma volume Z-score, returned by the model. The confidence level is equal to the exponential sum of the weighting function (calculated as a normality probability distribution of residuals in the variations of the markers) and is normalised between 0 and 1, so that values close to 0 have low confidence while values close to 1 have high confidence. The confidence level weights the variance that is used to calculate the Z-score associated to plasma volume shifts. Confidence in the plasma volume model prediction is, therefore, related to the uniformity of the plasma volume marker Z-scores.

By applying an adaptive Bayesian model, and the principles of the ABP, a longitudinal profile for [Hb] and OFF-score calculations for each individual subject was produced (specificities were set at both 99% and 99.9%). The individual [Hb] and OFF-score values were entered into the Bayesian model, which produced individualised reference limits. The individual estimations of plasma volume, derived from the panel of 8 plasma volume markers ([Hb], Tf, CRE, Ca, PLT, LDL, ALB, and TP) applied to an adaptive model, were then used to correct the individual ABP reference calculations for [Hb] and the OFF-score.

6.3 Results

Table 6.1 quantifies the mean change from baseline levels (using the B2 measure only) in [Hb] and plasma volume. The largest plasma volume mean change from baseline occurred at A8 in males (-9.6%) and A15 in females (-5.3%), calculated from the individual change values from baseline. The largest %change from baseline in plasma volume occurred concurrently with the largest %change value in [Hb]. The individual plasma volume response to altitude varied, and females presented with more variable change values (ranging from -19.7% at A3 to 15.9% at A15) in comparison to males who tended to present with plasma volume contractions from baseline levels (ranging from -26.8% at A8 to 9.0% at P42).

From a total of 283 observations from 34 subjects the number of Atypical Passport Findings (ATPFs) for [Hb] was reduced when the plasma volume model was applied. Without the
correction, 7 ATPFs of 5 subjects (99% set specificity) and 3 of 3 subjects (99.9% set specificity) were recorded. This was reduced to 6 of 3 subjects (99% specificity) and 1 (99.9% specificity) ATPF with the plasma volume correction (Table 6.2, the original [Hb] ATPF data are presented elsewhere).[10] However the OFF-score recorded an increased number of ATPFs with the inclusion of the plasma volume correction (at a set specificity of 99%). The ATPFs were increased from 9 of 8 subjects (99% set specificity) without the correction, to 13 of 11 subjects with the correction (Table 6.3). At a 99.9% set specificity, the OFF-score ATPFs were reduced from 4 of 4 subjects to 3 of 3 subjects. Two values were removed due to high abnormal chemistry values resulting from sampling errors (A15 from Subject 24 and P7 from Subject 29).
Table 6.1. Observed change values from baseline in [Hb] and plasma volume.

<table>
<thead>
<tr>
<th>Study Day</th>
<th>[Hb] (g/dL)</th>
<th>Plasma Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>%Δ (min, max)</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>14.8 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>A3</td>
<td>15.2 ± 0.8</td>
<td>3.3 (-6, 13.7)</td>
</tr>
<tr>
<td>A8</td>
<td>15.7 ± 0.8</td>
<td>6.7 (-1.3, 20.6)</td>
</tr>
<tr>
<td>A15</td>
<td>15.7 ± 0.8</td>
<td>6.7 (0, 19.2)</td>
</tr>
<tr>
<td>P1</td>
<td>15.6 ± 0.9</td>
<td>5.7 (-1.3, 17.1)</td>
</tr>
<tr>
<td>P7</td>
<td>15.4 ± 0.8</td>
<td>4.2 (-2.5, 13.9)</td>
</tr>
<tr>
<td>P21</td>
<td>15.2 ± 0.6</td>
<td>2.2 (-3.4, 8.7)</td>
</tr>
<tr>
<td>P42</td>
<td>15.0 ± 0.7</td>
<td>1.3 (-3.8, 12.3)</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>13.6 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>A3</td>
<td>14.0 ± 1.0</td>
<td>3.5 (-6.8, 15.1)</td>
</tr>
<tr>
<td>A8</td>
<td>14.2 ± 0.7</td>
<td>4.5 (-4.2, 10.1)</td>
</tr>
<tr>
<td>A15</td>
<td>14.4 ± 1.1</td>
<td>6.3 (-7.6, 18.5)</td>
</tr>
<tr>
<td>P1</td>
<td>14.4 ± 0.8</td>
<td>5.9 (-2.1, 15.3)</td>
</tr>
<tr>
<td>P7</td>
<td>14.1 ± 0.7</td>
<td>2.6 (-1.4, 8.5)</td>
</tr>
<tr>
<td>P21</td>
<td>13.8 ± 0.7</td>
<td>0.4 (-6.1, 8.7)</td>
</tr>
<tr>
<td>P42</td>
<td>13.8 ± 0.7</td>
<td>1.9 (-3.5, 12.2)</td>
</tr>
</tbody>
</table>

All placebo subjects (n = 9; 5 M, 4 F) have been removed to reduce the impact of differences in the erythropoietic response to altitude influencing [Hb] and thus plasma volume values.
Table 6.2. Atypical passport findings for [Hb] with and without the plasma volume correction

<table>
<thead>
<tr>
<th>Subject</th>
<th>Test</th>
<th>ATPF (upper or lower limit)</th>
<th>Confidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>A15</td>
<td>Lower</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>A3</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>A8</td>
<td>Upper *</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>A15</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S17</td>
<td>A3</td>
<td>Upper *</td>
<td></td>
</tr>
<tr>
<td>S21</td>
<td>A8</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S30</td>
<td>A15</td>
<td>Upper *</td>
<td></td>
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<tr>
<td><strong>With Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>A3</td>
<td>Upper</td>
<td>0</td>
</tr>
<tr>
<td>S8</td>
<td>A8</td>
<td>Upper *</td>
<td>0</td>
</tr>
<tr>
<td>S8</td>
<td>A15</td>
<td>Upper</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>A15</td>
<td>Upper</td>
<td>2</td>
</tr>
<tr>
<td>S1</td>
<td>P1</td>
<td>Upper</td>
<td>0</td>
</tr>
<tr>
<td>S25</td>
<td>P1</td>
<td>Upper</td>
<td>0</td>
</tr>
</tbody>
</table>

(*) Reference limits set at 99.9% specificity were also flagged. Italicics indicate subjects who recorded an ATPF only when the plasma volume correction was applied.
Table 6.3. Atypical passport findings for the OFF-score with and without the plasma volume correction

<table>
<thead>
<tr>
<th>Subject</th>
<th>Test</th>
<th>ATPF (upper or lower limit)</th>
<th>Confidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without Correction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>P7</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>B2</td>
<td>Lower *</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>P42</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>A15</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S13</td>
<td>A15</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>S15</td>
<td>B2</td>
<td>Upper *</td>
<td></td>
</tr>
<tr>
<td>S17</td>
<td>A3</td>
<td>Upper *</td>
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<td>A15</td>
<td>Upper</td>
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<td>S35</td>
<td>B2</td>
<td>Lower</td>
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(*) Reference limits set at 99.9% specificity were also flagged. Italics indicate subjects who recorded an ATPF only when the plasma volume correction was applied.
Figure 6.2 (Subject 11) gives an example of the plasma volume model’s function and describes the expected plasma volume contraction at A3, resulting in an increased [Hb]. Subsequent changes in plasma volume were also inversely related to [Hb] and the OFF-score. With the inclusion of the plasma volume correction, the specificity of the predicted reference ranges for [Hb] and the OFF-score were improved (Figure 6.2, panels A and E, respectively). For example, for Subject 11, at P7 the calculated Z-scores for PLT, LDL, CRE and Tfn were lower than the remaining four biomarkers (Figure 6.2, panel N), and therefore, the confidence calculation dropped to ~45% for this particular test (Figure 6.2, panel M).

One individual subject (Figure 6.3, Subject 8) presented with uncharacteristic results whereby [Hb] was the only plasma volume biomarker to respond to a plasma volume contraction observed between B2 and A8. From B2, A3 and A8 [Hb] increased from 14.6 to 16.6 and 17.6 g/dL, respectively. The calculated plasma volume decrease (using the CO-rebreathing method) at A3 and A8 was -19.4% and -26.8%, respectively. This volume shift was not reflected in any of the plasma volume biomarkers. Importantly, the confidence in the plasma volume estimation for A3 and A8 was 0%.

Subject 1 (female, IV group) recorded an increase in HbM towards the end of the altitude sojourn (Figure 6.4, panel B), presenting with increases in HbM greater than the female sample mean (between 33 to 67%). The increase in HbM was reflected with an increase in [Hb] at A15 and P1, which, as expected, was not reflected in any other plasma volume biomarker. This increase in [Hb], due to the HbM increase (rather than a plasma volume shift), resulted in a decline of the confidence to near 0% of the plasma volume calculation.
Figure 6.2. Subject 11. Red lines indicate the ABP adaptive model reference limits set at 99% (dashed lines) and 99.9% (solid lines) specificity (panels A, B, D to L). The green reference limits for [Hb] (A) and the OFF-score (E) represent the ABP reference limit calculations with the inclusion of the plasma volume correction. Panel C shows the red blood cell (RBC) (red line) and plasma volume (blue line) values calculated with the CO-rebreathing method. Panel M represents the confidence calculation in the plasma volume estimation. Panel N shows the z-scores for markers [Hb] (blue), Tfn (red), ALB (yellow), Ca (black), CRE (green), TP (pink), PLT (cyan), LDL (dashed blue). Panel O represents the plasma volume z-score calculations using the CO-rebreathing method (blue line) and plasma volume model (red line).
Figure 6.3. Subject 8. Refer to Figure 6.2 for panel descriptions. Note tests P7 and P21 are missing.
Figure 6.4. Subject 1. Refer to Figure 6.2 for panel descriptions.
6.4 Discussion

Exposure to hypoxia results in a number of inherent physiological adaptations, which alter an athlete’s hematological profile.\textsuperscript{[12]} The volumetric response to altitude is of interest here, and specifically, the influence of the initial haemoconcentration on the ABP markers, [Hb] and the OFF-score. A maximum mean decrease in plasma volume was observed concurrently with the maximum mean increase in [Hb] for both males and females, confirming the initial primary confounder of [Hb] fluctuations to be the plasma volume contraction. A few ATPFs were observed for [Hb] and the OFF-score values during and post-altitude, most likely the result of the initial plasma volume shift, as well as the expected increase in red cell production. Importantly, when the plasma volume correction was applied to the adaptive model, the reference limits were refined and the number of ATPFs for [Hb] was reduced. However, the number of ATPFs recorded for the OFF-score increased with the inclusion of the plasma volume correction (99% set specificity). It has to be highlighted that the study setting in this investigation presents a “worst case scenario” to test the functionality of the plasma volume model: Indeed, plasma volume is influenced by altitude, but at the same time, HbM will be impacted by the hypoxic exposure and the various iron supplementation protocols. While this is a possible scenario in certain athletes, the strongest (and most common) modulator of plasma volume in a normal setting is exercise alone.\textsuperscript{[13]}

A measureable increase in HbM has been reported after just 10 days at altitude,\textsuperscript{[14]} but typically does not present until a sufficient hypoxic dose \textsuperscript{[15]} has been achieved. Therefore, the initial plasma volume reduction upon arrival at altitude is most likely the primary factor influencing [Hb] fluctuations within the initial stages of altitude exposure.\textsuperscript{[16]} In our investigation, the initial haemoconcentration was not observed in all athletes, and there was substantial variability in the individual response to altitude (Table 6.1). Beidleman and colleagues also described variability in the individual response, where the reported standard error for [Hb] is large for the impact of the time spent at altitude (see Table 3 of Beidleman et al),\textsuperscript{[5]} suggesting considerable inter-individual variability at altitude. Additionally, the intermittent nature of the LHTL protocol,\textsuperscript{[12]} differences in training load,\textsuperscript{[17]} and the non-standardisation of blood collection times may have impacted the variable volumetric response reported here. Due to scheduling restrictions in the current study, the timing of the blood withdrawals and training programs were not standardised. Nevertheless, testing schedules can be random in an anti-doping setting, thus reflecting the practical application of the current results.
6.4.1 Frequency of atypical passport findings

When the plasma volume correction was applied to the ABP adaptive model, the predicted reference limits were narrowed and the number of ATPFs for [Hb] was reduced, which supports the model. With the plasma volume correction, four subjects no longer recorded ATPFs, however; Subject 1 and Subject 25 recorded ATPFs only when the plasma volume correction was applied (Table 6.2). This may be a reflection of the additional confounding effects of altitude on the ABP markers, namely an increased rate of erythropoiesis, which is reflected in the augmentation of reticulocytes.\textsuperscript{[18–20]} Subject 1 (Figure 6.4) and 25 recorded increases in RET% (which is not affected by plasma volume) at A3 and A8, respectively, indicating an increased red cell production influencing the [Hb] increase.

