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Blood Volume and Haemoglobin Mass Measurement in Medicine

by

Dr James Oliver Mark Plumb

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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This thesis focuses on measuring blood haemoglobin content and its relationship with physiological performance in human subjects. Haemoglobin concentration [Hb] is commonly measured in clinical practice. In contrast, total haemoglobin mass (tHb-mass) is rarely used since direct measurement methods have been expensive, inaccurate, or technically challenging. However, the modified carbon monoxide rebreathing method (oCOR) has the potential to measure tHb-mass rapidly and simply. The clinically important condition of anaemia is described and the different methods used to measure blood volume and tHb-mass preceding the oCOR technique's development are discussed. Subsequently, the oCOR technique is described and used to measure and characterise anaemia in different patient groups. Finally, the impact of intravenous iron on the correction of anaemia and its effect on physiological reserve is explored.

It was hypothesised that patients with greater physiological reserve, defined by exercise capacity measured using cardiopulmonary exercise testing (CPET), would be better able to withstand the physiological challenge of surgery. And secondly, that measurement and manipulation of tHb-mass would improve physiological reserve through improvement in measured exercise capacity. To test these, experiments were performed to refine the oCOR technique in different patient populations. Adaptations to the oCOR in conditions simulating mechanical ventilation during surgery or critical illness were explored.

Finally, a perioperative interventional pilot study testing whether intravenous iron could improve haemoglobin mass and CPET derived markers of cardiorespiratory fitness was undertaken.

It was demonstrated that the oCOR method was safe for measuring tHb-mass in patients with chronic liver disease and ascites without adjusting blood sample time points. In comparison with oxygen plus exercise, it was shown that oxygen alone could effectively clear carbon monoxide between tests to allow duplicate measurements within a short time frame. Finally, in a pilot interventional study, anaemic perioperative patients given intravenous iron had significant improvements in [Hb], tHb-mass and CPET derived markers of physical fitness.

The modified oCOR could illustrate the aetiology of anaemia in critically ill patients, accurately measure surgical blood loss and distinguish between dilutional and true anaemia in patients with heart failure. Further research should define normative data for tHb-mass for healthy subjects across different populations. Research into patient outcomes following oCOR directed perioperative optimisation is needed to support wider adoption. Pilot data from this thesis would ensure such perioperative studies were adequately powered.

Table of Contents

List of Tables

List of Figures

Declaration of Authorship

I, Dr James Oliver Mark Plumb

declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

Blood volume and haemoglobin mass measurement

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University.
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
3. Where I have consulted the published work of others, this is always clearly attributed.
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
5. I have acknowledged all main sources of help.
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.
7. Parts of this work have been published as: [please list references below]:

(1) Plumb JOM, Otto JM, Grocott MPW. “Blood doping” from Armstrong to prehabilitation: manipulation of blood to improve performance in athletes and physiological reserve in patients. Extrem Physiol Med. England; 2016;5:5.

(2) Plumb JOM, Otto JM, Kumar SB, et al. Application of the optimized carbon monoxide rebreathing method for the measurement of total haemoglobin mass in chronic liver disease. Physiol Rep. 2020;8(6):e14402. doi:10.14814/phy2.14402

(3) Plumb JOM, Kumar S, Otto J, Schmidt W, Richards T, Montgomery HE, et al. Replicating measurements of total hemoglobin mass (tHb-mass) within a single day: precision of measurement; feasibility and safety of using oxygen to expedite carbon monoxide clearance. Physiol Rep [Internet]. 2018 Sep [cited 2018 Sep 18];6(17):e13829. Available from: http://doi.wiley.com/10.14814/phy2.13829

(4) Krehl LM, Plumb JOM, Wachsmuth NB, Haupt S, Kumar SB, Otto JM, Schierbauer J, Grocott MPW, Montgomery HE, Schmidt WFJ. A carbon monoxide 'single breath' method to measure total haemoglobin mass: a feasibility study. Exp Physiol. 2021 Feb;106(2):567-575. doi: 10.1113/EP089076. Epub 2020 Dec 30. PMID: 33369791.

Signed:

Date: 06/12/2021

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Definitions and Abbreviations

The usual nomenclature for writing cardiac output in scientific texts is ‘CO’ however due to the frequency of wanting to use ‘CO’ for carbon monoxide gas in this thesis the following abbreviations are used:

CarO- cardiac output
CO- carbon monoxide

All other terms are explained in the ‘Glossary of Terms’ after the Appendices on page 201.

Chapter 1 - Introduction

1.1 Structure and questions of this thesis

The purpose of this chapter is to give a brief overview of the contents of this thesis.

This thesis centres on measuring the volume of the blood. It has a particular focus on the direct measurement of the total mass of circulating haemoglobin using carbon monoxide gas. All of the experiments found within this thesis were performed on human subjects, Chapter 5 & Chapter 8 on clinical patients and Chapter 6 & Chapter 7 on healthy volunteers. Each experiment used the optimised carbon monoxide re-breathing method (oCOR) as modified by Professors *Schmidt* and *Prommer* (5,6).

This thesis explored measuring total haemoglobin mass (tHb-mass) in a variety of settings. The aim was to explore the role of monitoring changes in tHb-mass perioperatively, with a particular focus on blood manipulation in the perioperative setting. This thesis focuses on the important perioperative disorder of anaemia and explores further the relationship between exercise capacity, anaemia, and blood volume measurement.

Questions are posed regarding the physiological extremes to which blood *could* be manipulated in the clinical setting. To frame this discussion experiments and lessons learned from elite sport are explored.

1.2 Background Chapters 2 & 3

Chapters 2 and 3 outline the background to the work. Chapter 2 focuses on the physiology of the blood and oxygen transport by haemoglobin. It then places the measurement of total haemoglobin mass in context (sections 2.2-2.5). Particularly, its relevance to perioperative care, which is explored further in section 3.9.1. The history of blood volume measurement is outlined. Chapter 3 is concerned with the global pandemic of anaemia.

The hypotheses, aims and objectives are clearly outlined at the end of Chapter 3 in section 3.12

1.3 Methods

Chapter 4 outlines the common methodology of the studies that follow in Chapter 5-Chapter 8. Common procedures and statistical analyses are discussed to avoid repetition in subsequent chapters. Each data driven chapter will have a methods section with methodology that was particular to that specific study/experiment.

1.4 Data chapters 5-8

Chapter 5 - Blood volume and tHb-mass in liver disease. **Application of the optimized carbon monoxide rebreathing method for the measurement of blood volume and haemoglobin mass in chronic liver disease** (2) This was a methodological study measuring blood volume and total haemoglobin mass in a group of patients with chronic liver disease and ascites.

Chapter 6 - The 'REMATcH' study. **Replicating measurements of total haemoglobin mass (tHb-mass) within a single day: Precision of measurement; feasibility and safety**

of using oxygen to expedite carbon monoxide clearance- REplicating MeAsurements of Total Haemoglobin (REMATcH) (3)

For widespread use in the clinical setting any measure of blood volume/total haemoglobin mass needs to be repeatable, precise, and accurate. Until now most authors had recommended that a minimum of twelve hours (when breathing room air) is left between repeat measurements due to safety concerns regarding inhalation of carbon monoxide gas. This study aimed to ascertain whether tHb-mass testing could be safely repeated within 3 hours if carboxyhaemoglobin levels were actively reduced by breathing supplemental oxygen alone (*PROC$_A$*).

Chapter 7 - A 'new method' for measuring tHb-mass. **A carbon monoxide 'single breath' method to measure total haemoglobin mass- a feasibility study**

In this chapter an experiment was undertaken in healthy volunteers to attempt to modify the traditional optimised carbon monoxide re-breathing method (oCOR). The established measurement technique for tHb-mass requires a spontaneously breathing non-intubated patient. The primary aim of this study was to assess feasibility of a new method for measuring tHb-mass in healthy participants simulating a procedure that *might* be used in a participant on a mechanical ventilator (*PROCnew*).

Chapter 8 - **The 'CAPOEIRA-I' study. Cardiopulmonary exercise testing before and after intravenous iron: a prospective clinical study.** Short title: CAPOEIRA-I STUDY. CArdio PulmOnary Exercise testing and IntRAvenous Iron

The final data chapter brought together many themes of the thesis. It was a pragmatic, prospective, pilot clinical perioperative trial, examining anaemic patients in the preoperative phase of their pathway. Specifically, we aimed to test the hypothesis that augmenting haemoglobin through intravenous iron therapy would improve preoperative physical fitness (cardiopulmonary exercise testing (CPET) variables).

1.5 Chapter 9 - Discussion and conclusions

The final chapter of this thesis discusses and concludes the body of work offering recommendations for future work.

The data driven chapters 5-7 have been published and these can be found in Appendix E. Chapter 8 has been submitted for publication and is currently undergoing peer review.

Chapter 2 - Background: Part 1: Blood composition and blood volume measurement

2.1 The important role of blood in the context of oxygen delivery and human performance

Humans are reliant upon oxygen for aerobic respiration. Blood, therefore, plays a fundamental role in facilitating this via oxygen transport (section 2.3). Measuring blood volume and its component parts has been the subject of scientific investigation for centuries (7,8) (section 2.6). The measurement of blood goes beyond mere scientific intrigue, after all:

> 'The life of the flesh is in the blood' Leviticus 17:11 (9)

Blood has played a central role in many religious ceremonies and rituals throughout the ages and today holds an especially significant place amongst the Jehovah's Witness community. Much of modern-day conservative patient blood management (PBM) strategy has been developed as a result of needing to accommodate the religious and cultural demands of this community. Physicians have been forced to learn adaptive strategies to safely manage patients who refuse blood products in the perioperative period or during critical illness (10,11).

Blood is a precious and expensive commodity. In the United Kingdom alone approximately 6000 units of blood are transfused each day (12). Whilst some drivers for reducing this figure are financial, many are in fact physiological with allogeneic blood itself having a host of significantly harmful effects (1).

The quest for the 'optimum' physiological condition with regard to the haematopoietic system has long been pursued by elite endurance athletes. Athletes and coaches are constantly pursuing *legal* means, such as training at altitude to augment oxygen carriage through an increase in haemoglobin content and thereby improving sea-level performance (1,13). However, it is well documented that illegal methods of blood manipulation have been a part of elite endurance sport for decades. The earliest reports of 'blood doping' in the scientific literature date back to 1945–1947 (14,15). The first alleged use in elite sport was in the 1960s, when a French four times Tour de France winner (1961–1964) was named as one of the first cyclists to use the technique (1,16).

Physiological reserve or resilience can be objectively measured with cardiopulmonary exercise testing (CPET). Measures of peak oxygen consumption ($\dot{V}O_{2peak}$) and the maximal oxygen consumption at the anaerobic threshold ($\dot{V}O_{2AT}$) have both been positively correlated with outcome following major surgery: less physically fit patients having a higher incidence of mortality and morbidity after surgery (1).

Much of the literature in this area is derived from studies reporting cardiopulmonary exercise testing (CPET) variables. The underlying hypothesis of these studies has been that patients with greater physiological reserve defined by CPET variables (most commonly $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$) are better able to withstand the physiological challenge of surgery. Given that the oxygen uptake variables $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$ are correlated with

[Hb], it may be that some of the physical fitness–outcome relationship is mediated through haemoglobin related effects rather than cardiorespiratory function (1).

As we learn more about the relationship between physical fitness, defined by CPET-derived variables, and responses to prehabilitation in different patient groups, it may be that lessons learned from elite athletes could be applied to improving outcomes in patients around the time of surgery (1).

Blood consists of 3 main cell types- erythrocytes, leukocytes and platelets suspended in a complex liquid plasma (section 2.1). Haemoglobin which is packed into erythrocytes can be measured directly rather than as a concentration suspended in plasma. The multitude of ways this is done are discussed in detail in section 2.6. Many disease states affect the ability of the blood to effectively transport oxygen. Be it a disorder of the haemoglobin molecule itself, for example sickle cell anaemia or an inability of the body to effectively circulate oxygenated blood, such as heart failure.

It is well established that having low levels of haemoglobin is detrimental within clinical medicine in a wide variety of settings, with a clear biological gradient demonstrating that mortality increases as [Hb] decreases (17–19). It is also well established that ethical (and unethical) manipulation of haemoglobin increases elite level endurance performance (1). There is some evidence, albeit less certain, that perioperative manipulation of the blood can lead to fewer red cell transfusions (20) but other studies have not shown any benefit (21,22) and there are studies ongoing to ascertain if perioperative outcomes are affected by blood manipulation with particular interest in the use of intravenous iron in the perioperative setting (23–25) see section 3.2.2.1.1 for further discussion.

Perioperatively blood volume, plasma volume, haemoglobin mass and hence haemoglobin concentration [Hb] are all subject to dynamic changes. Presently, the majority of clinicians are reliant upon concentration-based tests for assessing the oxygen carrying capacity of the blood. This is of course at best an estimate of oxygen carrying capacity. In certain circumstances it is well known that changes in plasma volume (PV) can take place, severe sepsis in an ICU setting, a patient going onto cardiopulmonary bypass or simply a renal patient undergoing dialysis. Due to the convenience and low cost of measuring [Hb] in a fully automated manner, the numbers generated are familiar to even the most junior of clinicians.

Deficiency of haemoglobin leading to ‘anaemia’ was defined by the World Health Organization (WHO) who classified anaemia in 1968 as $<120 g.l^{-1}$ for women and $<130 g.l^{-1}$ for men, the merits of this is are discussed in sections 3.1.1 and 3.1.2. There are still many unknown factors with regard to adequate levels of [Hb] in different patients, under different circumstances with much debate about ‘transfusion triggers’ being just one example (26,27). The level of ‘critical [Hb]’ varies from patient to patient depending on oxygen flux, the extent of adaptive responses and reserve capacity in various organs. This inter-individual variation also varies temporally in the same patient especially depending on PV shifts (1,10,19,28,29).

Plasma volume excess or true haemoglobin deficiency can be measured by measuring all component parts that make up the circulating blood volume. Total circulating blood volume (CBV) is composed of red cell volume (RCV) or erythrocyte volume (EV) and plasma volume (PV). Total haemoglobin mass (tHb-mass) represents the absolute mass of circulating haemoglobin in the body, the measured [Hb] being dependent upon tHb-mass and blood volume (BV) [sum of plasma volume (PV) and total red cell volume]. The proportion of oxygen carried in solution in plasma is trivial (0.3 ml per 100 ml of plasma)

under normal physiological conditions, whereas each gram of Hb binds up to 1.39 ml of oxygen (see 0) . Thus, tHb-mass is the principal determinant of total blood O_2-carrying capacity and may provide additional information regarding the clinical status of patients than that provided by [Hb] alone (1). Plasma volume is subject to constant fluctuation whereas total haemoglobin mass (tHb-mass) demonstrates stability for weeks to months (30–32). The correlation between physiological reserve as defined by CPET variables as explained above is much stronger when tHb-mass is used instead of [Hb] strengthening the premise that it is total circulating oxygen-carrying capacity that appears to be crucial for oxygen delivery rather than its concentration within plasma (33–35).

Since *Haldane* and *Smith* first used carbon monoxide gas to measure blood volume (BV) in the late 1800s there has been a continued evolvement and refinement of BV measurement (36,37). This thesis represents another small chapter in this evolution. There are financial and practical reasons why blood volume measurement is not commonly undertaken in the clinical setting and through the work contained in this thesis and further work a major objective is to try to change this. Section 2.5 and particularly 2.5.1 explore the potential added value of measuring tHb-mass over simply [Hb] and the possible implications for blood manipulation in the perioperative setting. These ideas are discussed further in Chapter 8.

Oxygen content equation

- Arterial oxygen content = bound oxygen + dissolved oxygen
- $CaO_2 = (1.34\text{-}1.39 \times [Hb] \times SaO_2 \times 0.01) + (0.0225 \times PaO_2)$

2.2 The composition of blood

Blood consists of three main cell types; erythrocytes, leukocytes and platelets suspended in a complex liquid plasma. In health, blood represents around 8% of total body weight and has an average volume of 5 litres in women and 5.5 litres in men. Due to 99% of cells within the blood being erythrocytes the *haematocrit (Hct)* or *packed cell volume (PCV)* represents the percentage of the total blood volume occupied by erythrocytes, with the plasma accounting for the remaining volume. Mean normal values for PCV are 42% and 45% and for plasma volume 58% and 55% in women and men respectively (See Figure 1) (38).

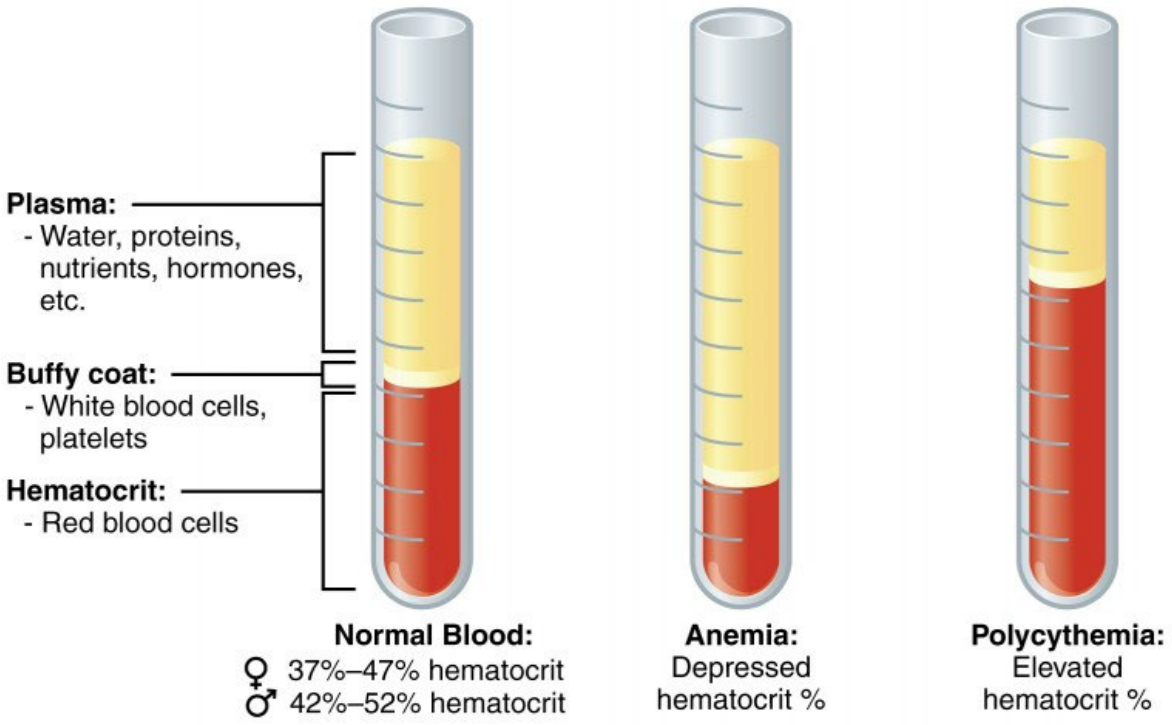

Figure 1: The composition of whole blood- Taken from (39)- Open source image

Haemoglobin is a conjugated metalloprotein molecule made of four polypeptide globin chains. Each contains a haem moiety which has an organic part (a protoporphyrin ring made up of four pyrrole rings) and a central iron ion in the ferrous state (Fe^{2+}) (40). More than 95% of adult haemoglobin is in the form of Haemoglobin A (HbA) with 2 α- and 2 β-globin chains. Each erythrocyte contains between 200 and 300 million molecules of haemoglobin almost excluding anything else; erythrocytes do not contain a nucleus, organelles, or ribosomes. As each haemoglobin molecule carries four oxygen molecules a single red blood cell can carry as many as one billion molecules of oxygen. Thus, a red blood cell is essentially a plasma membrane enclosed sac packed full of haemoglobin molecules.

Leukocytes are composed of granulocytes (neutrophils, eosinophils, and basophils) and non-granulocytes (lymphocytes and monocytes).

Platelets (thrombocytes) platelets are not actually cells but rather anucleate cytoplasmic fragments of megakaryocytes. Megakaryocytes are derived from hematopoietic stem cell precursor cells in the bone marrow.

2.3 Erythrocyte transport of oxygen

Red blood cells carry out the most vital of functions within the human body- those of oxygen loading in the lungs, carriage within the bloodstream and unloading at the tissues to facilitate aerobic cellular respiration. Oxidative phosphorylation to produce adenosine triphosphate (ATP), (the molecular unit of currency) being the primary product of aerobic glycolysis. ATP then facilitates cellular functions that are ultimately the end product of haemoglobin oxygen carriage. This is made possible by the fact that deoxygenated blood has a greater ability to carry and transport carbon dioxide dissolved in plasma in the form of bicarbonate and in carbaminohaemoglobin species within the erythrocyte than oxygenated blood does- this is known as the Haldane effect. The combination of the

Haldane effect and the Bohr effect (haemoglobin's ability to bind oxygen being inversely related to tissue pH and carbon dioxide concentration) result in oxygen binding and carbon dioxide release at the pulmonary capillaries and the opposite in the respiring tissues (41,42).

Carriage is of course dependent on the carrier (haemoglobin) and the delivery mechanism (the blood). Both can be affected in disease states, from disordered haemoglobin synthesis causing abnormal haemoglobin binding, such as in thalassemia to 'heart failure' which negatively affects the ability of the body to circulate haemoglobin. The effects on physical performance of these two entities (haemoglobin structure and blood flow) can be measured and are discussed in section 3.4.

As haemoglobin binds oxygen the protein undergoes a conformational change whereby its affinity to bind further oxygen molecules is increased. This cooperativity, the so named 'relaxed' 'R' and 'tense' 'T' states were described in the 1960s shortly after the protein structure was elucidated. *Monod* described this allosteric interaction and its ability to perform a homotropic positive co-operative interaction which explains the sigmoidal shape of the oxygen saturation curve (43). However things are more complex than this homotropic regulation alone (44).

There is a complex biochemical interplay that occurs within red blood cell that affects the binding characteristics of oxygen. The red cells regulate their own metabolism depending on the state of oxygenation, favouring glycolysis in low oxygen sates and the pentose phosphate pathway at high oxygen states. This occurs at the red cell membrane with the band 3 protein playing a critical role (45).

Hypoxia indices a vasodilatory response in the vasculature, these mediators come from within the red cell itself and include nitric oxide (NO) and red cell adenosine triphosphate (ATP). Haemoglobin serves as an oxido-reductase, inhibiting NO and promoting higher vessel tone when oxygenated and reducing nitrite to form NO and vasodilate when deoxygenated (44).

Cardiac output (CarO) expressed in $l.min^{-1}$ is the product of stroke volume (SV) x heart rate (HR). CarO clearly has a major influence on oxygen transport with many diseases affecting both cardiac rate and rhythm. Arguably, even more disease states affect cardiac filling, contractility, and the compliance of the cardiovascular system. The primary focus of this thesis is of haemoglobin and blood volume measurement and the factors that affect human performance as a result of manipulation of the body's total haemoglobin store (the total haemoglobin mass or red cell mass (RCM) and the implications for the classification and optimisation of anaemia. Whilst blood volume and haemoglobin content are extensively discussed in the context of human performance and patient outcomes, the plethora of other factors that affect erythrocyte transport such as blood vessel architecture, red cell flow properties and the subsequent effects on the endothelium (46) are not extensively discussed. Nor are diseases of the cardiovascular system such as heart failure, beyond its influence on [Hb] and tHb-mass measurement.