On the other hand, the OFF-score recorded an increased rate of ATPFs with the plasma volume correction (99% set specificity). The OFF-score is probably more sensitive to the increased rates of erythropoiesis at altitude due to the inclusion of RET% in the OFF-score algorithm. Therefore, when the plasma volume correction is applied in an altitude scenario, the resulting reference limits become too narrow, causing in an increased rate of false positives. However, with 99.9% specificity the OFF-score ATPFs were reduced by one when the plasma volume correction was applied (Table 6.3). The time between sample collections must also be considered. Blood collections acquired in quick succession, such as a few days apart, are at risk of the influence of sample autocorrelation and the time between measures was brief within this investigation.\textsuperscript{[10]}

Overall, applying the plasma volume correction to both [Hb] and the OFF-score was viable in a number of individuals who did not present with ATPFs with the inclusion of the plasma volume correction (only 2% and 5% of the 283 observations were atypical for [Hb] and the OFF-score, respectively). However, because of some false positives, further research into the plasma volume correction and its use within altitude scenarios is required.

6.4.2 The weighting function of the plasma volume model

The components of biomarker variance were higher than expected in some subjects, which resulted in a low confidence in the plasma volume estimation (calculated with the weighting function within the plasma volume model algorithm). If the plasma volume correction is to be considered for the ABP it is vital that the number of false positive ATPFs is kept to an
absOLUTE MINIMUM AND NOT INCREASED. AN IMPORTANT OBSERVATION FROM THIS INVESTIGATION WAS THE ASSOCIATION BETWEEN THE LOW CONFIDENCE VALUE AND UNCHARACTERISTIC CHEMISTRY VALUES. IN A HYPOTHETICAL ANTI-DOPING SCENARIO WHERE ONE OR MORE OF THE PLASMA VOLUME BIOMARKERS FLUCTUATE ABNORMALLY, RESULTING IN A LOW CONFIDENCE VALUE, IT IS RECOMMENDED THAT THE ABP ADAPTIVE MODEL EXCLUDES THE PLASMA VOLUME CORRECTION FOR THIS PARTICULAR OBSERVATION.


6.4.3 Limitations
Although the present study was performed in accordance to current WADA protocol and every effort was made to adhere strictly to the sample collection, handling and analysis protocols, it is important to recognise that the data presented here were collected under a research setting. For the plasma volume marker to be introduced into anti-doping practices, stringent protocols must be developed for the sample collection, handling, analysis, and
storage of the proposed chemistry markers, similar to those which are in place for the established ABP markers such as [Hb] or RET%. Additional factors that were not standardised within this investigation include diet, training and the timing of blood withdrawals. Further, the testing was performed during the Canberra winter, and a few subjects presented with Upper Respiratory Tract Infections. Finally, it must be mentioned that the likelihood of any of the subjects doping within this investigation is extremely low, yet cannot be excluded entirely. Of relevance however, each subject signed a statutory declaration prior to commencing the investigation, stating they have not and will not participate in any doping practices.

6.4.4 Conclusion and practical recommendations

The results presented here demonstrate the potential of the plasma volume correction to refine the ABP reference limit calculations by removing the influence of plasma volume on volumetric markers. It appears that the model is able to reduce the number of ATPFs on [Hb] in a reliable manner in most subjects. However, the multifaceted, individual physiological response to altitude confounded the results for some individuals. Nevertheless, the weighting function allowed an assessment of the outcome confidence, and could be included in any possible evaluation of an ABP. As such, it is recommended to include the confidence calculation when implementing the model, because it allows an appropriate weighting of the evidence added by the plasma volume correction. This investigation also highlights the importance of further research and development into a standardised protocol for the collection, handling, analysis and storage of the novel plasma volume biomarkers.

Acknowledgements

The authors wish to acknowledge the AIS Physiology staff for the nightly monitoring of the subjects during their stay at the altitude house and also the AIS medical staff for assisting in the administration of the IV iron supplementation.
6.5 References


Chapter 6: Validation of a blood marker for plasma volume


Conclusion: The need for an alternative method to determine intravascular volumes

This chapter is based on a *second submission* to the peer-reviewed journal, the European Journal of Sport Science:

Chapter 7: Conclusion

7.0 Abstract

It is well described that numerous environmental factors, including exercise, modulate plasma volume. These modulations prove problematic when a number of haematological markers are measured as a concentration in blood plasma. A primary example is haemoglobin concentration ([Hb]), a marker of erythropoiesis commonly used within medicine and also used to detect blood doping. Natural changes in plasma volume can confound [Hb] values when a volume change is detected rather than a true change in haemoglobin mass (HbM) (e.g. volume expansion resulting in a [Hb] decrease and pseudo-anemia vs. HbM decline resulting in anaemia). Currently, there is no simple solution to correct for plasma volume shifts, and this has proven problematic when monitoring volumetric health markers in clinical and anti-doping settings. This narrative review explores the influence that plasma volume shifts have on volumetric biomarkers, such as [Hb]. The progressive expansion in plasma volume observed during multi-day endurance events will be summarised, and the observed impact plasma volume variance has on concentration-based markers will be quantified. From this, the need for alternative methods to correct [Hb] for volume fluctuations is highlighted. Available methods for calculating intravascular volumes are then discussed, with a focus on a recently developed approach using a panel of ‘volume descriptive’ biomarkers from a standard blood test. Finally, the practical applications of this novel plasma volume blood test within both anti-doping and clinical settings will be examined.

Key words: Methodology, physiology, exercise, doping, health.
7.1 Background

Blood volume in humans is the sum total of red cell- and plasma- volume. Plasma is the liquid component of blood and plays a critical role in countless physiological processes. Both red cell volume and plasma volume fluctuate independently and are influenced by exercise, environmental stressors, trauma, and illness.[1] For example, exercise results in a plasma volume expansion, claimed to aid in muscle perfusion, increase stroke volume and cardiac output, and enhance the body’s thermoregulatory response.[1–3]

However, a number of haematologic markers are measured as a relative concentration in blood and are influenced by shifts in plasma volume. One such marker is haemoglobin concentration ([Hb]); Hb is an oxygen-carrying pigment commonly used to monitor human health, athletic performance, and to detect illegal blood doping in athletes. The concentration of circulating Hb in blood is dependent on the total mass of Hb (HbM) and the volume of plasma in which it is suspended. Therefore, such biomarkers are influenced by shifts in plasma volume, which can consequently confuse the interpretation of the biomarkers value within an anti-doping setting,[4] or influence the associated treatment strategy within a clinical setting.[5] For example, a low [Hb] measure can originate from an expansion in plasma volume (i.e. pseudo-anaemia) rather than a true decline in HbM (i.e. anaemia). Although methods to account for intravascular volume fluctuations are available, the techniques often lack accuracy and/or practicality, and frequently require the use of radioactive tracers.[6–9] In addition, there is currently no viable method to quantify plasma volume variations within anti-doping protocols. As a consequence, knowledge of the influence of plasma volume fluctuations on volumetric biomarkers is important for the accurate interpretation of haematologic profiles within both an anti-doping and clinical setting.

This narrative review explores the influence plasma volume fluctuations have on volumetric biomarkers, with a primary focus on [Hb]. First, the volume shifts observed during long-term endurance performances are presented, and the associated impact plasma volume variance has on concentration-based anti-doping markers is quantified. Second, the use of [Hb] as a health marker is discussed in patients with unstable intravascular volumes. Finally, the current methods available to determine intravascular volumes are considered, with a focus on a recently developed approach.
using a panel of ‘volume descriptive’ biomarkers from a standard blood test. The practical application of this novel blood test within anti-doping practices and the clinic will thus be examined.

7.2 Volume fluctuations confound anti-doping results

Within the Athlete’s Biological Passport (ABP), [Hb] is a primary marker used to detect illegal blood manipulation [e.g. blood transfusion or recombinant erythropoietin (rHuEPO) abuse to increase HbM and thus endurance performance]. The ABP relies on the longitudinal analysis of key biomarkers of erythropoiesis (e.g. [Hb], reticulocyte percentage, haematocrit (HCT)), and develops individualised reference limits, using Bayesian modeling, to detect blood manipulation. Initially, both intra-individual and inter-individual variance components are used to calculate preliminary (population-based) reference limits, however, as more individual information is provided, the reference calculations begin to solely rely upon intra-individual variance. Essentially, as more individual haematologic data is introduced to the ABP, more specific reference limits are developed. Currently, plasma volume fluctuations represent the majority of variance associated with the concentration-based ABP markers (once the inter-individual components are removed) (Figure 1).

Numerous conditions can influence plasma volume, including; the environment (i.e. heat or altitude), exercise intensity and duration, frequency of exercise bouts, posture, and/or hydration status. For example, exhaustive exercise typically results in an acute plasma volume contraction, followed by an expansion, with hypervolaemia being observed within minutes to hours of exercise cessation. These acute and natural shifts in plasma volume are capable of confounding an ABP profile (increasing [Hb]), thus, anti-doping authorities have attempted to curb the influence of acute volume fluctuations on the concentration-based ABP markers by ensuring all blood samples are collected 2 hours post-competition or post-exercise. However, this protocol only removes the acute effects of exercise, and does not necessarily remove the influence of the progressive plasma volume expansion, which has been observed during multi-day endurance events (Table 1). This is particularly problematic, as anti-doping testing during multi-day events is targeted extensively due to the presence of a
large number of athletes in a single location and the high profiles of events such as the cycling Grand Tours. Furthermore, endurance athletes train in ‘blocks’ of high intensity, which may result in the same (natural) progressive plasma volume expansion,[4] which could influence out-of-competition testing. In light of this knowledge, dishonest athletes could be illegally manipulating their plasma volume to mask doping effects. Recently, acute hyper-hydration (using 1000 mL of water) has been shown to mask the altered [Hb] ensuing from rHuEPO abuse.[13] It has also been suggested that athlete’s may be abusing artificial plasma volume expanders (e.g. mannitol, glycerol or hydroxyethyl starch) to deliberately mask the effects of illegal blood doping; many of these artificial expanders are, however, found on the World Anti-Doping Agency (WADA) prohibited list and are detectable.[14]

The variance component of [Hb] associated with plasma volume (refer to Figure 1) thus has the capacity to confuse the interpretation of an ABP profile, and could influence the ability of the ABP to detect a blood manipulation with certainty. On the other hand, it is of greater importance that the protection of the honest athlete is upheld and the rate of false positives (from, for example, a natural plasma volume contraction and increased [Hb]) is kept at an absolute minimum. Currently, any suspicious ABP profile is analysed by a panel of experts, who will consider the volumetric influences on a blood sample. Yet, there is currently no method available to directly quantify the influence of plasma volume within the ABP paradigm, and therefore, correct the concentration-based ABP markers, such as [Hb].
Figure 7.1. Variance components of [Hb]. Total variance is made up of inter-individual (light grey) and intra-individual (dark grey) components. Note: the chart represents general estimations of the contribution of each variance component to the overall variance of [Hb], these values are dependent on the population type and standardisation.\textsuperscript{[11]}
7.2.1 Progressive plasma volume expansions

An overview of the published plasma volume fluctuations and corresponding haematological fluctuations observed during multi-day endurance events is essential information to the anti-doping expert analysing a profile influenced by consecutive days of endurance exercise. A number of published studies (see Table 1) describe a progressive plasma volume expansion of up to ~20% during multi-day cycling competitions, which is inversely related to a decline in [Hb] of ~10%. It is suggested that [Hb] values are lower in-competition (14.1 g/dL) when compared to out-of-competition (15.0 g/dL) and pre-competition (14.9 g/dL) values.\textsuperscript{[15]} Plasma volume expansions of ~20 to 30% have also been observed in multi-day events involving running and skiing.\textsuperscript{[12,16,17]} Long-term plasma volume overcompensation, observed during extended endurance exercise, is most probably the result of an acute reduction in central venous pressure after successive bouts of exercise and a higher renal sodium retention due to changes in aldosterone and urodilatin secretion.\textsuperscript{[18]} Currently, the influence of multiple days of racing on red cell mass and reticulocytes is assumed to be minimal, as possible exercise-related adaptations of the erythropoietic system have most likely already occurred. Red cell mass and reticulocyte percentage (RET%) appear to remain stable during extended endurance events (Table 1).\textsuperscript{[19,20]} A plasma volume shift is thus expected to be a central factor, however, the time course, influence of rest days and the return to baseline levels post-competition must be considered. As such, descriptions of the published literature on these three factors are summarised below.
Table 7.1. Examples of the haematological changes observed during endurance cycling events.

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<th>ΔHCT (%)</th>
<th>ΔRET%</th>
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### Simulated cycling tour (5 stages)

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### Regio Tour, Germany (5 stages)

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### Chapter 7: Conclusion

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<td>7</td>
<td>19</td>
<td>-11.5%†</td>
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1. The observed change is in reference to the baseline value collected before the start of each respective competition. # These calculations have been debated.

2. †These values were calculated from the median, as presented in the original manuscript. ** Only the data from the sea level group is reported. ‡ Only %change values were available.
7.2.2 The time course of plasma volume expansion during multi-day tours

The time course of plasma volume expansion and the resulting haematologic fluctuations during consecutive days of demanding exercise is yet to be established. Some reports found a [Hb] decrease within 3 days,[19,21] 5 days [22] and 6 days [23] of starting a cycling tour (p < 0.05 reported by all studies; Table 1); whilst other studies observed a significant decline in [Hb] at competition day 12 (p < 0.05) [15,24] and competition day 20 (p < 0.05). [25] To our knowledge the relationship between the observed plasma volume expansion and the duration of a competition (in days) has not been formally investigated. However, studies investigating cycling tours of shorter duration (between 5 and 14 days) observed significant declines in [Hb] earlier in comparison to the findings of Corsetti et al (2012), Camanini et al (1988) and Morkeberg et al (2008), who studied tours of ~21 days. The observed differences may result from varying race conditions (temperature and altitude) or the timing of the sample collection (with posture is standardised).[1,3] Nevertheless, due to the expected (progressive) plasma volume expansion during multi-day racing an increased or even stable [Hb] value may be considered suspicious by anti-doping authorities.[26]

7.2.3 Rest days and the return to baseline levels post-competition

Between blocks of high intensity training or during multi-day competition, planned rest days are allotted throughout. Homeostasis is likely to be somewhat regained over the course of the rest day and a plasma volume contraction may occur. Thus, values collected one-day post rest are predicted to be closer to baseline values. However, to our knowledge, no research into the specific physiological response to rest days has yet been performed. In addition, published data on the time-course of the plasma volume reduction upon completion of an extended endurance event is limited. Such information is important from an anti-doping perspective as evidence suggests illegal autologous blood withdrawals are performed post-stage racing and stored for future reinfusion.[27] Of note, a plasma increase of ~22% on the final day of a 10-day cycling event was observed to be reverted within 2 days of recovery.[21] Likewise, Bejder and colleagues (2017) found that an increase in training load by ~250% for one week in highly trained male cyclists prompted a plasma volume expansion by ~10%, and reduction in [Hb] of ~6%. Plasma volume also returned to baseline levels within 2 days, while [Hb] required 4 days to return to baseline levels.[4] As such, it appears that an exercise-induced
plasma volume expansion is reverted as rapidly as it occurs; however, further research into the detail of the time course is needed.

7.2.4 Limitations and considerations

Table 1 presents results from various studies that have employed different blood collection, handling and analysis protocols. The environmental conditions, duration and intensity of racing prior to the blood collection also vary between studies. As such, a comparison between the results presented here must be done with care. In Table 1, eight studies followed blood collection and handling protocols recommended by anti-doping agencies. Additionally, it cannot be ascertained that the data presented within these studies is free of any blood manipulation by the athletes observed.

In conclusion, exercise is a key modulator of plasma volume, and the observed decline in [Hb] during multi-day endurance events are likely to be predominately under the influence of a progressive plasma volume expansion, and appears to be a universal and progressive response. However, more data is required in order to construct a clear time course of change. It is recommended that future studies are conducted which follow anti-doping sample collection guidelines in order to standardise the influences of intravascular volumes. Furthermore, data on rest days and the return to baseline following consecutive days of competition is required.
Chapter 7: Conclusion

7.3 Plasma volume and health

The knowledge gained through studying the effect of an endurance event on haematological modifications may in fact benefit the physician within a clinical setting. Such knowledge may help the sports physician in their diagnosis of iron deficiency, where the phenomenon of ‘sports anaemia’ is a common occurrence, resulting from dilution caused by a plasma volume expansion.[3] Generally, exercise-induced iron deficiency is thought to occur through mechanisms such as haemolysis, GI bleeding, haematuria, inflammation, and/or a negative iron balance;[29,30] however, shifts in plasma volume might be considered by the physician when establishing the rationale for low [Hb] as part of an investigative look at an athlete’s iron status. In addition to the athlete-based situation, this knowledge may also assist the physician in a wider clinical setting, since perioperative anaemia is a common problem associated with increased mortality and morbidity rates following surgery in hospitals.[31]

Traditionally, [Hb] is used to define anaemia (<130 g/L for men and <120 g/L for women),[32] however, in practice, the diluting effects of plasma volume shifts are not always considered. It is thus recommended that alternative measures are explored, not only in an anti-doping or sports setting, but also a clinical setting to quantify the influence of plasma volume on concentration-based biomarkers.

Recently, Otto and colleagues (2017) studied the correlation between HbM and [Hb] in five patient cohorts encompassing: healthy, surgical, inflammatory bowel, liver disease and heart disease patients. The results presented a correlation between HbM and [Hb] in healthy, surgical and inflammatory bowel patients (r = 0.687 to 0.871, p < 0.001), but a poor correlation existed for liver disease (r = 0.410, p = 0.11) and heart failure patients (r = 0.312, p = 0.16). In these latter patient cohorts, plasma volume explained most of the variance observed in [Hb] and changes in body water distribution are more likely to occur with liver disease and heart failure.[5] Therefore, such patients may be diagnosed with anaemia whilst having a normal or even supra-normal HbM due to a plasma volume expansion. Otto et al. (2017) concluded; “The traditional inference that anaemia generally reflects haemoglobin deficiency may be misleading, potentially resulting in inappropriate tests and therapeutic interventions to address ‘haemoglobin deficiency’ not ‘plasma volume excess’” (p.1477). The cause of a low [Hb] value has significant therapeutic implications, and is commonly used as a key clinical outcome predictor (e.g. a low [Hb] due to excess plasma volume often has a misguided prognosis). If a low [Hb] result is due to impaired Hb synthesis, increased erythrocyte loss, or erythrocyte destruction, the treatment goal will be to increase
erythropoiesis. However, if the cause stems from a plasma volume expansion, the treatment goal will be to correct fluid homeostasis. As such, knowledge of the influence of plasma volume fluctuations are of great importance, and methods to control for the effect from routine plasma analysis is warranted.

*In conclusion, the association between [Hb] and plasma volume must be carefully considered by physicians in both a sporting context, and in a wider clinical setting. Just as exercise prompts plasma volume shifts in athletes, some disease states will alter a patient’s intravascular volume. Direct measures of HbM, or alternatively, a measure for plasma volume to correct for volumetric fluctuations has significance, not only in improving athlete health monitoring, but also in enhancing patient monitoring in hospitals - with the potential to improve health outcomes.*

### 7.4 Current methods to determine intravascular volumes

Two approaches to monitoring intravascular volumes exist: first, classical dilution methods to estimate *absolute* volume, and second, indirect methods to estimate a *change* in intravascular volume. Dilution methods involve the injection of a tracer (e.g. a radioactive marker or dye) which binds to either plasma proteins (albumin) to measure the plasma space, or alternatively to the circulating red cells to estimate red cell volume using the dilution principle. From the known quantity of the applied tracer and the dilution of the tracer, calculations of the volume in question can be made (indicator concentration = total amount of indicator ÷ total volume). Indirect methods, such as the Dill and Costill method,[33] use pre/post [Hb] or HCT measures to calculate the change in plasma volume. The measurement errors associated with commonly used tracer techniques have previously been reviewed by Gore et al. (2005), although these techniques are reasonably accurate, the practicalities of applying these measures in a sports or clinical setting is questionable. Below is a brief description of three common methods (Evans blue dye, ⁵¹ Chromium-labeled red blood cells and carbon monoxide (CO)-rebreathing).

*Evans blue dye* has a strong affinity to plasma proteins and is an ideal marker for plasma space. However, the method is more appropriate when measurements are not influenced by postural or exercise-induced intravascular pressure changes, which may result in increased leakage of the dye from the vascular compartment (contributing to the larger measurement
error associated with the technique).\[^6\] As the Evans blue method assumes no change in plasma protein content, the test cannot be used when analysing short-term plasma volume shifts, which is mediated by the oncotic effect of a net increase/decrease of the intravascular protein content (causing water movement between the interstitial and intravascular space).\[^1\] The $^{51}$chromium-labelling technique of red blood cells is considered the gold-standard method within clinical practice, as red blood cells are believed to remain in the vasculature for longer than plasma proteins.\[^34\] The technique requires the collection of a venous sample, radioactive labeling of the sample’s red cells, re-injection, and then subsequent venous collections at set time points.\[^8\] With this approach blood volume is systematically underestimated, as erythrocytes in venous blood are concentrated relative to the numbers in the arteries and capillaries.\[^35\]

The CO-rebreathing method uses CO as a tracer to directly measure HbM performed by either a 2-min protocol\[^36\] or 10-min protocol\[^9\] (CO is an effective tracer due to its high affinity to Hb, in comparison to oxygen). With known values for Mean Corpuscular Haemoglobin Concentration (MCHC) and [Hb], intravascular volumes can be estimated using the equations below:

1. \[\text{Red cell volume} = \frac{\text{HbM (g)}}{\text{MCHC}} \times 100\]
2. \[\text{Blood volume} = \frac{\text{HbM (g)}}{100} \div \frac{\text{[Hb]}}{0.91}\]
3. \[\text{Plasma volume} = \text{Blood volume} - \text{Red cell volume}\]
4. \[\text{MCHC} = \frac{\text{[Hb]}}{\text{HCT}} \times 100\]

This method is commonly used in a sports research/health settings (e.g. during altitude training),\[^37\] and more recently, the application of this method to both anti-doping\[^38\] and clinical practices\[^5\] has been suggested. However, within anti-doping practices, this method is likely not viable due to a number of ethical and practical issues. First, CO, a potentially toxic gas, cannot be administered to healthy competing athletes, with reports of slight declines in aerobic capacity after CO-rebreathing.\[^36\] Second, the procedure requires the subject to be fully compliant (i.e. breathing in the entire bolus of CO, for example), thus providing opportunities for dishonest athletes to “cheat” the test. Finally, the tests cannot be standardised between laboratories, as a human, not a machine, is currently required to
perform the protocol. On the other hand, in a clinical setting it is clear that hospital staff would require specialist training in order to master the technique of this analysis method.

Although a number of techniques are available to directly measure intravascular volumes, the methods are often cumbersome, time-consuming and/or costly. Recently however, a novel approach to estimating intravascular volumes was suggested, which only requires a standard blood test, and estimates volumes through the observed variability of a panel of ‘volume descriptive’ biomarkers.\(^{[39]}\)

7.4.1 The use of common biomarkers to determine intravascular volumes

A number of previous investigations have suggested the use of biomarkers to determine intravascular volumes, for example, albumin\(^{[40]}\) or plasma pro-atrial natriuretic peptide (proANP).\(^{[4]}\) However, albumin has been found to account for only 20% of both a small (4%) and large (10%) expansion in plasma volume, occurring after acute fluid intake and increased training loads, respectively;\(^{[4,13]}\) proANP only accounted for \(~10\)% of plasma volume expansion, despite a large change in plasma volume of \(~10\)% induced by an increased training load.\(^{[4]}\) Total protein, gamma-glutamyltransferase and calcium have also been suggested, but these markers also lack accuracy independently.\(^{[41]}\) From these findings, it can be concluded that a single marker is unlikely to provide a robust estimation for plasma volume, and instead, a panel of ‘volume descriptive’ biomarkers may be more viable.

7.4.2 A newly developed plasma volume model

Recently, a panel of 8 and 15 common biomarkers was identified, which combined, accounted for 68% and 69% of plasma volume variance, respectively.\(^{[39]}\) The method developed allows for the calculation of absolute red cell-, blood- and plasma- volume from a simple blood test based on the consistency of change over time in the panel of concentration-based variables (after the removal of between subject variation by applying an individualised statistical approach). The model applies an adaptive Bayesian model on the set of volumetric biomarkers to calculate individual marker Z-scores. From these values, a Z-score for plasma volume can be established, and by comparing the calculated plasma volume Z-score to a pre-determined reference Z-score, a calculation of plasma volume change can be determined. The
absolute plasma volume at the time of blood collection can be calculated from the expected mean returned by the adaptive model together with the estimated Z-score and model confidence level calculation. In addition, estimates of HbM, red cell volume and blood volume can be obtained using the formulas stated above (equations 1 to 3), together with [Hb] and MCHC test results. To our knowledge only one study has attempted to validate this novel model in an altitude setting (n=34; 21 night simulated live high train low protocol, 3000m). The authors concluded that the plasma volume model is capable of reducing the impact of the plasma volume expansion (typically observed in the first days at altitude) on [Hb]. Moreover, the weighting function of the model is capable of identifying abnormal biomarker values (e.g. resulting from analytical error) by returning a low confidence value with the plasma volume estimation, lending further credibility to the plasma volume estimation. Further validation studies in various sports and clinical settings are highly recommended.

7.4.3 Requirements of the plasma volume model

Although this novel approach has the capacity to estimate intravascular volumes from a simple blood test, a number of requirements must be met. First, individual patient target values (the pre-determined reference z-score, refer above) must be defined for plasma volume during a stable state. These initial physiological targets may be established using a reference indicator dilution method (as those described above), however, anthropometric characteristics, such as body mass or estimations based on published population reference data may also be used to establish baseline values. Additionally, a baseline blood sample will also be required to calculate the individual distribution of the set of ‘volume descriptive’ biomarkers. If, however, only partial information is available in this initial step, the method, in theory, remains applicable. More data is still needed to establish the absolute minimal requirements of this initial step. Once these priors are established within the model, subsequent blood samples may be taken to estimate absolute intravascular volumes.