The oxygen content of the blood is principally affected by haemoglobin content, 98% of the oxygen carried in the blood is bound to haemoglobin. The equation used relies on haemoglobin concentration [Hb] which is easily and cheaply measurable (between £2.50 and £3.50 within the UK National Health Service (NHS)) (47). However, [Hb] measurement is not without issue and the merits of using [Hb] (which is fundamentally affected by plasma volume variation) in the equation used to calculate oxygen content (CaO_2) will be explored further in sections 2.5 & 2.6.

2.3.1 Carbon monoxide binding

Carbon monoxide (CO) forms carboxyhaemoglobin (or more accurately carbonmonoxy-Hb (COHb)) when CO combines with heme iron. The affinity is 200-300 times that of oxygen and it shifts the oxygen dissociation curve leftward as it promotes oxygen binding and prevents unloading at the tissues via changing haemoglobin into the 'R' state. CO competitively inhibits oxygen from binding to haemoglobin which explains why a person can have a normal partial pressure of oxygen but have severe tissue hypoxia (48).

CO binds to other heme-containing proteins, including myoglobin in heart and skeletal muscle, mitochondrial cytochrome c oxidase (COX; complex IV), and others (49). CO also inhibits activity at a mitochondrial level, it binds to the ferrous heme *a3* in the active cytochrome c oxidase (COX) which prevents oxidative phosphorylation. CO is preferred over oxygen for the COX enzyme (3-fold), so things are more extreme when there is tissue hypoxia with ATP production slowing down. A rescue mechanism via alternative electron transport chains attempts to make more ATP but this generates superoxide which can lead to further cellular damage (49) This is explained in Figure 2.

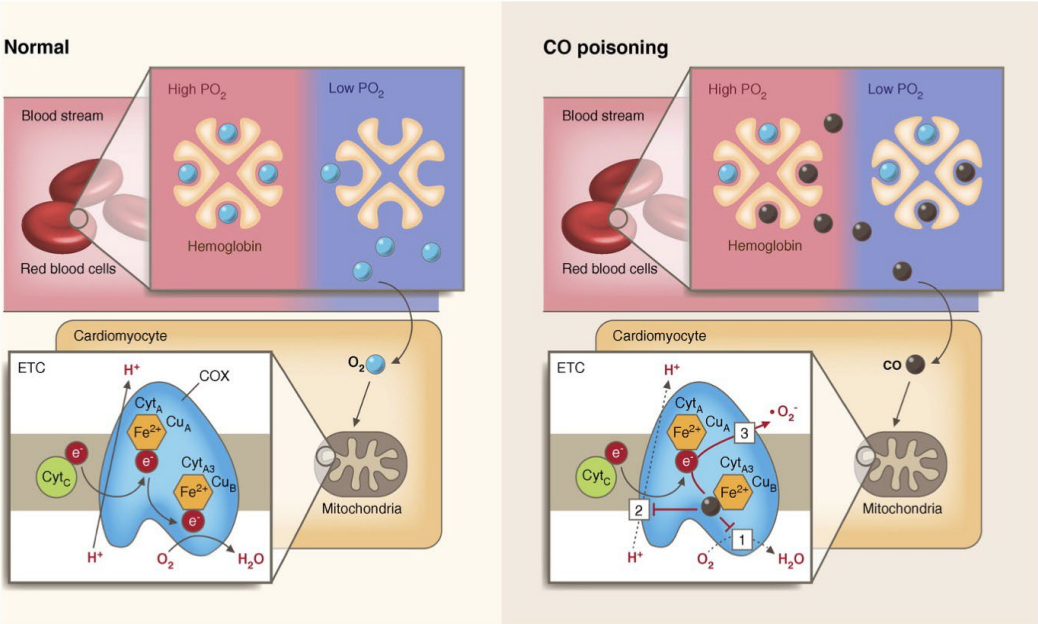

Figure 2: Hemoglobin (Hb) and mitochondrial effects of CO. Normal: Hb binds oxygen and delivers it to peripheral tissue with low PO2. Reduced cytochrome c (Cytc) transfers its electron (e−) to cytochrome c oxidase (COX) subunit 1 (CytA: binuclear center with heme *a* and copper [CuA]). The electron reduces oxygen (O2) at subunit 2 (CytA3: binuclear center with heme *a3* and copper [CuB]), forming water and transporting a proton (H+) through the inner mitochondrial membrane. CO toxicity: CO competitively binds to Hb with O2, reducing total oxygen carrying capacity by: (*1*) preferentially binding to CO instead of O2 (anemia-like effect); and (*2*) stabilizing the relaxed quaternary state of Hb, which binds to O2 with higher affinity and will not release it in low PO2 environment. CO binds competitively with O2 at the reduced

heme *a3* in subunit 2. This causes: (*1*) inhibition of the reduction of O2 to water (the end destination of electrons in the electron transport chain); (*2*) cessation of the transfer of H+ into the intermembrane space, shutting down ATP generation through ATP synthase; and (*3*) accumulation of electrons entering the electron transport chain through complexes I and III, which can produce superoxide, leading to deleterious effects. ETC = electron transport chain.

Jason J. Rose; Ling Wang; Qinzi Xu; Charles F. McTiernan; Sruti Shiva; Jesus Tejero; Mark T. Gladwin; *Am J Respir Crit Care Med* 195596-606. Am J Respir Crit Care Med, 2017. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

CO binding curves in the blood can be constructed. CO diffuses into all cells and is physically dissolved there according to the CO partial pressure. However, this partial pressure is very low, since almost all the CO is bound to the haemoglobin and the myoglobin which have a more than 200-fold higher affinity for CO over O_2. The affinity of myoglobin for CO is so high that only a relatively small amount of CO is dissolved in the muscle cell, for example. CO binds to ferrous enzymes and, in low concentrations, acts like NO as a signalling molecule.

2.3.2 Blood rheology

Rheology is the science of the deformation and flow of matter. All fluids resist, to a greater or lesser extent, attempts to alter their shape, and this resistance to flow is a measure of a fluid's viscosity (50). An in-depth explanation of the effect of blood rheology on oxygen carriage and oxygen delivery is beyond the scope of this thesis but for completeness a brief overview is given below. Blood rheology can be affected by different disease states, and this has important implications for flow and hence oxygen delivery.

From a rheological point of view, blood can be thought of as a two-phase liquid; it can also be considered as a solid-liquid suspension, with the cellular elements being the solid phase. However, blood can also be considered as a liquid-liquid emulsion based on the liquid-like behaviour of RBCs under shear. There are many factors which influence the apparent blood viscosity such as plasma viscosity, blood cell aggregation, PCV, and the mechanical properties of the cell wall. An important relationship exists between shear forces and the degree of red cell aggregation, high forces break up red cells which then reform under lower flow states. Haematocrit/PCV plays an important role and stories of doped professional cyclists setting their alarm clocks for 03:00 to pedal on a static trainer to avoid the dire consequences of static haemo-concentrated viscous blood are undoubtedly not an urban myth. Impairment of blood flow has significant implications for local tissue perfusion (51).

Blood is a non-Newtonian fluid; one cannot simply measure its viscosity but rather must measure it over a range of different shear stresses to produce a flow/viscosity curve. Plasma on the other hand is a Newtonian fluid so its viscosity is independent of shear forces. Blood flow is not only determined by cellular components and their relative viscosities but also how these cells behave under different conditions. For RBC their membrane is very important in how they flow through capillary beds. RBC have a

remarkable ability to conform their membranes which is especially important at higher shear rates (51).

There is clearly a very complex rheological balance to be struck with regard to supplying respiring tissues with oxygen via RBC. The optimum conditions to support flow and oxygen delivery are affected by numerous intra and extra cellular factors. In health and disease it is important to be aware of this when considering the factors that play a part in oxygen delivery at the extremes of physiology.

2.3.3 Altered oxygen affinity haemoglobins

Another thing that can affect oxygen binding and transport is genetic mutation of the globin genes. These can shift the oxygen dissociation curve either way depending on the mutation. Low affinity variants shift it to the right and high oxygen affinity shift it to the left (52). Thankfully these mutations are quite rare and will not be further explored in this work.

2.4 Laboratory reporting of a full blood count

Here I will outline the standard way in which a full blood count is reported at University Hospital Southampton (UHS) and the indices that were used throughout the thesis.

- Haemoglobin [Hb] concentration $g.l^{-1}$
- White blood cell count (WBC) $10^9.l^{-1}$
- Basophils $10^9.l^{-1}$
- Eosinophils $10^9.l^{-1}$
- Neutrophils $10^9.l^{-1}$
- Monocyctes $10^9.l^{-1}$
- Lymphocytes $10^9.l^{-1}$
- Platelets (Plt) $10^9.l^{-1}$
- Mena cellular haemoglobin (MCH) pg
- Mean cellular haemoglobin concertation (MCHC) $g.l^{-1}$
- Mean cellular volume (MCV) fL
- Packed cell volume (PCV) L/L

Packed cell volume (PCV): This is a directly measured value obtained from centrifuging blood in a microhematocrit tube in a microhematocrit centrifuge. The PCV is measured as the height of the red cell column in a microhematocrit tube after centrifugation. The SI unit is L/L, and this is simply *% ÷ 100 = L/L*

2.5 Total Haemoglobin Mass (tHb-mass) measurement in context

The different components of blood can be measured. Traditionally, haemoglobin concentration [Hb] is measured giving a value in grams per litre ($g.l^{-1}$) and is used as a surrogate for the blood oxygen carrying capacity that is used in the oxygen content equation (0). In fact, haemoglobin concentration measurement is the most common laboratory test in the world (53). [Hb] and Hct are familiar to clinicians of all specialities and clinical decision making often hinges on their values. The concentration of haemoglobin in the circulation ([Hb]) is determined by its total circulating mass (tHb-mass), and the plasma volume (PV) in which it is suspended. PV is rarely measured in clinical medicine, largely due to the technical difficulties of doing so (see section 2.4) (54). However, a low [Hb] may be due to a reduced amount of haemoglobin (absolute mass of circulating haemoglobin; tHb-mass) or an increased volume of dilution (plasma volume). Thus, [Hb] may be stable and tHb-mass low in the context of acute bleeding, [Hb] normal or elevated but tHb-mass low or normal in the context of dehydration, or [Hb] low but tHb-mass normal or even high in the context of excess plasma volume (fluid). Therefore, the use of [Hb] to define blood oxygen carrying capacity may be misleading under some circumstances. Clinical data examining the relationship between [Hb] and tHb-mass is discussed in section 3.9.

Total haemoglobin mass (tHb-mass) represents the absolute mass (in grams) of circulating haemoglobin in the body, the measured [Hb] being dependent upon tHb-mass and blood volume (BV) (sum of plasma volume (PV) and total red cell volume). The proportion of oxygen carried in solution in plasma is trivial (0.3 ml per 100 ml of plasma- see section 0) under normal physiological conditions, whereas each gram of Hb binds up to 1.34-1.39 ml of oxygen; the value of Huffner's constant being variably reported depending on measurement technique, the original description being: 1.34 $ml.g^{-1}$ (41,55,56). However, due to abnormal haemoglobin and the presence of carboxyhaemoglobin and other species like methaemoglobin the value is actually closer to 1.31 $ml.g^{-1}$ (57). Thus, tHb-mass is the principal determinant of total blood O_2-carrying capacity and may provide additional information regarding the clinical status of patients than that provided by [Hb] alone.

The measurement of blood volume is not new, and the history is explored in section 2.6. The wealth of literature from the sports medicine community regarding blood volume and tHb-mass measurement is explored in section 3.4.3.

2.5.1 Haemoglobin and tHb-mass as measures of oxygen content

Although it is well described that [Hb] is subject to fluctuations secondary to changes in plasma volume (27,28,34,58), there have been no randomised controlled trials using red cell mass (tHb-mass) as a transfusion trigger. This is despite a recognition that [Hb] is in constant flux, particularly as noted during cardiopulmonary bypass and has an inverse relationship with intravascular volume (27). Measures of tHb-mass are not widely used clinically (see section 3.9). Calls to change this, particularly in the context of transfusion triggers have recently been made (1,27) and recent work (see section 3.9) has begun to shed light on the clinical utility of tHb-mass measurement (28,34,59–63). Much of this thesis aims to add to this increasing body of work.

The quest for a simple test to guide the therapeutic management of acute bleeding, chronic anaemia, disorders of plasma volume (heart failure, chronic kidney disease and chronic

liver disease) and/or haemoglobin synthesis (e.g., JAK2 positive polycythaemia), or to guide the use of erythrocyte stimulating agents (ESAs) or red cell transfusion/manipulation continues. The bulk of this thesis will focus on measuring tHb-mass using the optimized carbon monoxide rebreathing method (oCOR) as first described by Professor Walter *Schmidt* and Dr Nicole *Prommer* in 2005 (5) (see sections 2.6.1 & 2.6.6).

Recently work has been done using a panel of biomarkers to describe vascular volumes from a 'simple blood test'(59,64). The driver has been to improve the accuracy and applicability of the Athlete's Biological Passport (ABP) to try to eliminate blood manipulation in elite sport. Since the introduction of the ABP in 2008 there is evidence that its presence alone has reduced the practise of 'blood doping' (64,65).

2.5.2 Blood volume equation

Total circulating blood volume (CBV) is composed of red cell volume (RCV) or erythrocyte volume (EV) and plasma volume (PV):

- BV= RCV + PV

Blood volume can be measured using several techniques. RCV is primarily composed of haemoglobin molecules which occupy approximately one third of its volume (30). It is possible to measure these via a number of different techniques that will be discussed below in section 2.4. Due to haematocrit and haemoglobin concentration being relatively simple and cheap to measure they are used throughout the world in all aspects of scientific and clinical practice. However, plasma volume is subject to constant fluctuation whereas total haemoglobin mass (tHb-mass) demonstrates stability for weeks to months (30–32).

2.6 Blood volume measurement

The measurement of the blood's volume is not new, having been studied for well over a century (8). Perhaps the earliest attempt was made by *Vierordt* in 1858 who bled an animal and waited for the 'tissue juices' to restore the lost fluid volume and then determined the dilution of the red cells (7). The early descriptions seem at first sight to be somewhat brutal but nonetheless were scientifically quite refined. *Welcker* bled animals to death and then washed out their blood vessels and minced their organs to extract all the haemoglobin. He then left them in water and compared the haemoglobin in the first blood, the washings and the extracts bringing them together to calculate that the blood volume was $1/13^{th}$ of the body weight. The technique was then used by *Bischoff* on two criminals (66). These direct methods, whilst academically interesting are clearly not especially useful in respiring humans.

The experiments conducted by *Haldane* and *Smith* in the late 1800s were quite remarkable in their findings (36,37); they used carbon monoxide gas for the first time in human subjects to measure blood volume. They used carmine colorimetry to work out carboxyhaemoglobin levels in the blood and for the first time were able to calculate the red cell volume in human subjects, they even considered the loss of carbon monoxide to the tissues and even to muscles which they could not account for, a subject that has subsequently been revisited multiple times (6). The commonly held dogma at the time was that the red cell volume was around 8% of body weight. Their experiments dispelled this, calculating that it was closer to 5%. However, it is not entirely clear in the paper if they

simply meant red cell mass as opposed to blood volume. It was also the first time that obesity was noted not to result in an increase in haemoglobin weight per excess kilogram.

> 'The fat *seems* to be so much dead weight, so far as the blood is concerned' (37).

The technique was later criticised due to the sampling time being too short to have allowed complete mixing within the entire circulation resulting in an underestimation of blood volume. *Douglas's* experiments resulted in values closer to the 1/12th or 1/13th of total body weight using the same technique as described by *Haldane* (67).

In 1919 *Harold Salvesen* described a modification on *Haldane's* technique. He neatly classified the methods used to measure blood volume into *direct* and *indirect* but then went further to describe 2 broad groups of indirect methods:

Group 1: A known amount of 'an easily determinable' substance is introduced into the circulatory system for sufficient time for 'thorough mixing' to occur and then its concentration is determined. When one knows the original amount given it is possible to work out that which is bound to red blood cells (66).

Group 2: The blood is either diluted or concentrated in various ways and the blood volume calculated from the variation in the content of haemoglobin or red cells (66).

Essential criterion for blood volume measurement based on the principle of dilution was well described by *Thomsen* et al. They described 3 criteria which must be fulfilled by any substance that is proposed.

1- The indicator substance must have reached uniform concentration in the cell mass or plasma in all aspects of the vascular compartment before the sample to be measured is taken.
2- The substance must not leave the blood stream during the mixing period or if it does then the rate of disappearance must be known.
3- The indicator must be precisely and accurately measured (68).

In this section the different methods of blood volume measurement will be described.

2.6.1 Carbon monoxide to measure haemoglobin mass and blood volume

The basic principle involved in CO rebreathing is that a small amount of CO gas is inhaled diluted in oxygen. This is then re-breathed for a specified time period, in which the majority of the CO binds to haemoglobin in the bloodstream. Via the dilution principle it is then possible to calculate the total number of haemoglobin molecules based on the number of absorbed CO molecules and the resulting change in blood carboxyhaemoglobin. As above the test in one form or another had been used for over a hundred years, various modifications and improvements have led to a modern day test that is both accurate and precise (5,8,69,70). The first record of carbon monoxide gas used as a tracer to measure blood volume parameters dates back to 1882 with *Gréhant* and *Quinquad* (71) who measured it in dogs and then *Haldane* and *Smith* who were the first to use CO in humans to measure blood volume as described above (37). The technique was modified in 1919 by *Van Slyke* and *Salvesen* who pointed out that other scientists had struggled to replicate the methods described by Haldane (72). They described a gasometrical method that directly measured CO gas in the blood as opposed to the calorimetric method described by

Haldane. They achieved this by separating carbon monoxide and oxygen from haemoglobin using ferricyanide and then removing the gases in a Toricellian vacuum in the 'Van Slyke apparatus' used for gas analysis (72). They measured blood volume on rabbits and then in humans using their modification. However, despite reports to the contrary, none of the methods described were reliable enough due to the wide variation of carboxyhaemoglobin measurements in the blood (68).

Work in the late 1940s and early 1950s by *Gemzell* and *Sjöstrand* further modified the technique noting that in previous experiments, particularly those described by *Haldane* (37) and *Gréhant* the techniques used for the determination of carboxyhaemoglobin (COHb) were unreliable and subject to gross variation. They were:

> *'neither dependable nor directly comparable'* (73).

Sjöstrand also mentioned that it had not been widely considered that baseline levels of COHb varied amongst individuals, especially if they were known to smoke tobacco. *Sjöstrand* noted factors that to this day are still a cause for some debate and indeed form part of one of the main experiments of this thesis described in Chapter 5; those of adequate mixing of carbon dioxide within the entire blood prior to sampling and the uptake of CO gas to myoglobin. It was also noted that the levels inhaled by the subjects in the experiments by Haldane were probably not safe for clinical subjects and *Sjöstrand* used more modest doses of CO (10-30ml) (73). The calculations described by *Sjöstrand* are not dissimilar to those used today (5,69,73). *Sjöstrand's* experiments relied upon the CO saturation of Hb being measured indirectly using the partial pressure of CO in the expired air prior to administration of CO and then again afterward. This assumed a standard dissociation curve for Hb and CO (Haldane's M factor). However, the technique was deemed unreliable (74). In the 1950s further modifications in the precision of CO detection within a blood sample were reported by *Linderholm* and *Sjöstrand* (75).

In 1963 *Pugh* reported data from Nepal measuring 6 subjects' blood volume using CO re-breathing apparatus similar to that described by *Van Slyke* (72). The reported repeatability was reasonable and the results did not appear to be dramatically affected by the altitude (4650m) (76).

> *"The fact that in both investigations haemoglobin concentration stayed constant or rose only very slowly after the first few months, while red cell volume rose continuously, shows clearly that plasma volume changes play an important part in the regulation of haemoglobin concentration at altitude."* (76)

Pugh elegantly demonstrated the effect of PV changes on haematopoiesis at altitude. The rapid changes that they had witnessed in [Hb] changes which had been put down to haemolysis or extreme erythropoietic stimulation were for the first time shown to be more likely related to PV fluctuations; a subject that would come to dominate discussion about the ABP many years later (31,77,78).

The technical precision and complexity of efforts to improve the precision of CO detection in blood sampling during this period 1945-1970 were staggering and enormous credit is due to the scientists who painstakingly undertook those experiments. The methodology described in the refinements is detailed and is even described as 'frustrating' (79). It was however evident that widespread adoption of such techniques was unlikely (74).

The ability to measure carboxyhaemoglobin using diode-array spectrophotometry was in many ways game changing for the determination of tHb-mass via the CO breathing

technique (80). Work in the early 1990s by *Thomsen* et al paved the way for further refinement. Thomsen compared his modified CO re-breathing procedure using diode-array spectrophotometry to measure COHb and compared measurements with ^{99m}Tc-labelled erythrocytes, ^{125}I-albumin and albumin labelled with T1824 (Evans Blue). BV was very similar when the ^{99m}Tc-labelled erythrocytes were compared to CO-re-breathing (4557 ml (3251-6576 ml) compared with 4527 ml (3390-6527 ml) (r=0.97))(68). The plasma volume measurements were noted to be lower by the two methods used to tag red cells compared to the albumin techniques, with the best correlation being between the CO method and the 2 albumin techniques. The explanation given is due to the biphasic disappearance of albumin after injection and that early bidirectional diffusion in the peripheral tissues is not adequately taken into account by either albumin method, therefore they will always slightly over estimate PV (68).

The work of *Burge* and *Skinner* in 1995 modifying CO re-breathing and also clearly describing equipment and standardising a protocol for testing forms the basis for the different methods still used today (74). Their work built on earlier work from *Christensen* et al who described the coefficient of variation and precision of the CO rebreathing method for determination of blood volume based on differing values of the change in carboxyhaemoglobin from baseline to the end of the rebreathing period (81), the Delta change (ΔCOHb%). The details of the testing procedures, for example the number of samples that were required to be processed and the effect this had on precision was discussed by both authors for the first time (74,82). Compared to other methods at the time *Burge* and *Skinner* demonstrated a test - retest precision of 0.8% (coefficient of variation) between tests in 7 individuals which was lower than any other method previously reported (74). They also reported the ability to detect changes in tHb-mass of around 1.5% after bloodletting experiments (74).

However, the method described by *Burge* and *Skinner* still required a subject to rest for twenty minutes, have two antecubital cannulae inserted and then undergo a rebreathing procedure taking at least fourteen minutes. The equipment used was bulky and required the heavier elements to be suspended using chains (Figure 3).