7.4.4 Practical applications of the plasma volume model

The estimation of plasma volume can be used to normalise analytical test results performed on blood plasma or serum, which are returned as a concentration. For example, if the
calculated plasma volume Z-score indicates a haemodilution 28% above expected plasma volume level, the concentration-based marker (e.g. \([Hb]\)) is divided by 1.28. It is recommended that normalisation be performed after the application of the adaptive Bayesian model to determine individualised distributions and reference limits of the concentration-based marker. Otherwise, a direct estimation of HbM can be made. The model can also be used to continually monitor plasma volume, red cell volume or blood volume with multiple blood samples. Finally, the estimated intravascular volumes can also be used to specify an intervention that aims to normalise intravascular volumes based on the target(s) defined by the initial baseline testing. The proposed method may be run on any device with a microprocessor, for example, a computer, smart phone, tablet or internet server, with the results returned in a fraction of a second, even for more complex situations.

The concept of a blood test to directly determine intravascular volumes has the potential to significantly impact the monitoring of athlete blood samples influenced by a plasma volume shift, or the assessment of anti-doping samples, which have been illegally contaminated with a plasma volume expander. Within clinical practice, the plasma volume model has the potential to improve patient management by improved monitoring of intravascular volumes and the ability to easily detect cases of pseudo-anemia. Nevertheless, the stability of the recently developed 'volume descriptive' biomarkers in different sporting scenarios must be validated, and more research into the stability of the model within clinical practice is needed.

7.5 Conclusion

Knowledge of intravascular volumes and the influence of plasma volume fluctuations on haematological markers, such as [Hb], is fundamental when examining athlete samples for anti-doping purposes and for general health monitoring in both a sports and a clinical setting. The current methods available to monitor intravascular volumes are, however, sub-optimal. Nevertheless, new techniques are emerging with the potential to simplify and enhance our ability to monitor intravascular volumes, with the potential to optimise anti-doping analysis and improve patient care in hospitals.
Chapter 7: Conclusion

7.6 References


Chapter 7: Conclusion


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Summary
8.0 Summary

The Athlete’s Biological Passport (ABP) is an important anti-doping tool that is able to circumvent the need for direct detection of a banned substance.\[1\] This is of significance, as dishonest athletes are continually developing performance enhancing substances and techniques that are undetectable with traditional anti-doping methods. The blood module of the ABP detects illegal haematological manipulation by establishing individualised reference intervals for key biomarkers of erythropoiesis.\[2\] This allows for the detection of irregular fluctuations that are characteristic of doping. However, the blood module of the ABP currently relies on a number of biomarkers measured as a concentration in blood plasma. Recently, two studies have shown that plasma volume shifts resulting from hyper-hydration\[3\] or an increased training load\[4\] can either mask doping, or result in false positive ABP results. Furthermore, a number of plasma volume expanders are currently banned by the World Anti-Doping Agency (WADA) to discriminate athletes attempting to mask blood manipulation.\[5\] Consequently, the variance associated with plasma volume is currently a major confounder within the volumetric biomarkers of the ABP.\[3,4\] Consequently, the primary aim of this thesis was to develop a novel method to reduce the component of variance associated with plasma volume.

Currently, the ABP protocol requires athletes to refrain from exercise up to 2 hr prior to sample collection. This is enforced, in part, to remove the influence of an exercise-induced plasma volume shift on the test results. However, a number of alternative environmental factors are known to also influence vascular volumes, one of which is an ascent to altitude. As altitude is a common technique used by athletes to improve endurance performance, the preliminary investigation of this thesis (Chapter 3) was focused on describing the haematological fluctuations observed at elevations above 1200 m. A meta-analysis of the observed variations in key ABP markers (Haemoglobin Concentration ([Hb]), Reticulocyte Percentage (RET%) and the OFF-score) was performed using the raw data of 17 altitude studies. Altitude dose and baseline measures were identified as the key variables associated with the subsequent haematological change values both during- and post-altitude. The predicted plasma volume contraction (and increased [Hb]) upon ascent to high altitude was observed within two days. Yet, this phenomenon was only observed in studies applying a Live High: Train High (LHTH) protocol. Upon return to sea level, [Hb] returned to baseline levels within two weeks, while RET% values declined below baseline, which was influenced by altitude dose. The overall individual response to altitude was highly variable.
Nevertheless, the influence of an altitude-induced plasma volume shift on the concentration-based biomarkers, [Hb] and the OFF-score, is apparent.

The principal investigation of this thesis (Chapter 4) was focused on identifying a panel of ‘volume descriptive’ biomarkers that were obtained from a simple blood test. Over a 6-month period, 33 healthy males were monitored on a monthly basis. Venous blood samples (serum and whole blood) were collected concurrently with the CO-rebreathing manoeuvre,\textsuperscript{6} which provided a gold-standard measure for plasma volume. The variance of a large panel of common chemistry markers (all with a propensity to present with low levels of biological variance) were analysed and matched against the calculated plasma volume variance. Finally, after the removal of between-subject variance with a Bayesian statistical model, multivariate analysis identified a panel of 8 and 15 biomarkers which, when combined, described absolute vascular volumes and explained \(\sim 68\%\) of plasma volume variance. The final multi-parametric plasma volume model contained a weighting function, which allowed for isolated abnormalities in one biomarker. It is hypothesised that with this weighting function, the plasma volume model will remain stable across different testing conditions, for example, with pathology. Of note, it is of extreme importance that the inclusion of the plasma volume correction to the ABP does not increase the rate of false positives. As such, the weighting function is a vital aspect of the model.

The subsequent investigation of this thesis (Chapter 5) focused on the aforementioned models application within the ABP. Here, the same group of 33 healthy male subjects performed a maximal exercise test on a cycle ergometer, designed to promote a maximal plasma volume shift. This test was performed on completion of the 6-month monitoring period (described above). Venous blood samples were collected immediately and 1 hr post-exercise. The ABP adaptive model was then applied to the data to create individualised reference limits for [Hb] and the OFF-score, both with and without the inclusion of the plasma volume correction. As expected, the maximal exercise effort resulted in an increased number of atypical [Hb] and OFF-score values in the samples collected immediately post-exercise. However, with the inclusion of the plasma volume correction to the ABP reference calculations, the number of Atypical Passport Findings (ATPFs) post-exercise was reduced. Importantly, with the plasma volume correction, the number of ATPFs without exercise did not change. Therefore, with the inclusion of the plasma volume correction, the ABPs sensitivity was considerably improved with no concurrent loss of specificity.
The final investigation of this thesis (Chapter 6) was designed to apply the new model to a “real life” scenario that encompassed a natural altitude-induced plasma volume shift. Here, 34 endurance athletes completed a 21 night simulated Live High: Train Low (LHTL) altitude training protocol (14 hr/day at 3000 m). Venous blood samples were collected twice pre-altitude, at days 3, 8, and 15 of altitude exposure, and at days 1, 7, 24, and 42 post-altitude. When the plasma volume correction was applied to individual [Hb] reference calculations, the number of ATPFs was reduced from 7 of 5 subjects to 6 of 3 subjects. However, the application of the plasma volume correction to the OFF-score during an altitude scenario increased the number of ATPFs (from 9 to 13, 99% specificity). This is most likely the result of the narrowed reference limit calculations (with the inclusion of the plasma volume correction) combined with the increase in erythropoiesis in response to the hypoxic environment, which collectively influenced the RET% component of the OFF-score calculation. Importantly, the weighting function of the plasma volume model was capable of identifying all isolated abnormalities in the data by returning a low confidence value. Although the multifaceted response to altitude confounded some individual results, the plasma volume correction remains valid for [Hb] in an altitude scenario.

8.1 Conclusion

The collection of studies presented within this thesis explored the influence of plasma volume fluctuations on volumetric ABP markers. The primary focus was on developing a novel blood test capable of correcting for plasma volume shifts and estimating absolute blood-, red cell- and plasma- volumes. The principal findings are as follows:

- A meta-analysis of the altitude literature described an initial plasma volume fluctuation in the first two days of natural, LHHT altitude exposure.
- Altitude dose and baseline values are key confounders to the resulting change values in [Hb], RET% and the OFF-score both during- and post-altitude.
- A set of 8 and 15 biomarkers were identified, describing 68% and 69% of plasma volume variance, respectively.
- When applied to the ABP, the plasma volume correction improved the sensitivity of the ABP reference limits with no loss of specificity.
• The application of the plasma volume correction to the ABP reference limit calculations within an altitude setting are valid for [Hb].
• The increased specificity of the ABP reference limits with the inclusion of the plasma volume correction may not be applied to the OFF-score when altitude is also a factor.

8.2 Practical applications

This thesis describes the development of a novel multi-parametric approach to estimate vascular volumes. With the additional weighting function, it is hypothesised that the model will remain stable even if a single biomarker is irregular, for example, due to pathology. The model’s capacity to describe ~68% of plasma volume variance and capacity to identify abnormal readings (with the model’s weighting function) makes this approach extremely attractive within an anti-doping setting. The application of the plasma volume correction to the current ABP paradigm has the potential to significantly reduce the confounding effects of plasma volume fluctuations on volumetric ABP markers. Furthermore, the weighting function of the plasma volume model will minimise or remove the probability of the plasma volume correction returning a false positive result.

Beyond the anti-doping setting, it is possible that this model may also be applied to other sport science research practices. For example, the model can be used to accurately and simply monitor an athlete’s hydration status during intensive exercise, or heat and altitude training to ensure optimal performance. A simple blood test to estimate vascular volume may also provide opportunities to expand our understanding of the thermoregulatory responses to extreme heat within a sports research setting.

In addition, the model developed within this thesis offers a simple solution to improve volume management in hospital patients. Knowledge of a patient’s absolute vascular volume provides fundamental information in developing appropriate treatment goals. At present, there is no simple or accurate method available to achieve this. Volumetric markers of erythropoiesis, such as [Hb], are also commonly used when monitoring clinical patients. However, the confounding effects of plasma volume fluctuations also influence these values. Therefore, accurate information on a patient’s absolute vascular volume, or true [Hb] value (with the influence of plasma volume removed), could reduce the rates of morbidity and mortality in hospitals. Therefore, the approach described within this thesis to estimate absolute vascular volumes or correct concentration-based biomarkers for volume
fluctuations (using a simple blood test) has the capacity to significantly improve patient care in hospitals. It is hypothesised that, with the weighting function, the plasma volume model described here will remain stable in patients whose physiology may be irregular. However, further research is needed on the application of the plasma volume model within such clinical settings.

8.3 Limitations

Study 1. The athlete’s response to hypoxia: A meta-analysis of the influence of altitude exposure on key biomarkers of erythropoiesis

- Sample collection was not standardised between studies.
- A range of different haematology analysers were used between studies (studies performed post 2000 were chosen to reduce the variability stemming from analyser technology).
- Data on training load was not collected or standardised.
- It cannot be confirmed that all participants were clean of doping.

Study 2. The use of biomarkers to describe plasma-, red cell- and blood volume from a simple blood test

- The study was restricted to male participants only.
- No data on patients with a pathology were collected and therefore, future data is required if the model is to be applied within a hospital setting.

Study 3. A step towards removing plasma volume variance from the Athlete’s Biological Passport: the use of biomarkers to describe vascular volumes from a simple blood test

- The study was restricted to male participants only.
- The participants were ‘healthy’ to ‘well-trained’.
- Minimal elite athlete data was collected.
- It cannot be confirmed that all participants were clean of doping.
Study 4. Validation of a blood marker for plasma volume in endurance athletes during a live high: train low altitude camp

- Diet, subject training programs and blood withdrawal times were not standardised.
- A number of subjects suffered from upper respiratory tract infection.
- Although every effort was made to adhere to strict World Anti-doping Agency protocols for blood sample collection, handling and analysis, no protocol is yet in place for the chemistry analysis. As such, stringent protocols must be developed before the plasma volume model may be applied within anti-doping practices.
- It cannot be confirmed that all participants were clean of doping.

8.4 Directions for future research

The results from this thesis provide the foundations for an alternative approach to calculate vascular volumes. However, for this approach to be applied to an anti-doping, sports science, or clinical setting, further research is recommended as follows:

- For the collection, handling, analysis and storage of the novel ‘volume descriptive’ biomarkers, standardised protocols must be established to meet anti-doping standards and regulations.
- For wider use, the model should be validated within:
  a) Different athletic populations (e.g. team sports)
  b) Various environmental conditions (e.g. heat-induced dehydration)
  c) Subjects who are masking the effects of blood manipulations with illicit plasma volume expanders or with hyper-hydration
- For use within clinical patients, the stability of the ‘volume descriptive’ biomarkers must be established (e.g. with chronic kidney failure patients)

In conclusion, the results presented within this thesis, when strengthened with the aforementioned recommendations, will provide a promising advancement in our capacity to monitor vascular volumes with ease and accuracy.
8.5 References


Appendices

Appendix A
Supplementary material: Study 1 (Chapter 3)

Appendix B
Supplementary material: Study 2 (Chapter 4)

Appendix C
Participant information sheets, consent forms, ethics approvals, patent approval

Appendix D
Raw data
Appendix A

Supplementary material

Study 1 (Chapter 3)

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During altitude: comparison of exponential component of models

Standardised values of day, etc have a stdev = 1.

Figure II. Comparison of the standardised values for day at altitude, altitude hours and km.hr in relation to the observed change values in [Hb].
Appendix A: Study 1 Supplementary material

Extreme Values

**Figures III – V** represent all data points observed during altitude for [Hb], sqrt(RET%) and OFF-score, respectively.

**Figures VI – VIII** represent all data points observed post altitude for [Hb], sqrt(RET%) and OFF-score, respectively.

![Graph of [Hb] (during altitude)](image)

*Each symbol represents up to 2 observations.*

Figure III. [Hb] values during altitude.
Appendix A: Study 1 Supplementary material

\( \text{sqrt(\% retics)} \) (during altitude)

Each symbol represents up to 2 observations.

Figure IV. Sqrt(RET\%) values during altitude.

OFFscores (during altitude)

Figure V. OFF-score values during altitude.
Appendix A: Study 1 Supplementary material

[Figure VI] [Hb] values post altitude.

Each symbol represents up to 2 observations.

[Figure VII] Sqrt(RET%) values post altitude

Each symbol represents up to 2 observations.
Each symbol represents up to 2 observations.

Figure VIII. OFF-score values post altitude.
### Table 1. Cut-offs for delta-values based on normal distribution (one-sided cut-offs).

<table>
<thead>
<tr>
<th>Variable</th>
<th>1 in 100 cut-offs</th>
<th>1 in 1000 cut-offs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td><strong>During altitude</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Hb] (LHTH &lt; 3days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.66</td>
<td>2.10</td>
</tr>
<tr>
<td>Males</td>
<td>-0.57</td>
<td>2.17</td>
</tr>
<tr>
<td>Females</td>
<td>-0.72</td>
<td>1.29</td>
</tr>
<tr>
<td>[Hb] other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-1.22</td>
<td>2.19</td>
</tr>
<tr>
<td>Males</td>
<td>-1.32</td>
<td>2.25</td>
</tr>
<tr>
<td>Females</td>
<td>-0.89</td>
<td>2.00</td>
</tr>
<tr>
<td>Sqrt(RET%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.285</td>
<td>0.395</td>
</tr>
<tr>
<td>Males</td>
<td>-0.267</td>
<td>0.354</td>
</tr>
<tr>
<td>Females</td>
<td>-0.319</td>
<td>0.497</td>
</tr>
<tr>
<td>OFF score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-25.39</td>
<td>28.02</td>
</tr>
<tr>
<td>Males</td>
<td>-24.61</td>
<td>27.76</td>
</tr>
<tr>
<td>Females</td>
<td>-27.64</td>
<td>28.80</td>
</tr>
<tr>
<td><strong>Post-altitude</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Hb]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-1.44</td>
<td>2.07</td>
</tr>
<tr>
<td>Males</td>
<td>-1.50</td>
<td>2.07</td>
</tr>
<tr>
<td>Females</td>
<td>-1.27</td>
<td>2.05</td>
</tr>
<tr>
<td>Sqrt(RET%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.410</td>
<td>0.312</td>
</tr>
<tr>
<td>Males</td>
<td>-0.408</td>
<td>0.296</td>
</tr>
<tr>
<td>Females</td>
<td>-0.417</td>
<td>0.359</td>
</tr>
</tbody>
</table>
### OFF score

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>-22.00</td>
<td>34.24</td>
<td>-31.27</td>
<td>43.50</td>
</tr>
<tr>
<td>Males</td>
<td>-21.20</td>
<td>33.89</td>
<td>-30.27</td>
<td>42.97</td>
</tr>
<tr>
<td>Females</td>
<td>-24.58</td>
<td>35.40</td>
<td>-34.45</td>
<td>45.27</td>
</tr>
</tbody>
</table>
Table II. Within- and between-subject standard deviations (95% CI) for [Hb], sqrt(RET%) and OFF-score values during- and post-altitude. Additional control data included.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Altitude Phase</th>
<th>WS SD</th>
<th>BS SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>During (n=345)</td>
<td>0.46 (0.40, 0.52)</td>
<td>0.82 (0.71, 0.94)</td>
</tr>
<tr>
<td>[Hb]</td>
<td>Post (n=284)</td>
<td>0.43 (0.37, 0.49)</td>
<td>0.82 (0.72, 0.92)</td>
</tr>
<tr>
<td></td>
<td>Control (n=174)</td>
<td>0.45 (0.39, 0.51)</td>
<td>0.80 (0.65, 0.99)</td>
</tr>
<tr>
<td>sqrt(RET%)</td>
<td>During** (n=295)</td>
<td>*0.15 (0.14, 0.17)</td>
<td>M 0.14 (0.13, 0.16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F 0.17 (0.14, 0.21)</td>
</tr>
<tr>
<td></td>
<td>Post* (n=258)</td>
<td>*0.14 (0.13, 0.15)</td>
<td>M 0.13 (0.12, 0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F 0.16 (0.13, 0.19)</td>
</tr>
<tr>
<td></td>
<td>Control (n=175)</td>
<td>0.14 (0.12, 0.15)</td>
<td>0.17 (0.14, 0.22)</td>
</tr>
<tr>
<td>OFF-score</td>
<td>During** (n=277)</td>
<td>*6.91 (5.81, 8.22)</td>
<td>M 6.56 (5.42, 7.92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F 7.81 (6.08, 10.04)</td>
</tr>
<tr>
<td></td>
<td>Post** (n=257)</td>
<td>*7.39 (6.41, 8.52)</td>
<td>M 7.00 (5.97, 8.22)</td>
</tr>
<tr>
<td></td>
<td>Control (n=148)</td>
<td>9.65 (8.43, 11.04)</td>
<td>14.37 (11.27, 18.32)</td>
</tr>
</tbody>
</table>

*The model applied allowed for different within-subject SDs for males and females.

# For males and females combined.

Note: The WS and BS SDs are calculated from the raw values (as opposed to delta values). The WS SDs correspond to those calculated with the delta values presented in the main text, with the exception of sqrt(RET%), this may reflect some ill-conditioning between models.
Table III. Models: parameter estimates with (p-values) and (95% CIs) during altitude.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>intercept</th>
<th>sex</th>
<th>pre</th>
<th>kmHr</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>delta[Hb]^a</td>
<td>5.277</td>
<td>-4.994^{(1)} (&lt;0.001)</td>
<td>-0.304^{(2)} (0.002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 52)</td>
<td>(&lt;0.001)</td>
<td>(-7.717, -2.271)</td>
<td>(-0.485, -0.122)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.599, 7.956)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>delta[Hb]^b</td>
<td>2.401</td>
<td>-0.170 (0.003)</td>
<td>0.945[1-e^{-0.0022kmHr}] (&lt;0.001)</td>
<td>-0.439[Hb3g&gt;2]^{(3)} (0.008)</td>
<td></td>
</tr>
<tr>
<td>(n = 345)</td>
<td>(0.004)</td>
<td>(-0.281, -0.059)</td>
<td>(0.687, 1.203)</td>
<td>(-0.762, -0.117)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.776, 4.027)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>deltaSqrt(RET%)</td>
<td>1.314</td>
<td>0.089 (0.005)</td>
<td>-0.359 (&lt;0.001)</td>
<td>-0.823[ln(kmHr)]^{0.148}</td>
<td></td>
</tr>
<tr>
<td>(n = 295)</td>
<td>(0.193)</td>
<td>(0.028, 0.150)</td>
<td>(-0.484, -0.234)</td>
<td>(-1.942, 0.296)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(-0.672, 3.301)</td>
<td></td>
<td></td>
<td>(+0.200[ln(kmHr)]^{2} (0.060)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(-0.009, 0.408)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.0147[ln(kmHr)]^{3} (0.024)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(-0.0274, -0.0020)</td>
<td></td>
</tr>
<tr>
<td>deltaOFFscore</td>
<td>103.17</td>
<td>-8.72 (0.001)</td>
<td>-0.334 (&lt;0.001)</td>
<td>-26.95[ln(kmHr)]^{(2)} (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>(n = 277)</td>
<td>(&lt;0.001)</td>
<td>(-13.80, -3.63)</td>
<td>(-0.469, -0.198)</td>
<td>(-38.41, -15.49)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(68.20, 138.13)</td>
<td></td>
<td></td>
<td>2.641[ln(kmHr)]^{2} (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.604, 3.678)</td>
<td></td>
</tr>
</tbody>
</table>

^a first two days at altitude for LHTH athletes

^b all other [Hb] results during altitude

^{(1)} sex as a factor with males as the reference level. Elsewhere (equivalently) sex has been treated as a variable with male=1, female=2.

^{(2)} males only

^{(3)} pre-altitude [Hb] values > 16 for males and >14.4 for females
### Table IV. Models: parameter estimates with (p-values) and (95% CIs) post altitude.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>intercept</th>
<th>sex</th>
<th>pre</th>
<th>Days post-altitude (Dpost)</th>
<th>other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Delta [Hb]</strong></td>
<td>6.669</td>
<td>-0.582 (&lt;0.001)</td>
<td>-0.410</td>
<td>0.863 [e^{(0.126 \times Dpost)}]</td>
<td></td>
</tr>
<tr>
<td>(n = 284)</td>
<td>(&lt;0.001)</td>
<td>(-0.828, -0.336)</td>
<td>(&lt;0.001)</td>
<td>(&lt;0.001)</td>
<td>(5.119, 8.219)</td>
</tr>
<tr>
<td></td>
<td>(5.119, 8.219)</td>
<td>(-0.501, -0.319)</td>
<td></td>
<td>(0.594, 1.132)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.537</td>
<td>0.063</td>
<td>-0.455</td>
<td>-0.018Dpost</td>
<td>-0.084 \times (age ≥ 20)</td>
</tr>
<tr>
<td>(n = 258)</td>
<td>(&lt;0.001)</td>
<td>(0.014)</td>
<td>(&lt;0.001)</td>
<td>(0.003)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>(0.395, 0.679)</td>
<td>(0.013, 0.113)</td>
<td>(-0.564, -0.346)</td>
<td>(-0.029, -0.006)</td>
<td>(-0.126, -0.041)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0086Dpost2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.005)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.0026, 0.0146)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.0012 \times \ln(kmHr) \times Dpost</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.009)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(-0.0021, -0.0003)</td>
<td></td>
</tr>
<tr>
<td><strong>Delta OFFscore</strong></td>
<td>66.04</td>
<td>-12.41 (&lt;0.001)</td>
<td>-0.565</td>
<td>0.236</td>
<td>-0.084 \times (age ≥ 20)</td>
</tr>
<tr>
<td>(n = 257)</td>
<td>(&lt;0.001)</td>
<td>(-16.57, -8.25)</td>
<td>(&lt;0.001)</td>
<td>(0.083)</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>(53.26, 78.82)</td>
<td>(-0.674, -0.455)</td>
<td></td>
<td>(-0.031, 0.503)</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Supplementary material

Study 2 (Chapter 4)

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Steps to estimate plasma volume from a simple blood test

Note: steps 1.1 to 1.2 describe the development of target measures. These steps represent the preferred approach to obtain prior patient information, however, the method remains applicable when only partial information from steps 1.1 to 1.2 is available.

(1.1) Define physiological targets for red cell, plasma and blood volumes. These targets should be defined when the patient is known to present a well-controlled balance of vascular volumes. Targets can be defined with classic indicator-dilution methods such as the CO-rebreathing method, described above. Alternatively, targets may be calculated from anthropometric characteristics (body mass, fat free body mass, height, age and sex). Body mass correlates with HbM ($R^2=0.61$) (Fig. 1) and targets for HbM can be defined based on normal averages of HbM per kilogram of fat free mass. The latter target for HbM can be further derived in a target for red cell volumes, given a population average of 33 g/dL for MCHC, or if available, a patient specific value for MHCH.

Red cell, plasma and blood volumes can be calculated as follows:

\[
\text{Red Cell Volume (mL)} = \frac{\text{HbM (g)}}{\text{MCHC (g/L)}} \times 100
\]

\[
\text{Blood Volume (mL)} = \frac{\text{HbM (g)}}{100} \times \frac{\text{[Hb] (g/dL)}}{0.91}
\]

\[
\text{Plasma Volume (mL)} = \text{Blood volume (mL)} - \text{Red cell volume (mL)}
\]

Alternatively, a clinician can define targets using conventional estimation methods based on population references, or with the medical history of the patient.

(1.2) Individual distributions, using the adaptive Bayesian model, are calculated for the following markers: [Hb], Tfn, CRE, Ca, PLT, LDL, ALB and TP (Set 1), and optionally, CHOL, TG, T4, WBC, PCT, NEUT and MONO (Set 2). The adaptive Bayesian model consists in a hierarchical Bayesian network that allows the derivation of individual distributions for the markers described...
above, and in turn develops individual reference ranges for that marker at a pre-defined specificity level (eg. 99% or 99.9%). Two sets of blood results are required to calculate an individual distribution; ideally the two blood samples are collected 5 days apart. Again, these measures should be defined when the patient is known to present with a well-controlled balance of vascular volumes.

(2.1) Blood sample(s), including a full blood sample (preferably with EDTA as the anticoagulation agent) and a serum sample, are collected from the patient. Set 1 (8 markers) or Set 2 (15 markers) biomarker panels are calculated, together with MCHC.