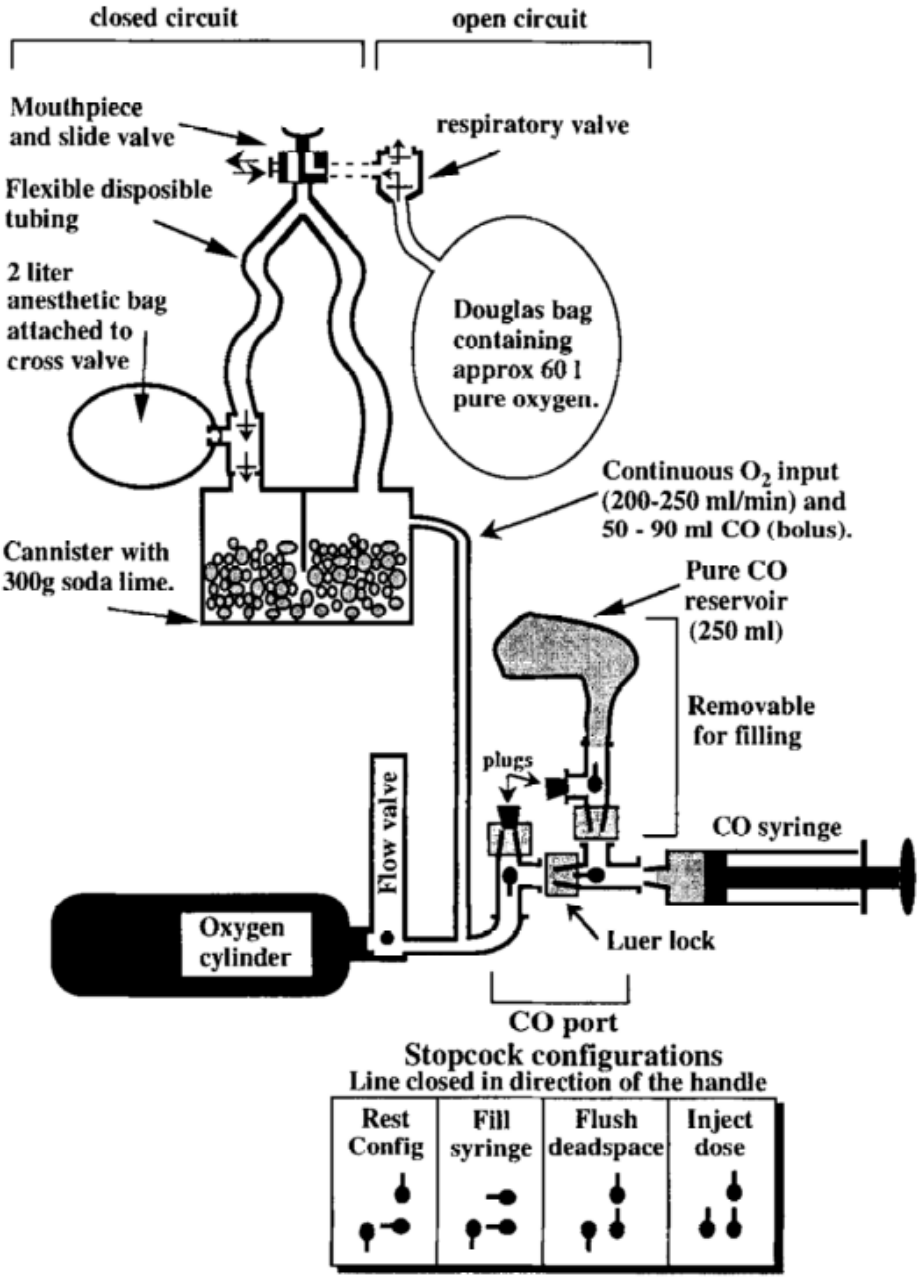

FIG. 1. Open- and closed-circuit breathing systems connected via respiratory and manual 3-way slide valves, respectively. CO port consists of a CO reservoir, a syringe, and three 3-way stopcocks. *Bottom right*: stopcock configurations (Config) for filling CO syringe, flushing CO port dead space, and injecting bolus CO dose into re-breathing system.

Figure 3: Equipment for the CO-rebreathing method used to measure haemoglobin mass and blood volume described by *Burge* and *Skinner* (74) (reproduced from the American Physiological Society who do not require permission for reproduction in a thesis)

Schmidt and *Prommer* published their 'optimized carbon monoxide rebreathing method' (oCOR) in 2005. They strove for a test that was simple, convenient for athlete or patient but retained the levels of precision that had been demonstrated by *Burge* and *Skinner*. The modifications are described in detail in Chapter 4 of this thesis as this technique has been used in all the experiments that form the subject matter of this thesis. Briefly, by using a CO bolus they were able to markedly simplify the procedure without reducing validity or reliability (5). The equipment used is shown in Figure 4.

Figure 4: Example of the apparatus developed by *Schmidt* and *Prommer* for the measurement of tHb-mass. Photograph used with permission.

Not all current researchers agree however, and some are still using methodology broadly in-line with that described by *Burge* and *Skinner*. However, these same authors concede that although the 2-minute rebreathing procedure described by *Schmidt* and *Prommer* might not represent an 'optimization', the precision is in line with the 10-minute rebreathing procedure described by *Burge* and *Skinner*. With both the 2-minute so called 'optimized' procedure and the traditional 10-minute procedure both having a typical error (TE) (83) of measurement of 1.2 and 1.3% respectively in a recent study when they were compared (69,70).

A recent review of total haemoglobin mass measurement using carbon monoxide rebreathing has called the 1980 'gold standard' accreditation given to radioactive labelling techniques into question (84). The reason for this is that carbon monoxide rebreathing not only has comparable levels of accuracy but has superior precision (69).

2.6.2 Dye techniques

Using a dye or tracer injected into the blood stream to measure BV is also not new. Prior to the 1930s all manner of substances had been suggested and even tried; including saline solution, isotonic glucose, Bayliss' solution (a colloid preparation of gum Arabic from the sap of the Acacia tree), suggested by physiologist *William Bayliss* (85,86)), foreign serum and antitoxin (87).

The principle of any indicator dilution technique relies on the criteria described above in 2.4, namely that the substance stays within the circulation throughout the measurement period, that it does not disappear due to binding, disintegration, degradation, or excretion. The metabolisation half-life must be known. Plasma volume is calculated according to the following equation:

$$\text{Volume}_{\text{plasma}} \text{ (PV) x concentration marker}_{t=0} = \text{Volume}_{\text{injected dye}} \text{ x Concentration}_{\text{injected dye}}$$

To ascertain the concentration of dye at the virtual t=0 one must log transform the concentrations ascertained at given measured time points which produces a straight line and the intercept of the y axis at time zero gives the concentration at t=0 (88).

In 1915 *Keith* et al used 'vital red'/Congo red' and determined the degree of dilution of the samples using colorimetric readings against a known standard. This was modified further by *Whipple* et al who noted that any such dye must be non-toxic and not be permanently stored within any cells (89). *Whipple* et al also experimented with many other dyes demonstrating three broad categories of dye; group 1 being dyes that remained in the plasma for long enough to be usefully measured. They also describe the first use of Evan's Blue (T-1824) a blue azo dye and commented that it was slightly superior for measuring BV (89). Whipple's work is noteworthy for many reasons, not least the attention to detail and extent of the experiments that were done to measure BV. The group was one of the first to question the notion that the ratio of red blood cells to plasma volume remained constant; at the time this had not been challenged and their insights were fascinating. A quote from 1921 summarises their thoughts:

> *"Many investigators consider the whole subject of blood volume to be in a state of hopeless confusion"*(90)

They further went on to dismiss isotonic saline as a tracer due to its fast passage out of the bloodstream (90).

Smith modified the technique so that repeat measurements could be made at short intervals in the same subject (89). These methods however suffered with the precision problems related to manual colorimetric assessments and wide inter-observer variations. Of note, Smith was particularly interested in blood volume measurement due to wanting to obverse the changes that occurred with haemorrhage/acute shock. In much the same way part of this thesis aims to develop BV measurement techniques to expand its use within the perioperative sphere. 'Congo red' was also used by *Powers* et al but has long since been withdrawn due to its carcinogenic properties (87). Evans blue (T-1825) seemed to become the most commonly used dye.

In 1959 Iron dextran was tried but shortly afterwards reports of severe anaphylaxis were reported (91). The aim was to find a tracer that was not radioactive and could be used more widely in smaller hospitals without the facilities to handle radioactive tracers. The problem of 'lipaemia' is discussed with regard to using Evans's blue dye. *MacKenzie* et al studied

Evans blue versus iron dextran and pointed out some of the benefits of using iron dextran; namely, the lower volumes of blood that needed to be drawn and the faster time to elucidate BV with fewer reagents being required (92).

T_{1824} (Evans blue) is an azo dye with a high affinity for serum albumin, prior to the development of radioisotopes it was the most used plasma dilution marker (88). Indocyanine green (cardio-green) has also been used however is less stable and is eliminated faster than Evans blue, it was also prone to allergic reactions so was rarely used clinically. However, in the 1990s and early 2000s it was used in clinical studies as it was felt to be a lot quicker and more suitable for the intensive care setting (93,94). Evans blue has been used extensively in the clinical arena (95–100). Evans blue is not as precise as other methods for the determination of plasma volume but with care can be used in the clinical setting (8). Evans blue eventually fell out of favour due to reports of sensitivity reactions and unacceptable levels of discolouration in addition to carcinogenic activity as seen in animals. However, due to radioisotopes being unacceptable for use in women of child bearing age and children it had a resurgence in the 1990s (7).

2.6.3 Radiolabelling techniques

Over time many different radioisotopes have been used to tag either red blood cells or large plasma proteins (predominately albumin). For red blood cell measurement chromium-51 (^{51}Cr), Technetium-99m (^{99m}Tc), Phosphorus-32 (^{32}P,) iron-59 (^{59}Fe), have all been used and ^{125}iodine (^{125}I) and ^{131}iodine (^{131}I) have been used for plasma volume.

Radiolabelling either red blood cells or plasma proteins first occurred over 70 years ago using radioisotopes of iron and iodine (54,101–103). For red cells ^{51}Cr quickly became the standard isotope. The International Committee for Standardization in Haematology (ICSH) published guidance in 1980 for the measurement of red cell volume and plasma volume and advised sodium radiochromate (^{51}Cr) or sodium pertechnetate (^{99m}Tc) for the measurement of red cell volume and radioiodine-labelled human serum albumin for the measurement of plasma volume based on:

> *"their reliability, reproducibility, and ease of operation in routine clinical use"* (84).

However, this has recently been questioned (69) (see 2.6.1) as carbon monoxide rebreathing methods are as good, if not superior and lack the technical and safety issues of radiolabelled substances. Of note, the ICSH has not updated these guidelines since 1980. One of the principle clinical uses for the measurement of blood volume and particularly RCV/EV and red cell mass/tHb-mass was to make the diagnosis or polycythaemia rubra vera (now commonly known simply as polycythaemia vera (PolV)). However, current clinical practise in haematology does not recommend this practise, although the subject is still debated. One of the reasons for this has been the discovery of the JAK2 exon 12 mutation (see section 3.9.2.2) and the acceptance of the fact that in the vast majority of cases measuring Hct/PCV is enough to make the diagnosis. However, the measurement of red cell mass (tHb-mass) is still in the major criteria for diagnosis according to the WHO guidance (104).

Iodine radiolabelled albumin for the measurement of PV was an improvement with regard to precision and accuracy as compared to the dye techniques that were commonly used prior to its introduction. It circumvents the problem of overestimation of PV that was prevalent with all the dye techniques due to transcapillary leakage, as might be seen in oedema, ascites, or proteinuric states.

It is important to realise that red cell mass/tHb-mass is not directly measured by any of the radionucleotide labelling techniques (54,84). Red cell mass (tHb-mass) is derived from BV and PCV. This is evident from the following formula:

RCM (tHb-mass) = $(S)(D)(Vi)(B)^{-1}(PCV)$

> *"Where S is the count rate of the diluted labelled red cells, and D is the dilution; Vi is the volume of labelled blood injected and B is the count rate of the post-injection blood specimen. In this formula, BV is the product of the first four terms, i.e., $(S)(D)(Vi)(B)^{-1}$. The calculation simplifies to RCM = (BV)(PCV), or, when results are expressed in mL/kg, $RCM = (BV)(PCV)(Wt)^{-1}$. Thus, RCM is, quite like the mean corpuscular hemoglobin concentration, not a variable that is measured, but a complex ratio 1. lnject ("dose") 1 ml containing 10,000 black balls that is calculated from three measured primary variables* (54,84)."