(2.2) The adaptive Bayesian Model is applied to the set of biomarkers calculated in step 2.1 to derive Z-scores for each individual marker. These Z-scores represent individual variations over individual means. This process is critical to remove inter-individual variations and highlights the variations associated with changes in plasma volume.

The individual Z-score for each marker, $M_i$, is determined by:

$$Z(M) = \frac{M(i) - VAR(i,j)}{\sqrt{ME(i,j)}}$$

Where $ME(i,j)$ is the individual mean for subject $j$, $VAR(i,j)$ is the individual variance for subject $j$ and $M(i)$ represents the value for the respective marker at time $i$.

(2.3) The individual Z-scores are then combined using a weighting function derived from the known variations of each maker with plasma volume as well as from the consistency between all Z-scores. The outcome is an estimation of the variations in plasma volume, given as a Z-score. This step comprises of the following:
- Establishing a first estimate of the Z-score \((Z(Mi)\) estimate\) associated with plasma volume calculated by multiplying the sum of the individual Z-scores of each biomarker by the respective markers coefficient (Table 1).

- Determining the residuals \((R)\) in the variations associated to each observation, \(Mi\), using the following equation:

\[
R(Mi) = Z\text{scores of all biomarkers} - \sum (Z(Mi)\) estimate\) \times (respectivemarker'\) coefficient\)

- Establishing a weighting function associated to the consistency between variations in each marker, \(M\), calculated as the normality probability distribution of the residuals in the variation of the markers.

- Calculating the Z-score associated to plasma volume by weighting the estimate of the Z-score \((Z(Mi)\) estimate\) with the weighting function.

A weighting function associated to the consistency between the variations in each marker is calculated as the normality probability distribution of the residuals in the variations of the markers. The second and final estimate Z-score associated to plasma volume shifts is calculated similarly as above, namely as the sum of the Z-scores computed for each biomarker multiplied by the markers’ coefficient (Table 1), except the calculation is further weighted by the weighting function computed from the residuals.

A Z-score higher than 0 indicates haemodilution, with values greater than 2.3 indicating a strong haemodilution. Conversely, values less than 0 indicate haemoconcentration and values less than -2.3 indicate a strong haemoconcentration (for a specificity of 99%, other specificities may be chosen as considered appropriate).

(2.4) A confidence level is associated to the Z-score. The confidence level is equal to the exponential of the sum of the weighting function. This confidence level is normalised between 0 and 1 so that values close to 0 have a low confidence while values close to 1 have a high confidence. The variance used to calculate the Z-score associated with plasma volume is finally weighted by this confidence level.

(2.5) The plasma volume at the time of blood collection is calculated from the
Appendix B: Study 2 Supplementary material

expected mean returned by the adaptive model together with the estimated Z-score and confidence level. Estimates of HbM can be further obtained using the formulae given in step (1.1), together with the original [Hb] and MCHC values.

Application of the Plasma Volume Model

1. Estimation of plasma volume at time of blood collection
2. Estimated plasma volume can be used to specify an intervention based on targets of plasma volume
3. The estimated red cell mass and corresponding HbM can be used to specify an intervention on red cell mass based on pre-defined targets.
4. Steps 2.1 to 2.5 can be repeated for the continuous monitoring of red cell, plasma and blood volumes over time, as well as for the continuous monitoring of red cell mass and HbM.
### Table I. Coefficients for both set 1 and set 2 plasma volume biomarkers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Set 1 marker coefficient</th>
<th>Set 2 marker coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Hb] (g/dL)</td>
<td>0.43</td>
<td>0.30</td>
</tr>
<tr>
<td>Tfn (mg)</td>
<td>0.32</td>
<td>0.23</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>0.38</td>
<td>0.25</td>
</tr>
<tr>
<td>Ca (mmol/L)</td>
<td>0.31</td>
<td>0.20</td>
</tr>
<tr>
<td>CRE (U/L)</td>
<td>0.33</td>
<td>0.23</td>
</tr>
<tr>
<td>PLT (x10³/μL)</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td>TP (g/L)</td>
<td>0.47</td>
<td>0.31</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td>CHOL (mmol/L)</td>
<td>NA</td>
<td>0.12</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>NA</td>
<td>0.064</td>
</tr>
<tr>
<td>T4 (μg/dL)</td>
<td>NA</td>
<td>0.082</td>
</tr>
<tr>
<td>WBC (x10/μL)</td>
<td>NA</td>
<td>0.48</td>
</tr>
<tr>
<td>PCT (x10⁻²%)</td>
<td>NA</td>
<td>0.28</td>
</tr>
<tr>
<td>NEUT (x10/μL)</td>
<td>NA</td>
<td>0.19</td>
</tr>
<tr>
<td>MONO (x10/μL)</td>
<td>NA</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Since the markers are correlated with each other, their respective coefficients are different if selected from set 1 or set 2. Haemoglobin concentration ([Hb]), Transferrin (Tfn), Albumin (ALB), Calcium (Ca), Creatinine (CRE), Platelet count (PLT), Total Protein (TP), Low-density lipoprotein (LDL), Cholesterol (CHOL), Triglyceride (TG), Thyroxine (T4), White Blood Cells (WBC), Plateletcrit (PCT), Neutrophils (NEUT), and Monocytes (MONO). Refer to [https://www.westgard.com/biodatabase1.htm](https://www.westgard.com/biodatabase1.htm).
Fig. I. Relation between haemoglobin mass measured by the CO-rebreathing method for 33 healthy subjects as a function of body mass. Points represent the average of 9 values measured over a 6-month period, the bars the standard deviations (X-axis: body mass, Y-axis: haemoglobin mass). Linear regression: Haemoglobin mass [g] = 11*Body mass [kg] + 50, \( R^2 = 0.61 \).
Fig. II to XVII. Application of the plasma volume marker. Top: measured variations in plasma volume (black) and variations in plasma volume marker (grey) (using Set 1). Bottom: confidence in plasma volume marker. Test number indicates months 1-6; test 7 represents post-exercise; test 8 represents 1hr post-exercise.

Fig. II. Application of the plasma volume marker. Subject 2 is missing a monthly value.
Fig. III. Application of the plasma volume marker. Subject 6 is missing a monthly value.
Fig. IV. Application of the plasma volume marker.
Fig. V. Application of the plasma volume marker.
Fig. VI. Application of the plasma volume marker.
Fig. VII. Application of the plasma volume marker.
Fig. VIII. Application of the plasma volume marker.
Fig. IX. Application of the plasma volume marker.
Fig. X. Application of the plasma volume marker. Subject 19 is missing a monthly value.
Fig. XI. Application of the plasma volume marker. Subject 22 is missing a monthly value.
Fig. XII. Application of the plasma volume marker.
Fig. XIII. Application of the plasma volume marker.
Fig. XIV. Application of the plasma volume marker.
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Fig. XV. Application of the plasma volume marker. Subject 29 is missing a monthly value.
Fig. XVI. Application of the plasma volume marker.
Fig. XVII. Application of the plasma volume marker.
Appendix C: Participant information, consent, ethics approvals

Appendix C

Participant information sheets, consent forms, ethics approvals, patent approval

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Appendix C: Participant information, consent, ethics approvals

Our Ref: RA/4/1/8714

19 October 2016

Dr Peter Peeling
School of Sport Science, Exercise and Health
MBDP: M408

Dear Doctor Peeling

HUMAN RESEARCH ETHICS OFFICE – EXEMPTION FROM ETHICS REVIEW

Altitude exposure and the Athlete’s Biological Passport: A review of the effect of hypoxia on current markers and associated iron variables

Based on the information you have provided to the Human Ethics office in relation to the above project, the described activity has been assessed as exempt from ethics review at the University of Western Australia.

However, should there be any significant changes to the protocol, you must contact the HREO to determine whether your exempt status remains valid or whether you will be required to submit an application for ethics approval.

If you have any queries please contact the Human Ethics office at humanethics@uwa.edu.au.

Please ensure that you quote the file reference – RA/4/1/8714 – and the associated project title in all future correspondence.

Yours sincerely

Dr Caixia Li
Manager, Human Ethics

<table>
<thead>
<tr>
<th>Name</th>
<th>Faculty / School</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Peter Peeling</td>
<td>School of Sport Science, Exercise and Health</td>
<td>Chief Investigator</td>
</tr>
<tr>
<td>Professor Brian Dawson</td>
<td>School of Sport Science, Exercise and Health</td>
<td>Co-Investigator</td>
</tr>
</tbody>
</table>

Student(s): Louisa Lobigs
**Appendix C: Participant information, consent, ethics approvals**

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**Written Informed Consent**

<table>
<thead>
<tr>
<th>IRB number: .................................</th>
<th>Principal Investigator:  Y. O. Schumacher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Title: Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data</td>
<td>IC Version Date: 14. January 2013</td>
</tr>
</tbody>
</table>

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**INTRODUCTION**

We invite you to take part in a research study called "Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data". You were contacted as a possible participant in this study because you fall in the category of our research spectrum. Please take your time to read this form, ask any questions you may have and make your decision. We encourage you to discuss your decision with your family, friends and doctor(s).

---

**WHAT IS THE PURPOSE OF THIS STUDY?**

This study is being done to investigate the variability of blood variables used for the indirect detection of doping in the so-called "Athlete Biological Passport" (ABP). With the ABP, not a doping substance itself, but its effect on the body is detected through (longitudinal) repetitive measurement of certain biological markers. Other than from doping, the major influence on the markers used in the ABP comes from variations in body water, the so called plasma volume. Variations in body water will influence most measures used in the ABP, as these are typically measured as concentrations. Thus, increases in body water will dilute these measures. It is therefore sometimes difficult to distinguish natural variations in blood markers, which occur in every human, from doping induced changes. In athletes, plasma volume variations of up to 20% have been reported. Plasma volume also plays a key role in the adaptation of the organism to heat and exercise in general.

The purpose of this study is to investigate the variation of plasma volume and ABP markers during long term sporting activity. Using mathematical methods developed from these data, it could be possible in the future to normalize ABP data for the natural variability and thereby differentiate between natural variation and doping effects.

---

**WHAT ELSE SHOULD I KNOW ABOUT THIS RESEARCH STUDY?**

It is important that you read and understand several points that apply to all who take part in our studies:

- Taking part in the study is entirely voluntary and refusal to participate will not affect any rights or benefits you normally have;
- You may or may not benefit from taking part in the study, but knowledge may be gained from your participation that may help others; and
- You may stop being in the study at any time without any penalty or losing any of the benefits you would have normally received.

The nature of the study, the benefits, risks, discomforts and other information about the study are discussed further below. If any new information is learned, at any time during the research, which might affect your participation in the study, we will tell you. We urge you to ask any questions you have about this study with the staff members who explain it to you and with your own advisors prior to agreeing to participate.
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: ......................... Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume shift from high-throughput longitudinal biological data
IC Version Date: 14. January 2013

WHO IS IN CHARGE OF THIS STUDY?

The principal investigator of this study is Dr. Yorck Olaf Schumacher. It is done as a cooperative project between Aspetar, Aspire and the Anti-Doping Laboratory Qatar. The research is being sponsored by the World Anti-Doping Agency (WADA) and the Aspire Zone Foundation.

WHO CANNOT PARTICIPATE IN THIS STUDY?

You cannot be in this study if any of the following apply to you:

- Unfit to play sports
- Anaemia
- Polycythaemia
- Haemophilia or other clotting disorders
- Smoking
- Low blood pressure (repeated, long lasting periods of systolic measures below 90 mm Hg)
- Surgery within the last 4 months
- Altitude sojourn at more than 2500m for more than 1 week in the three weeks preceding the study.
- Subjects with acute illnesses or diseases
- Subjects with reduced ability to communicate
- Subjects with ongoing drug abuse
- Subjects that are unable to provide written informed consent

WHAT IF I AM PRESENTLY PARTICIPATING IN ANOTHER RESEARCH STUDY?

Are you presently participating in any other research studies?   Yes ☐  No ☐

If yes, please state which study (ies) ____________________________

While participating in this study, you should not take part in any other research project without approval from the people in charge of each study. This is to protect you from possible injury arising from such things as extra blood drawing or similar hazards.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 30 people will take part in this study.

Guardian initial: ____________________________

Page Number: 2 of 7
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: .................................  Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data
IC Version Date: 14. January 2013

WHAT HAPPENS IF I AGREE TO BE IN THE STUDY?

If you agree to participate in this study, you will be monitored on a monthly base over 6 months while performing your normal training routines. At the end of the 6 months (within a period of 2 weeks), you will be submitted to one short-term plasma volume challenge in a heat chamber (exercise on a bicycle in the heat). Once every month during these 6 months, you will undergo several medical tests, which are outlined below. The schedule of these tests will be adapted individually to suit your other commitments.

The procedures that are research related are:

- Venous Blood Sampling
  On each occasion, you will be asked to provide a venous blood sample from a forearm vein. Several sample tubes with a total volume of about 15 ml will be obtained.