This is better explained graphically (see Figure 5).

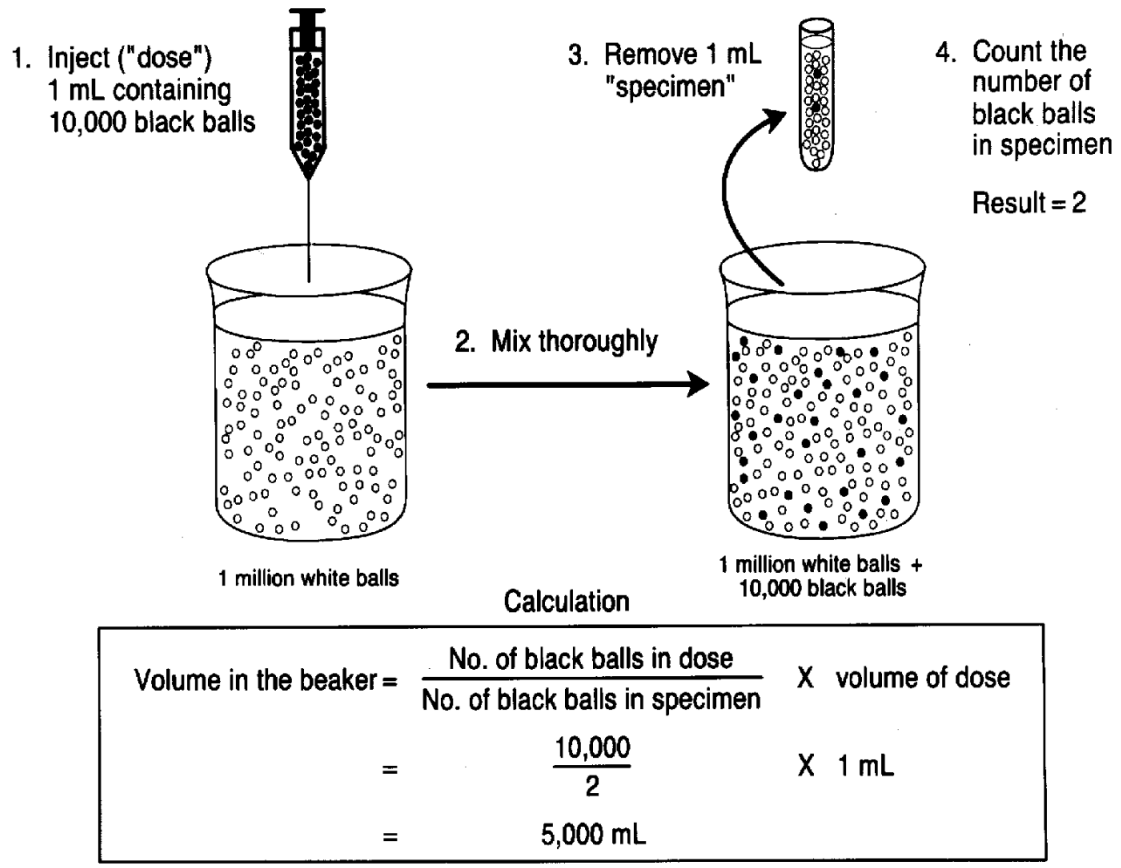

Figure 5: The dilution principle in measurement of a fluid volume such as blood volume is illustrated using a large beaker containing a million white balls (red cells) suspended in a solution of unknown volume. To this is added 1 mL of a fluid containing 10,000 black balls (^{51}Cr-labeled erythrocytes) in suspension. The contents of the beaker are thoroughly mixed, and a 1 mL sample is removed, and the number of black balls is counted. Result = 2. The volume of the fluid and suspended white balls is 10,000 ÷ 2 = 5000 mL. From this measurement, nothing whatever is learned about the number of white balls in the beaker. The result would be the same if there were a thousand

white balls or 10 million white balls in suspension in the fluid in the beaker (105). Used with permission from RightsLink®

There is an inherent irony in the measurement of Hct/PCV to calculate red cell mass/tHb-mass which is then used to evaluate PCV. This somewhat circular logic is eloquently explained by Fairbank at the turn of the current century who advocated for the use of the radioisotope measurements as routine for the diagnosis of PV to be stopped due to their inherent difficulties, cost and lack of precision amongst inexperienced laboratories (54). Fairbank expressed this somewhat circular logic as such:

> *"Yet, this strange logic, in the ICSH method, is embraced by nearly all of those who study P.vera."*(54)

2.6.4 Pitfalls when measuring blood volume

2.6.4.1 Red cell volume versus. total circulating volume of blood cells

Total blood volume is derived by summing simultaneous estimates of RCV and PV on the assumption that the volume of red cells is virtually the same as that of the total circulating blood cells. However, in some conditions, such as leukaemia, the volume of circulating leukocytes may constitute a substantial fraction of the total circulating blood cells.
The total blood volume will then be underestimated if the sum of the RCV and PV is used to estimate blood volume (84).

2.6.4.2 Whole body haematocrit versus. venous haematocrit

Haematocrit (Hct) or packed cell volume (PCV) is not uniform throughout the blood vessels. Also, it does not consider the plasma that is bound to the endothelial layer of blood vessels. Estimations have been used to overcome this and a correction factor to account for whole body haematocrit versus. venous haematocrit (0.91) are often employed with any modern attempts to quantify blood volume using Hct (54,70,74). The ratio however is not necessarily constant (106) which is particularly important when RCV/EV is measured indirectly.

2.6.4.3 Plasma volume variation

Plasma volume varies not only daily but also between days in a way that tHb-mass does not (28,30,107,108). *Jones* et al observed wide variation in both [Hb] ($143 g.l^{-1}$) and Hct/PCV (0.048). The differences clearly being secondary to plasma volume variation (108). The considerable fluctuations seen in PV are secondary to postural changes and the varying state of hydration/food ingestion that occurs throughout the day. BV and PV both being lower in the morning. It has been suggested that this daily variation is not widely appreciated by clinicians (54). In athletes even more factors affect PV variation: heat, exercise, altitude, and time of the athletic season. Shifts greater than 10% can easily be observed (64). This fact has not however been lost in the war on doping in elite sport with some authors advocating measurements of tHb-mass to be included in the athlete's biological passport (ABP) (1,31,64).

2.6.5 Lack of 'normal' ranges

There is not a 'normal' laboratory range for tHb-mass in health or in disease. The majority of measurements using the oCOR have been performed in athletes or healthy volunteers. This work gives researchers a reasonably clear idea of a tHb-mass that might be seen in an elite endurance athlete but is less useful for a patient laying in an intensive care unit. This thesis may go some way to expanding the measurements taken in clinical subjects. The quote from the ICSH in 1980 unfortunately holds true today:

> "In practice no method is available for predicting accurately the blood volume of a given normal individual, so that only fairly large deviations from normal values of red cell volume can be established" (84).

2.6.6 Blood volume measurement in 2021

Like many fundamental truths in life and particularly within clinical medicine a certainty of 'truth' is that the passage of time will often render past 'truths' to be frankly untrue. Data from scientific studies often underestimate significant anomalies (109) and perhaps we ought to embrace uncertainty more often that we do (110). The measurement of blood volume has vexed clinicians for centuries. Enormous progression has been made in the last 150 years as documented in this section. So where does this leave blood volume measurement?

It is fair to conclude that the ICSH guidelines from 1980 have been superseded by the refinements in the measurement of tHb-mass using carbon monoxide re-breathing (5,69,70). The direct measurement of red cell mass or total haemoglobin mass (t-Hb-mass) using carbon monoxide rebreathing using with the method described by *Burge* and *Skinner* (74) or that described by *Schmidt* and *Prommer* (5) has a typical error (and therefore greater precision) lower than any of the radio-nucleotide techniques that measure RCV/EV volume. Also, it measures tHb-mass directly rather than being a derived measurement with the fundamental issues inherent with this and the variation in PV described in section 2.6.4.3. It is also much easier to perform and has been shown to be suitable for measurement in clinical subjects (28,34).

Meta-analysis in 2005 demonstrated that the most precise measures for tHb-mass and red cell volume (RCV) were CO rebreathing and ^{51}Cr respectively. Both having short term errors (between measurement separated by 24 hours) of ~2.5%. The only other techniques with enough data for analysis were Evans blue dye and CO rebreathing for RCV both of which had less precise short term errors – approximately three fold greater (8).

A recent study compared the two major modern adaptations of the CO rebreathing technique as described by *Burge* and *Skinner* (74) and *Schmidt* and *Prommer* respectively (5,6) with the indocyanine green (ICG; for plasma volume) and the sodium fluorescein (SoF; for red blood cell volume) methods. They found that CO rebreathing via either method demonstrated a very low typical error (1.31 (*Schmidt*) and 1.91 (*Burge*)) and concluded that both were valid for detecting small changes in tHb-mass (a 3% change in tHb-mass was detectable) (70). The key focus of the above study was to ascertain if the CO rebreathing methods could precisely measure PV compared to the ICG method. No difference between ICG and CO re-breathing derived PV could be established when a whole body/venous haematocrit correction factor of 0.91 was applied (p= 0.11, **r**=0.43, mean difference -340 ± 612mL. The precision, however, was much greater with the CO rebreathing methods.

Due to its ease of use, relative safety and good precision carbon monoxide rebreathing is seemingly the most suitable test for the measurement of all BV parameters and is unequivocally the most superior way to measure tHb-mass.

The decision to use the oCOR exclusively during the work of this thesis was based around our belief that it is the best test to measure blood volume with the highest level of precision. The other techniques described are more expensive, require a more complex laboratory set up and involve radiolabelled samples. Pragmatically, locally I had purchased the equipment and had been specifically trained by the inventor of the technique.

We did contemplate comparative studies and or using controls but ultimately it was decided not to pursue those studies. That does not mean that I believe that other techniques lack merit within the field.

2.7 Accuracy versus precision

Any scientific test is prone to error. Error can be broadly separated into systematic and random error. Systematic errors (in principle) can be corrected for and shift all measurements in a systematic way, for example a weighing scale that has not been calibrated correctly and constantly reads 0.5g too high. Random errors are caused by unknown or unpredictable changes in a measurement, and they can't be eliminated entirely although one can reduce their effect. For example, one investigator reads 26.3 °C from a solution and another comes moments later and reads 26.4°C. This type of error can be minimised by taking multiple samples and using the mean value (111).

Secondary to error any scientific test will therefore have a degree of accuracy and a degree of precision. It is important to distinguish between the two as a test could be very precise but wildly inaccurate and vice versa.

Accuracy refers to the closeness of a measured value to its 'true value, often this is compared to a known standard or known value. With regard to BV measurement in humans there is no direct method that can be employed during life. Regarding tHb-mass this is a difficult concept because the 'normal ranges' have not been adequately described. When comparing measurement devices and particularly when comparing a new device, a 'gold standard' device or technique will often be used as a reference point. As discussed in section 2.5.1 the current gold standard reference of using radioactive labelling of red cells/albumin for the measurement of RCV and PV respectively is based on consensus (as opposed to rigorous scientific evidence) from 1980 and since both techniques are based on the dilution principle both suffer from the same methodological errors as CO rebreathing. Work described above by *Thomsen* et al however is reassuring with regard to the correlation between CO re-breathing derived intravascular volumes and those directly measured from radioactive labelling techniques (68,69). This has also been shown in the clinical setting with a good correlation between the ^{51}Cr method and a carbon monoxide method (albeit not a rebreathing method described by either *Burge* or *Schmidt*) of r=0.97 (112).

Precision is used synonymously with reproducibility and refers to the closeness of two or more measurements to each other. This is particularly important when measuring a variable before or after an intervention designed to change that variable. Measures of reliability will be discussed in the methods in Chapter 4 however, briefly in respect of the measurement of tHb-mass a reliability statistic described by Hopkins is commonly used to compare techniques. This is expressed as the typical error (TE) which usually requires at

least 2 separate measurements to be taken (usually around 24 hours apart, a time which there should be no appreciable changes in tHb-mass). The measurement error includes random error (analytic error arising from using the method-specific apparatus and intra-individual biological variation) but not systematic error (bias) (8,83). A TE of <2.5% being common amongst expert laboratories using CO re-breathing. Interestingly this is superior to radiolabelling techniques with a TE of 3.4% by modelling and 2.8% from a meta-analysis (8)

2.8 Accuracy and precision applied to carbon monoxide re-breathing techniques to measure blood volume and total haemoglobin mass

2.8.1 Positioning and preparation of the subject

Most authors have performed CO rebreathing procedures in the sitting or semi recumbent position with a rest period prior to sampling to allow plasma volume to settle (5,31,113–115). However, recently one research group has questioned the accuracy of this. They have raised the issue that there is no transfer of CO between red blood cells; so, for a red blood cell to be 'tagged' with CO it needs to come into contact with it in the pulmonary circulation. It is said that in the supine position blood turnover in the large veins of the lower limb can take up to thirty minutes and that sampling in the seated position may therefore underestimate tHb-mass. This group now use whole body tilting when measuring tHb-mass. This is fine for research purposes but impractical in the clinical setting (69,116,117). However, the study was small, and this procedure has not yet been adopted by other researchers in the field. Other research groups have tested this hypothesis and found that although whole body circulatory mixing of CO is slightly delayed in the sitting position it has negligible effects on measured COHb% at six minutes (unpublished personal communication with Professor Walter *Schmidt* 30/08/2018). The adequacy of the original description of the oCOR in clinical patients with chronic liver disease resulting in ascites is tested in Chapter 5.

2.8.2 Leaks in the system

Measuring the volume of CO gas that is contained within the spirometer is crucial to avoid measurement error. Any gas that leaks during the re-breathing process must be detected using portable CO detection devices. Leaks could result in an artificially low ΔCOHb% and a subsequent overestimation of tHb-mass (118). *Ryan* et al demonstrated that with increasing leaks test-retest measurement error dramatically increased, a minor leak was defined as <30ppm for less than 5 seconds. No leak had a measurement error of 1.9% (1.6-2.3), minor leak 3.6% (2.6-6.1) and major leak 9.3% (6.3-17.6) (118). There is no doubt that this is one of the most significant technical errors with any form of CO rebreathing technique.

2.8.3 Type of blood drawn and timing of samples

Carbon monoxide enters the bloodstream via the lungs and binds competitively to Hb forming COHb. Additionally, some CO diffuses to other compartments, for example binding to myoglobin in muscle. A certain time period after inhalation an equilibrium is reached and sampling from this time onwards can be used to measure tHb-mass (113).

As discussed in section 2.6.1 complete circulatory mixing of COHb, meaning that all vascular compartments have the same COHb% is vital for the accurate measurement of tHb-mass. Values obtained in capillary blood for COHb% are slightly higher than in venous blood post CO inhalation. Debate in the literature exists as to the optimum sampling time points to ensure that complete mixing has occurred. In the original description by *Schmidt* and *Prommer* capillary samples were taken at 4 and 6 minutes and the mean value was used to calculate tHb-mass, the '5 minute value' (5). Subsequent studies used minutes 6 and 8 taking the mean '7-minute value' and this is standard practice in the laboratory of Professor *Schmidt* (personal communication and attendance at the laboratory to learn the technique). Refinements made by *Schmidt* and *Prommer* in 2007 confirmed this slightly later time point, samples drawn at 6,8 or 10 minutes from either capillary or venous systems were deemed sufficient to allow complete mixing within the blood (6). This is further explored in Chapter 5 with a study involving patients with cirrhosis of the liver who had disordered circulatory dynamics.

Using the oCOR as described by *Schmidt*, *Gore* et al determined that minutes 8 and 10 were the best times using capillary samples and applying a 2% correction factor for loss to myoglobin due to the fact that complete mixing had occurred by minute 10 (119). In venous samples the timings were adequate at 6 minutes with complete mixing at this time demonstrated. However, they still recommended adjusting the original timing proposed by *Schmidt* and *Prommer*. They acknowledge that reliability is not affected by later sampling times but recognise the importance in subjects where mixing might be disordered such as peripheral vascular disease (119).

Due to the discrepancies in time points described in the literature *Garvican* et al set out to study the CO uptake kinetics comparing the oCOR and the method of *Burge* and *Skinner*. *Garvican* found that complete mixing had not occurred in all subjects at the recommended '7 minute' time period using the oCOR method. They noted that muscle blood flow was not measured therefore it was impossible to be sure if subjects had differences in peripheral circulation that may have affected the mixing time of COHb. Factors such as capillary density and muscles mass were postulated as variables that could affect this. *Garvican* concluded that:

> "an understanding of the influence of blood volume distribution on ΔCOHb% at the time of sampling is of critical importance for the accuracy of both methods" (113)

Garvican et al recommended that researchers ascertain the circulatory mixing time ('t_{mix}') and make adjustments when circulatory mixing time might be prolonged in cases of poor peripheral circulation. Provided there is no reason to suspect delay in t_{mix} Garvican concluded that the '7 minute' sampling time point was adequate for the oCOR. However, sampling at minute 10 would ensure this for the vast majority of subjects (113). This has recently been explored in polycythaemic native altitude dwellers with chronic mountain sickness or 'Monge's disease and was found to be prolonged, presumably due to poor peripheral circulation (120). *Alhgrim* et al found that the same was true in patients with heart failure and an ejection fraction (EF) of <30% (121). However, this had debateable clinical significance (see Chapter 5).

One of the principle aims of this thesis was to ascertain if the sampling timepoints as recommended by *Schmidt* and *Prommer* were adequate for patients with chronic liver disease and known disorders of the circulatory system as a result of portal hypertension and ascites: see Chapter 5.

2.8.4 Haemoximeters and the number of replicate samples obtained

Measurement error from blood gas analysers vary between manufacturers, with most modern haemoximeters reporting values of COHb to 0.01-0.1% resolution. This is the major source of analytical error (122). Multiple measures of each sample attenuate this error as a function of $\sqrt{n}$ replicates (83,123). *Burge* and *Skinner* used 4 replicates whereas *Schmidt* and *Prommer* initially used duplicate and in subsequent refinements they used triplicate measures (5,6). *Gore* et al investigated this for the purposes of introducing the oCOR into anti-doping detection laboratories and recommended 5 samples based on a ΔCOHb of >5.5% for the oCOR and >6.5% for the *Burge* and *Skinner* method. They demonstrated that an analytical error of <1% could be achieved by using 5 replicate samples and achieving a ΔCOHb of >5/5% and >6% respectively (123). Their findings are best displayed graphically, and a figure taken from their study is shown below (Figure 6), ΔCOHb% is shown on the x axis and analyser error on the y axis. As is clearly demonstrated, as the number of samples increases so the analytical error decreases. A balance between safety and convenience clearly must be struck and this is particularly relevant in the clinical arena.

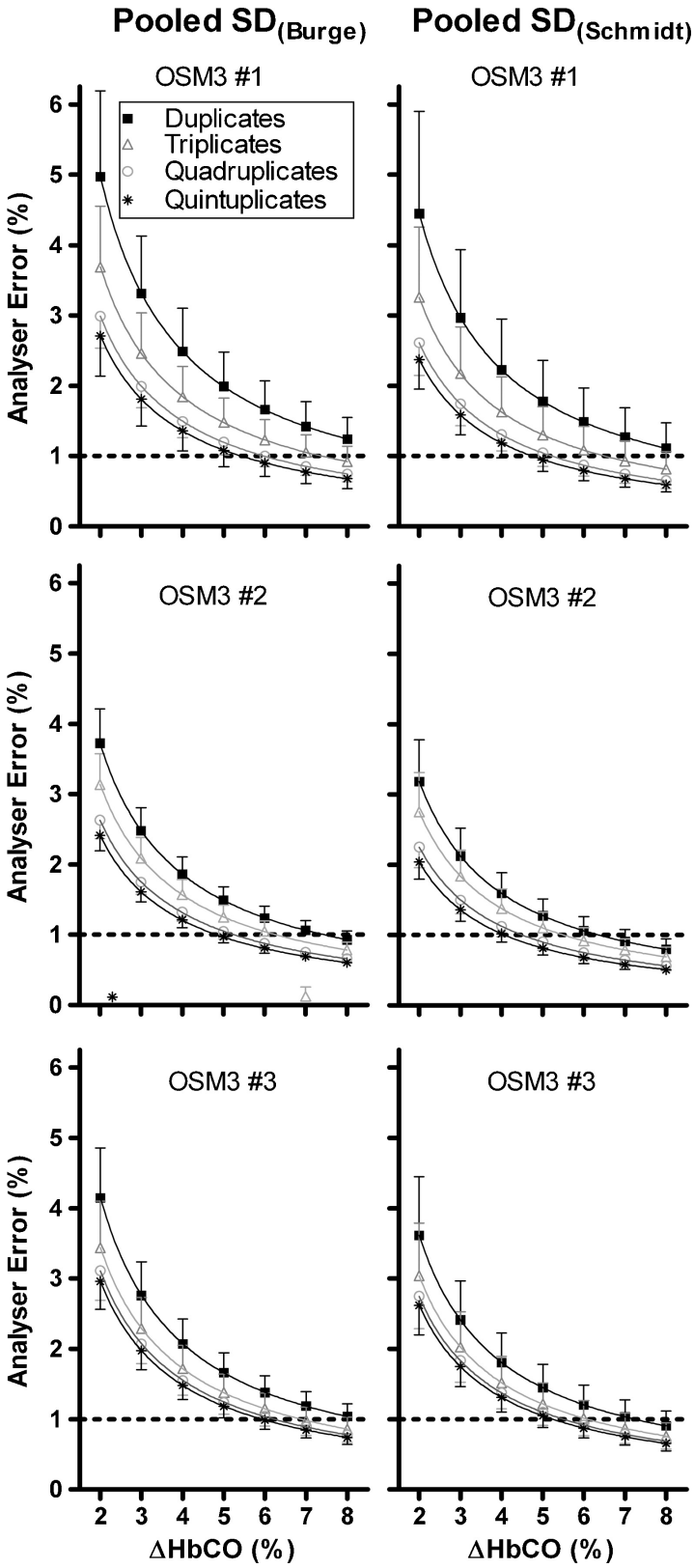

Figure 6: Theoretical analyser error as a function of the change in percent carboxyhaemoglobin (ΔCOHb) for three different OSM3 analysers. Values are means and error bars are 90% confidence limits; these are polled SDs from Table 1 expressed as a percentage of the Δ%COHb. Taken from *Alexander* et al (Figure 1) (123)- permission to use from Elsevier- licence number- 4417690617060.

These experiments were repeated by *Turner* et al who also showed that increasing the number of replicate samples improved precision (122). *Turner* et al concluded that three replicate samples struck a good balance between precision, time efficiency, and cost when using a slightly newer blood gas (the Radiometer^{TM} ABL-80 model) analyser compared to much of the early work in the field which had used the Radiometer^{TM} OSM-3 model. Importantly, this study also demonstrated that the newer haemoximeter module in the ABL-80 was more precise than its predecessor the OSM-3. It is important to identify a single laboratory's own analytical error based on the number of samples used for CO rebreathing and the precision of the haemoximeter (122).