- Haemoglobin mass determination
  The measurement of your body’s total content of haemoglobin will be performed using a method called “CO rebreathing method”. In this procedure, a small amount of a gas tracer substance is introduced in your organism via inhalation. A very small quantity of carbon monoxide is used for this purpose. Carbon monoxide will stick to your red blood cells and through the amount of gas attached to your blood cells (which will be measured via capillary blood samples taken from your earlobe), we will be able to calculate your body’s content in haemoglobin. (The carbon monoxide will leave your body within 5 hours after the procedure).

HOW LONG WILL I BE IN THE STUDY?

You will be in the study for 6 months and 2 weeks.

The investigator may decide to take you off this study if it is believed to be in your best interest, you fail to follow instructions, new information becomes known about the safety of the study, or for other reasons the investigator or sponsor believes are important.

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the investigator and your regular doctor first.

If you suddenly withdraw from the study, we may not be able to use any of the information gathered from your participation.
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: .................................................. Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data IC Version Date: 14. January 2013

WHAT ARE THE RISKS AND SIDE EFFECTS OF THIS STUDY?

If you decide to participate in this study, you should know there may be risks. You should discuss these with the investigator and/or your regular doctor and you are encouraged to speak with your family and friends about any potential risks before making a decision. Potential risks and side effects related to this study include:

*Haemoglobin mass determination (CO rebreathing method)*
None, other than the possible toxicity of CO (a maximal level of 4-6% will be reached during the study). In the organism, CO has a half life time of approximately 5 hours.
- General side effects of CO: Destruction of Red Blood cells (dose dependent haemolytic agent)
- Acute Toxicity at CO-levels of >2000ppm
- CO-Hb Level < 20%: Headache, Dizziness, visual impairment, nausea, impaired physical performance.
- CO-Hb Level 20-30%: additional impairment of the cognitive skills
- CO-Hb Level >30%: Loss of consciousness, respiratory inhibition, cardiovascular complications
- Possible damage to unborn children.

*Phlebotomy (blood sampling from forearm)*
Bleeding from the phlebotomy site, injuries of surrounding structures such as nerves.

As part of this study, you will be involving in genetic/ proteomic testing. Risks of being in genetic testing include the misuse of personal, genetic information. Although rare, misuse of such information has caused problems for persons related to their employment and/or their life and/or health insurance and other benefits or entitlements. Also, there is a risk that being in a genetics study can cause psychological distress or experience tension with other family members. Although there can be no absolute guarantees, every reasonable effort will be made to keep your personally identifiable information secret so that there will be no misuse. Even when the information is kept secret, if you are asked if you have ever been tested for a genetic disorder, answering “yes” could cause benefits to be denied or could cause other problems including discrimination.

For more information about risks and side effects, please ask Dr. Y.O. Schumacher.

ARE THERE ANY BENEFITS TO TAKING PART IN THE STUDY?

This study is not designed to provide direct benefits to any participants.

Guardian initial: ___________________________
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: .................. Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume
shifts from high-throughput longitudinal biological data
IC Version Date: 14. January 2013

WHAT ABOUT CONFIDENTIALITY?

Your personal health information (PHI) will be kept private to the extent allowed by law. You will not be identified by name in any publications resulting from this study.

WILL I BE PAID FOR PARTICIPATING IN THIS STUDY?

You will be paid for being in this study, AMOUNT REMAINS TO BE DETERMINED. Materials and information obtained from you in this research may be used for commercial or non-commercial purposes. It is our policy not to provide additional financial compensation to you should this occur.

WHAT ARE THE COSTS?

You do not have to pay anything to be in this study. You will not be charged for any procedures or investigations that are part of this research study.

WHAT IF I’M INJURED OR BECOME ILL DURING THE STUDY?

We will make every effort to prevent injuries and illness from being in the study. The research methodology has been designed to minimize the likelihood of an injury occurring during participation in this study. Aspetar will, within its scope of practice provide emergency care and reasonably treat any injury sustained as a direct outcome of participation in the study.

The participant will be transferred to Hamad Medical Corporation or an appropriate alternative treatment site for any medical intervention required by the participant that falls outside of Aspetar scope of practice.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

- You have the right to be told about the nature and purpose of the study;
- You have the right to be given an explanation of the exactly what will be done in the study and given a description of potential risks, discomforts, or benefits that can reasonably be expected;
- You have the right to ask any questions you may have about the study;
- You have the right to decide whether or not to be in the study without anyone misleading or deceiving you; and
- You have the right to receive a copy of this consent form.

Guardian initial: __________________________

Page Number: 5 of 7
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: ......................... Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume
shifts from high-throughput longitudinal biological data IC Version Date: 14. January 2013

By signing this form, you will not give up any legal rights you may have as a research participant. You may choose not to take part in or leave the study at any time. We will tell you about new information that may affect your health, welfare, or willingness to be in this study.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact the investigator, Dr. Yorck Olaf Schumacher at 3302 5807. If you are having a medical emergency, you should go directly to the nearest emergency room.

For questions about your rights as a research participant, contact the Office of Research or the principal investigator.

Consent for studies involving genetic material (DNA / RNA)

To protect you against the risk of breaching confidentiality, all samples for genetic, proteomic or metabolomics analysis will be coded and stored without identifying information. There will be a strictly confidential record of these samples, which will remain locked in secure places.

Efforts will be made to protect your personal genetic information to the extent allowed by law. Medical records of research study participants are stored and kept according to legal requirements. You will not be identified in any reports or publications resulting from this study.
Appendix C: Participant information, consent, ethics approvals

Written Informed Consent

IRB number: …………………………
Principal Investigator: Y. O. Schumacher
Project Title: Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data
IC Version Date: 14. January 2013

Agreement for the use of samples for genetic testing:

I permit coded use of my blood samples for the proposed study, and I specify the use of the samples in the following manner (please check only one of the following):

☐ I permit anonymized (samples cannot be linked to subject) use of my blood samples for other studies without contact.
☐ I permit further contact to seek permission to do further studies on my samples.
☐ I do not allow use of my blood samples for further studies.

SIGNATURES
As a representative of this study, I have explained the purpose, the procedures, the possible benefits and risks that are involved in this research study. Any questions that have been raised have been answered to the individual’s satisfaction.

__________________________
Signature of Person Obtaining Consent

__________________________
Date of Signature

I, the undersigned have been informed about this study’s purpose, procedures, possible benefits and risks, and I have received a copy of this consent. I have been given the opportunity to ask questions before I sign, and I have been told that I can ask other questions at any time. I voluntarily agree to be in this study. I am free to stop being in the study at any time without need to justify my decision and if I stop being in the study I understand it will not in any way affect my future treatment or medical management at Aspetar. I agree to cooperate with Dr. Yorck Olaf Schumacher and the research staff and to tell them immediately if I experience any unexpected or unusual symptoms.

__________________________
Participant’s Signature

__________________________
Date of Signature

__________________________
Signature of Witness

__________________________
Date of Signature

__________________________
Signature of Legally Authorized Representative (When Appropriate)

__________________________
Date of Signature

__________________________
Relationship to Participant (When Appropriate)

__________________________
Date of Signature

Guardian Initial: ____________________________
Dr Yorck O. Schumacher,
Sports Medicine Physician,
Aspetar, Orthopaedic and Sports Medicine Hospital
P.O. Box 29222 Doha, Qatar
19 March, 2013

RE: IRB REVIEW
RESEARCH PROPOSAL APPROVAL
IRB Project Number: 2013-002
Protocol Title: “Derivation of a marker of plasma volume shifts from high-throughput longitudinal biological data”.
Approved Location: Shafallah Medical Genetics Center

Dear Dr. Schumacher,

The Shafallah Institutional Review Board has reviewed and approved your research proposal that was submitted for the above referenced protocol (2013-002), and informed consent documents (English versions), at its full board meeting on February 27, 2013. Approval of this study is valid March 19, 2013 through March 18, 2014. If the study will continue beyond the expiration date, please submit a continuation request form forty five (45) days, prior to the expiration date, to allow the IRB sufficient time to review and approve the request. Please refer to the SMGC Office of Research, website to review the “Principle Investigator responsibilities” on www.SMGC.org.qa
If you have any question, please contact me at 4956160 or via e-mail alshabanf@smgc.org.qa

Sincerely

Fouad Al Shaban, MD, MSc, PhD,
Senior Research Scientist,
Institutional Review Board Coordinator,
Shafallah Medical Genetics Center

Attachments: copy of approved consent forms.
Appendix C: Participant information, consent, ethics approvals

Patent Application

BioKaizen Laboratory SA has filed a patent to the World Intellectual Property Organization (WIPO) that includes claims about the plasma volume blood test described within this thesis.

Information and documents referring to the patent application can be found in the link below.

INFORMATION TO PARTICIPANTS

Research Title:

**Confounders of the Blood Passport - what is the effect when iron and altitude are combined?**

**Principal Researcher:**
Dr Laura Lewis,
Physiology, Australian Institute of Sport

We would like to invite you to participate in this original research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

**Aim:**

The primary aim of this research project is to quantify the effects of iron supplementation at altitude on the blood markers which are usually collected for the Athlete Blood Passport.

The effect of living at altitude and training at sea level (also known as Live high, train Low) altitude is of interest particular in an anti-doping context. One of the ways the body responds to living at altitude, is to make more red blood cells. Anti-doping authorities often track changes in numbers of red blood cells as a method of blood doping detection. A “biological passport” is created for each athlete, with the two key variables used being haemoglobin concentration ([Hb] = a measure of blood thickness) and percent reticulocytes (RET% = young red blood cells), both of which can increase with altitude exposure. It is therefore, very important to understand the magnitude of red blood cell changes that occur naturally in response to altitude training, so that innocent athletes are not falsely accused of cheating. We will also be using the changes in red blood cells observed in this study to help improve anti-doping detection methods.
Appendix C: Participant information, consent, ethics approvals

A secondary aim is to investigate the combined effect of intravenous iron supplementation and altitude training in athletes. Specifically, we will be comparing how different iron supplementation protocols (oral iron, intravenous iron (IV) and placebo) influence the physiological adaptations to altitude exposure.

Benefits:

The study will also assist anti-doping authorities to improve the Athlete Blood Passport, by providing important information on the “normal” response to altitude training.

Who we are recruiting?

We are looking for male and female endurance-trained athletes aged 16-50. Athletes must not be taking any form of iron supplementation for 4 weeks prior to commencing the study. Unfortunately, if you have any of the following conditions, we will not be able to include you in the study:

- Previously documented hypersensitivity to iron
- Anaemia not caused by simple iron deficiency
- Iron overload (haemochromatosis, haemeosiderosis)
- Osler-Rendu-Weber syndrome
- Chronic polyarthritis
- Infectious renal complaints in acute phase
- Uncontrolled hyperparathyroidism
- Decompensated hepatic cirrhosis
- Infectious hepatitis
- Pregnancy
- Severe infection or inflammation of the kidney or liver
- Low iron binding capacity
- Polycythaemia
- HCT >50%
- Hypertension (>140/90)
- Recent blood transfusion or blood donation

Participants must be prepared to sign a statutory declaration, stating that they have not previously used a prohibited substance or practice. Participants should be aware that AIS staff
are obligated to report abnormal results to the Australian Sports Anti-Doping Authority (ASADA).

**What is involved?**

Endurance-trained athletes, who fit the criteria detailed below, will be invited to participate in the study, involving a 3 week Live High, Train Low Altitude camp. After a medical health screen, you will be randomly assigned to one of three supplementation protocols: 1. IV iron supplementation, 2. Oral Supplementation or 3. Placebo. You will not know which group you are in until after the study has finished, so will be receive both oral supplementation and a course of IV injections during the study.

*Oral Iron Supplementation:*

The oral iron supplementation group will be supplemented with iron tablets (Ferrograd C brand) which are to be taken at the prescribed dose (either 1 or 2 x daily depending on individual) for 6 weeks. It is important that you take the iron supplement as prescribed – do not exceed the prescribed dose. If you forget to take the supplement on one day, do not take double the dose to ‘catch up’ but instead record the day and inform the study research team. Ferrograd C also contains a high dose of vitamin C which improves iron absorption by by your body. The placebo and IV groups will be given a glucose pill to ingest daily.

*IV Iron Supplementation:*

On up to 3 occasions, you will be asked to attend the AIS medical clinic to receive your injection. Either IV Iron (FERINJECT) or placebo (normal saline) will be injected via an indwelling cannula (plastic tube) inserted into a forearm vein. Your arm will be placed through a curtain during this time, and the syringe will be taped over so you will not be aware of which treatment you are being given. The total dose of iron given intravenously over the supplementation period will be carefully determined based on your current iron stores and body weight. In rare cases, IV iron supplementation may cause hypersensitivity and anaphylactoid (shock) reactions that can be potentially fatal. A full description of the risks involved is included later in this information sheet but it is important that you discuss the potential risks with the study team before participating.

**Supplements/Medication use**

Appendix C: Participant information, consent, ethics approvals

(http://www.ausport.gov.au/ais/nutrition/supplements/overview). Ferinject is not prohibited under the WADA 2015 Prohibited list. However if you have any concerns about the status or use of this substance or method please raise these concerns with the principal researcher or AIS Ethics Committee Secretary. Alternatively you may wish to check the “check your substances” database at https://checksubstances.asada.gov.au”. Ferinject is approved for use by Therapeutic Goods Administration (TGA). The Ferinject used in this study will be obtained directly from our Pharmacy suppliers in pure form and has been checked to ensure it is not contaminated. Expiry dates and product ingredients are checked by at least two members of medical staff before administration.

**How long will the study last?**

The total length of the study is 11 weeks, including 3 weeks of LHTL altitude training plus pre and post testing. During this time you will be asked to continue your normal training program.

You will be required to sleep in the AIS Altitude House for 3 weeks. The AIS Altitude house has been specially constructed to simulate 3000 m of altitude. Our altitude house comprises 5 modest rooms and a kitchen and it simulates altitude by displacing some of the oxygen in the room with nitrogen. Throughout each night the house you will be monitored by an altitude house supervisor stationed about 15 m from the house. All meals will be provided on site. You will be asked to spend 14 hours per day inside the AIS altitude house. Your training will be performed outside, in your usual environment.

You will be asked to attend the physiology laboratory two weeks prior to your stay in the AIS altitude house and 3 times over 6 weeks following your stay. A number of other tests will be performed during the 3 week LHTL period. The testing requirements and time required for each test are detailed below. For each treadmill-testing day, we ask that you avoid strenuous exercise, alcohol and caffeine for the preceding 24 hours.

**TESTING REQUIREMENTS**

<table>
<thead>
<tr>
<th>Test Description</th>
<th>Time Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical screening questionnaire and explanation</td>
<td>15 min</td>
</tr>
<tr>
<td>Haemoglobin mass (-14, -1 day prior to LHTL; weekly during LHTL; 1,3,6 weeks post LHTL)</td>
<td>15 min</td>
</tr>
<tr>
<td>Blood test (-14, -1 day prior to LHTL; weekly during LHTL; 1,3,6 weeks post LHTL)</td>
<td>15 min</td>
</tr>
<tr>
<td>VO2 max test on treadmill or cycle ergometer (Pre and post LHTL)</td>
<td>1 hour</td>
</tr>
</tbody>
</table>
Appendix C: Participant information, consent, ethics approvals

<table>
<thead>
<tr>
<th>LHTL altitude camp (3 weeks)</th>
<th>14 hr per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV injections (up to three) and supervision (-12, -1 day prior to LHTL, day 10 of LHTL)</td>
<td>45 min</td>
</tr>
</tbody>
</table>

Total time for pre testing: 4 hours
Total time for each IV injection session: 45 min
Total time for post testing: 3 hours

**TEST DETAILS**
If you would like to participate in this research project you will be asked to attend the AIS before the study begins so that the full details of the experiment can be explained. At this initial visit you will be shown all the facilities, made familiar with the testing procedures, and meet the AIS staff involved with this study. At this point, if you are interested in participating, you will receive a blood test to assess your haemoglobin and iron levels and determine your suitability for the study. These results will be available to you whether or not you choose to participate further.

**Medical Screen**
An AIS doctor will meet with you to assess your medical history, review your blood test results, check for any known blood problems, and ascertain if you have high blood pressure or any other risk factors. These include iron deficiency anaemia, pregnancy, altitude training within 1 month of start of study, hypertension, polycythaemia, previously documented hypersensitivity to iron, anaemia not caused by simple iron deficiency, iron overload (haemochromatosis, haemeosiderosis), Osler-Rendu-Weber syndrome, chronic polyarthritis, infectious renal complaints in acute phase or uncontrolled hyperparathyroidism. If you are deemed fit to participate in the study, we will continue with baseline testing.

**Laboratory tests**
A treadmill or cycle ergometer test will be conducted at baseline for assessment of your aerobic fitness (VO$_2$max).

- The standard incremental test involves 4-5 x 4-minute periods of continuous running at increasing speeds (typically 11-16 km/hr for females and 13-18 km/hr for males). You will be required to wear a headpiece, mouth piece and nose clip throughout the test in order to collect expired gas, as well as a heart rate monitor around your chest to record your heart rate. Fingertip capillary blood lactate (5 µl) will be sampled after each stage.
Appendix C: Participant information, consent, ethics approvals

After a 5-minute rest, running will resume with speed increasing by 0.5 km/hr every 30s for 5 minutes, then gradient will be increased by 0.5% every 30s until volitional exhaustion. Expired gas and heart rate will be measured continuously and fingertip capillary blood (100 µl) will be sampled 3 minutes after the test to assess blood lactate and buffering capacity. The total running time for the whole test is typically 25-30 minutes. If the test is performed on the cycle ergometer, the test involves cycling for 3 minutes at a constant workload which is increased by 25 W every 3 mins until exhaustion.

You may experience temporary heavy breathing and muscular fatigue immediately upon completion of the final maximal step of the treadmill test. These feelings of discomfort will subside within 1 or 2 minutes and are commonly experienced in racing and training.

Total haemoglobin mass - Carbon Monoxide (CO) rebreathing test
Your total haemoglobin mass (amount of red blood cells in your body) will be measured throughout the study. A small quantity of carbon monoxide (<100 ml) is re-breathed at rest with 3.0-3.5 L of pure oxygen for 2 minutes. Capillary blood (200 µl) will be sampled from the fingertip at 0 and 7 minutes to measure carboxyhaemoglobin in the blood which allows calculation of total haemoglobin mass. This is a routine test in our laboratory and has been performed on hundreds of athletes from a range of sports.

Blood sampling
A qualified phlebotomist will take a small blood sample (~10 ml, which equates to about 2 teaspoons) at each time-point. Your blood will be analysed for a range of markers including iron status, young red blood cells and proteins in the blood that are activated during erythropoiesis. These results will help us to monitor and understand how your body is responding to iron supplementation and altitude exposure. These results will be entered into the Blood Module of the Athlete Biological Passport (ABP) but do not constitute any official form of drug testing. Your name will not be provided to anti-doping authorities.

Adverse Effects and Withdrawal:
You are not obliged to participate in this study. Your participation in this research study is entirely voluntary and you have the right to withdraw at any time, without disadvantage. If your results yield any concerning or abnormal results we will discuss these with you immediately and offer appropriate medical follow up, if required. The ABP profile created
Appendix C: Participant information, consent, ethics approvals

using your results is for research purposes only and will not be provided to anti-doping authorities.

1. **IV Iron supplementation** – All IV injections will be performed by an AIS medical doctor in the AIS Medicine rooms. Although the risks are low, it is important to be aware that IV iron preparations can cause hypersensitivity reactions including anaphylactoid reactions, which in severe cases can potentially be fatal. Therefore, you will be required to remain within AIS medicine, under medical supervision for 15 minutes following each iron injection. Cardiopulmonary resuscitation equipment will be available at all times. If allergic reactions or signs of intolerance occur during administration, the treatment will be stopped immediately. The injection will involve a butterfly needle being inserted into a forearm vein, which the soluble iron is then injected into. The needle will then remain in place whilst you are supervised in case of any adverse reactions.

   With all IV injections there is a small risk of some leakage outside of the vein which can lead to skin irritation and brown discolouration. If any leakage occurs, the injection will be terminated immediately.

   The following side effects may be experienced from FERINJECT: headache, dizziness, nausea, abdominal pain, constipation, diarrhoea, rash, anxiety and injection site reaction. Incidence of any side effects should be reported to the research team. Full product details and associated risks will be provided to you and you are encouraged to raise any questions or concerns with the research team at any time. **You must not take additional iron supplements during the study period.**

2. **Sleeping at Altitude** - Most athletes comment that they feel absolutely no different from normal when they walk into the Altitude House. Because the Altitude House does not involve any alteration in the barometric pressure there is no sensation of a “sudden change” when you walk inside. At 2000 m, the altitude simulated is not too different from that to which you are commonly exposed during an international plane flight. However, at 3000 m it is possible that you may experience symptoms of altitude sickness. The symptoms of altitude sickness include:

   - headache
   - dizziness
   - nausea
   - shortness of breath
   - persistent coughing
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- chest pain
- abdominal pain

If you experience any of these symptoms at any time during or after spending time in the Altitude House you must immediately report them to the supervisor. On reporting such symptoms, you will promptly be removed from the Altitude House.

Participants should be aware that the symptoms of altitude sickness can mimic those of anxiety and panic attacks. Participants should report any concerns to the research team or to their GP.

3. Testing procedures - The research team has extensive experience in the testing procedures involved in this study and we are not aware of any participant who has experienced a problem in either the short or long term. However, there are some risks involved in any experiment using these procedures, as follows:

- Infection – in any situation where the skin is penetrated there is a risk of infection. In this project all blood sampling procedures will use sterile materials so that the risk of infection is minimal. Infection has never occurred in our experience. You must not participate in this study if you have a history of bleeding disorders or thrombosis.

- Bruising – it is possible that you will suffer some bruising around the blood sampling sites. Any bruising that does occur should clear up within a few days.

- CO-rebreathing test - The total amount of CO introduced into the rebreathing circuit is harmless and is equivalent to being in a ‘smoky’ room for a few hours. Carbon monoxide is harmful in large volumes but our procedure using a 100 millilitre syringe to deliver the CO into the rebreathing system means that it is impossible to endanger you. The research team has performed several thousand of these tests without any adverse effects. You can train normally directly after the CO test without any noticeable effects. The half-life of CO in blood is approx 2.5 hours, therefore, more than 90% of the CO in your blood will be gone after 10 hours.

4. Oral iron supplementation – Iron supplementation is usually prescribed to athletes exposed to altitude in order to support the body’s increased drive to make red blood cells. In some cases iron supplementation can cause disturbances in the gut such as
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abdominal pain, nausea, constipation or diarrhoea, vomiting, rash or dark coloured faeces. If you experience any of these side effects please speak to the research team about ways to minimise or reduce these effects. The following may decrease the absorption of iron when used concurrently. Therefore you should try not to take your iron supplement within one hour before or two hours after taking:

- antacids
- calcium supplements
- tea and coffee
- eggs
- milk or milk containing products
- cereals, whole grain breads or dietary fibre
- medicines containing bicarbonates, carbonates and phosphates

**Anti-doping research**

The present study is an important step in the fight against doping in sport. The World Anti-doping Authority (WADA) is aware and supportive of our research and will be provided with a full report following the completion of the study. If out of competition testing is conducted during the study, participants are advised to notify officials of their involvement in the study at the time of testing.

**Confidentiality:**

You will be provided with a full copy of your own results on completion of the study, as well as the general findings of the study. At this point we will also inform you as to which treatment group you were allocated to. No data from any other individual participant will be provided to you by the researchers. Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by a password. You will be identified by number only and when results are presented (in publications and at conferences) we will only make reference to group changes not individuals.

**Ethics Approval:**

The study has been approved by the Australian Institute of Sport ethics committee. If you have any concerns, you should contact the secretary of the AIS Ethics Committee on 02 6214 1577.
Further information:

Please contact the principal researcher, Laura Garvican-Lewis if you are interested in participating in the study or if you have any further questions:

Laura Lewis, AIS Physiology
‘INFORMED CONSENT’ FORM (Adult)

Project Title: Confounders of the Blood Passport - what is the effect when iron and altitude are combined?"

Principal Researchers: Dr Laura Garvican-Lewis, Dr Greg Lovell, Professor Chris Gore, Dr Daniel Eichner, Dr Peter Peeling and Dr David Hughes.

This is to certify that I, ____________________________ hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Dr Laura Garvican-Lewis.

The investigation and my part in the investigation have been defined and fully explained to me by Dr Laura Garvican-Lewis and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

• I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.

• I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.

• I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.

• I understand that I am free to withdraw my data from analysis without disadvantage to myself.
• I understand that any data or answers to questions will remain confidential with regard to my identity.

• I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.

• I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

*Privacy Statement:* The information submitted will be managed in accordance with the ASC Privacy Policy.

☐ I consent to the ASC keeping my personal information.

Signature of Subject: _______________________________ Date: ___/___/___

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: _____________________________ Date: ___/___/___
Appendix C: Participant information, consent, ethics approvals

TO: Dr Laura Garvican-Lewis
FROM: Ms Helene Rushby
SUBJECT: Approval from AIS Ethics Committee
DATE: 14th January 2016

On the 13th October 2015, the AIS Ethics Committee gave consideration to your submission titled “Confounders of the Blood Passport - what is the effect when iron and altitude are combined”. The Committee noted that following adequate responses from the Research Team on concerns raised, there are no ethical reasons why this study shouldn’t now go ahead.

Project 20151001 is approved

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,
Any adverse events that may occur,

Researchers are required to submit annual status reports and final reports to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the “Guidelines” for ethics submissions.

Please note the approval for this submission expires on the 31st December 2017 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don’t hesitate to contact me on (02) 6214 1577

[Signature]
Secretary, AIS EC
Appendix D

Raw data

A link for the raw data from studies 1 to 4 is provided below.

**Study 1 (Chapter 3)**

https://drive.google.com/open?id=19XgnTE0MlmiYWhYAM7BNtbaWQM_4Huso

**Study 2 (Chapter 4) and Study 3 (Chapter 5)**

https://drive.google.com/open?id=16iq0ltRXN5R8zxU8NVqlnzjnktEBmEYz

**Study 4 (Chapter 6)**

https://drive.google.com/open?id=1AjRutVFspw-LqTS0hwmMxU304_JYPHzO