2.8.5 Carbon monoxide dose used

The crux of the issue when calculating the 'dose' of CO is again, striking a balance between precision and safety. However, 'safe levels' of COHb are not exclusively about peak COHb% but duration of high blood levels is equally, if not more important (124). The safety limit of 10% is based on previous work by our group and Professor *Schmidt* who developed the oCOR test. This level allows a significant margin of safety before subjects are likely to experience any significant level of CO toxicity. Previous researchers have also used this level (28,34,125,126). Levels greater than 5% can produce symptoms such as mild headaches and when levels start to exceed 20% most individuals start to experience headaches and visual evoked potentials start to change (127,128). However, some authors would argue that symptoms do not really start until levels exceed 25% (129) and others would argue that it is the duration rather than the peak level that is most significant (130).

The accepted rise in carboxyhaemoglobin (ΔCOHb%) for the oCOR is variably required (for precision in tHb-mass measurement) to exceed an absolute value of 5% (typically 0.5–1.5% at rest rising to 5.0–7.0% post CO rebreathing) (5,122), or to reach values of 5.5%-6% (8). Unpublished data from our own laboratory (*Plumb, Grocott*) would suggest that values over 4.0% are essential for adequate reliability. Work by *Turner et al* who showed that low doses (0.6 ml·kg^{-1}) resulting in a ΔCOHb% of only 3.4% ± 0.4 resulted in similar tHb-mass values compared to using 1.0 ml·kg^{-1} (791 vs. 788 grams respectively). They demonstrated that it may in fact be allowable to go lower than a ΔCOHb% of 4% at the expense of decreased accuracy (125). In healthy subjects, a dose of 1 ml·kg^{-1} has been shown to be adequate and safe (125). We have safely and effectively used 0.4-1ml. kg^{-1} in patients (2,28,34), and the only other clinical study to provide data reported using doses of 0.7 ml·kg^{-1} for women and 0.8 ml.kg^{-1} for men (63) and yielded a mean ΔCOHb% of 4.5% in patients with coronary artery disease. Other clinical studies did not report the dose used (60,61). Dosing in clinical subjects is explored further in Chapter 5.

Chapter 3 - Background Part 2: Anaemia, haemoglobin, blood volume measurement and blood manipulation; in both athletes and patients

3.1 Anaemia- definition and relevance in perioperative medicine

Anaemia was defined in the background (Chapter 2) according to the WHO criteria. In this section its relevance in modern clinical medicine is explored, the WHO classification is questioned and the association between anaemia and perioperative outcomes are explored in section 3.2. The relevance to research aligned with the measurement of blood volume and tHb-mass measurement is intrinsically linked to that examining subjects who are 'anaemic', or who have other disorders of the hematopoietic system and hence an understanding is fundamental to the content of this thesis.

Anaemia is important because it is extremely common with an estimated global prevalence of 24.8%, affecting 1.62 billion people (131). The WHO estimated in 2002 that iron deficiency anaemia (IDA) was within the top 10 most important factors contributing to the burden of disease globally that leads to increased morbidity and mortality (131). It is more common in women and children (132). The WHO also point out that although haemoglobin concentration is used to define 'anaemia' it does nothing to delineate the causative factors, which are often multifactorial (133). Iron deficiency is the leading cause accounting for approximately 50% of cases, with hookworm infection being the leading culprit in the developing world (133). In adult men and post-menopausal women in the developed world iron deficiency anaemia (IDA) occurs in approximately 2-5% of the population (131,134). The prevalence is much higher in certain groups, see below and Table 1. The remainder of this section and this thesis will only focus on anaemia in the perioperative setting.

Anaemia is the commonest haematological problem in the world (135) and has a proven association with poor outcomes in a number of disorders (17,136–140) see Table 1 below and Table 14 and Table 15 in Appendix A. A meta-analysis examining the prevalence of anaemia in surgery covering studies between January 1966 and February 2003 was published in 2004 (141) calling for much needed studies on anaemia and outcomes in perioperative care. Table 1 shows a selection of studies since the turn of the century in perioperative medicine detailing the prevalence of anaemia, which is significantly greater than that of the 'general population'(142). These large cohort studies eclipsed the population sizes of the studies in the systematic review from 2004 (141) and definitively demonstrated the *associations* predicted by the work of *Carson* et al and some of the studies in the systematic review by *Shander* et al that anaemia was associated with mortality and that a clear biological gradient had been demonstrated with poorer outcomes seen with worsening [Hb] (17–19,141). Studies from the *Shander* et al meta-analysis assessing anaemia and transfusion requirements in surgical patients are presented in Appendix A. Studies examining anaemia and outcome in the perioperative setting are presented in Table 15.

Study	*Cohort*	*Study Population*	*Prevalence of anaemia*
Baron – BJA 2014 (18)	Non-cardiac, non – neurological surgery	46, 539	28.7%
Saager- Anesth Analg 2013 (143)	Non-cardiac surgery	574,860	25.3%
Gupta- Ann Surgery 2013 (144)	Patients over 65 elective vascular surgery	31,857	47%
Ranucci- Ann Thorac Surg 2012 (145)	Cardiac surgery Only classified severe anaemia those with Hct <30	13,843	2.98%#
Musallam- Lancet 2011 (17)	Non-cardiac surgery	227, 425	30.44%
Van Straten- Circulation 2009 (146)	Cardiac surgery	10,025	16%
Beattie- Anaesthesiology 2009 (147)	Non-cardiac surgery	7,759	39.5%
Karkouti- Circulation 2008 (148)	Cardiac surgery	3500	26%
Kulier- Circulation 2007 (149)	Cardiac surgery	4804	28.1% Male 35.9% Female
Wu JAMA 2007 (150)	Non-cardiac surgery	310,311	

*Broadly based on WHO definition of anaemia, < 130 g/L for men and <120 g/L for non-pregnant women.
This study was not designed to report an overall prevalence but to compare a propensity matched group of severely anaemic patients with a Hct<30.

Table 1: Prevalence of preoperative anaemia* - used with permission © *Plumb* et al. 2016 (1)

The clinical importance and utility of blood volume measurement as an experimental entity gains true value when it is applied to patients or healthy subjects with disorders of the haemopoietic system or where there are changes in blood volume secondary to interventions such as ascent to altitude, differing training volumes (for athletes) or a change in disease state, bleeding, fluid shifts or use of erythrocyte stimulating agents (ESAs) or nutritional supplements (patients).

3.1.1 Is the WHO definition of anaemia 'correct'?

The link between iron deficiency and anaemia can be attributed to the work of *Helen Mackay* in the 1920s (151). The World health Organisation (WHO) classified anaemia in 1968 (from the WHO scientific group on nutritional anaemias in Geneva March 1967) as $<120 g.l^{-1}$ for non-pregnant women and $<130 g.l^{-1}$ for men (152). The report makes an interesting statement that is underappreciated by many clinicians-

> "Anaemia is considered a late manifestation of nutritional deficiency, and even mild anaemia is not the earliest sign of such deficiency."

The inference being that treating the cause is more important than reacting to the manifest reduction in circulating haemoglobin resulting in anaemia. Interestingly fifty years later the concept of non-anaemic iron deficiency (NAID) or iron deficiency without anaemia (IDWA) is still in its infancy in the minds of clinicians with no definitive evidence as to

whether treating this is beneficial or not (137). Equally, it is not clear how to manage IDWA patients with discretion as to whether upper and lower gastrointestinal endoscopy is warranted being left up to the individual clinician (134).

The definition of anaemia is extremely important as the vast majority of clinical studies and especially large epidemiological cohorts have used the WHO definition in their methodology. The fact that anaemia has been linked with poor outcomes in a variety of different disorders has brought this into sharp focus in recent years (17–19,137,138,142,153). The problem being that the data presented in the WHO report was minimal and of poor quality (136). It is pointed out by *Beutler* et al that it was not the intention of the WHO committee at the time to have developed such a hallowed set of criteria for the diagnosis of anaemia but unfortunately, that is exactly what happened.

Work by *Beutler* and colleagues has attempted to use modern large registries to define the normal lower limits for [Hb] more eloquently They used the NHANES-III (the third US National Health and Nutrition Examination Survey) database, and the Scripps-Kaiser database. This allowed them to not only account for patients who were clearly anaemic (both databases had ferritin and transferrin saturation levels) but also account for the effect of race (only in the Scripps Kaiser database). They were also able to consider whether excessive alcohol intake, diabetes mellitus, renal failure, or inflammatory markers (C-reactive protein in NHANES, erythrocyte sedimentation rate in Scripps-Kaiser), elevated serum ferritin, or leukocytosis affected the haemoglobin concentrations in the 2 populations (136).

Despite evidence that the levels defined by the WHO may actually be inappropriately low they are still widely accepted within clinical practice, this is particularly relevant to non-Caucasian individuals, possibly in part due to the fact that the gene frequency for α-thalassemia is extraordinarily high in the black population (136,137). Cynically, this may have something to do with the burden and immense cost that would come with raising the lower limits, secondary to the increase in investigation and treatment that would inevitably ensue.

3.1.2 Should anaemia be defined by the same haemoglobin concentration in men and women?

In the perioperative community calls have been made to treat men and women the same (with regard to classifying anaemia) when it comes to major surgery where blood loss is a distinct possibility (137,142). Due to the fact the women have a lower circulating blood volume the propensity of developing post-operative anaemia after major surgery is higher and relative blood losses have a greater proportional effect. This of course ignores the issues of plasma volume variance as discussed in section 2.6.4.3 and does not address the need to quantify genuine haemoglobin mass deficiency rather than plasma volume excess. This is not helped by the fact that there is currently no accepted reference range of ‘normal’ for total haemoglobin or red cell mass as there are simply not the large cohorts of data that are available for routinely tested haematological tests run by automated analysers. Perhaps the holy grail of blood volume and haemoglobin mass studies is the development of an accurate and precise test that is fully automated.

3.2 Anaemia and outcome in the perioperative setting

3.2.1 Is it the anaemia or the underlying disease that causes detrimental outcomes in the perioperative setting?

In many ways this is the fundamental question and one which may determine the focus of any therapy to treat perioperative anaemia. The answer currently remains unknown. Critically, the difference between association and causality must be stressed. Much of the data in the perioperative arena focuses on large retrospective cohort studies which by definition cannot speak to causality. International and national bodies (NICE & NHSBT) recognise that poor outcomes are associated with its presence and have produced guidance for the management of preoperative anaemia (137,154).

It was not until the 1970s that anaemia leading to negative patient outcomes was questioned in the perioperative setting. It was recognised that adequately powered RCTs would be required to answer the question (155). The assumption made by *Lunn* et al and many at the time was that 'anaemia' was simply a reflection of the severity of the underlying disease which caused the anaemia to occur in the first place.

It has only been very recently that this has been questioned and it remains unclear if anaemia in and of itself is an independent risk factor for poor outcomes (156). As recently as 2015 a leading perioperative research group determined that we were no further along:

> "It remains unclear whether anaemia is an independent risk factor for poor outcome or simply a marker of underlying chronic disease. However, red cell transfusion is much more frequent amongst anaemic patients" (156).

It is well known that even a single unit transfusion has a negative effect on outcome and the overall evidence base for red cell transfusion in the perioperative (non-cardiac) setting is weak. In a large retrospective cohort study from ACS-NSQIP (USA)- 941,496 operations were analysed in patients from 173 hospitals. This was a very important study as it was the first non-cardiac study showing that even a single unit transfusion was worse for mortality and major morbidity and, that this was dose dependent after a single unit (157). A meta-analysis published in 2012 in colorectal cancer patients determined that red cell transfusion resulted in worse outcomes not limited to worsening mortality but importantly to cancer progression (158). Large patient blood management programmes that have resulted in a reduction in red cell transfusion have also shown associations with decreased mortality, length of stay, hospital acquired infection, myocardial infarction and stroke (159).

Two large cohort studies shed further light on this. A secondary analysis of a large prospective European cohort study – the (EUSOS) study (160) was analysed for the prevalence of anaemia and multivariable logistic regression analysis was performed to understand the effects of preoperative haemoglobin [Hb] levels on in-hospital mortality (18). Along with another large retrospective cohort study an association was found between preoperative [Hb] level and mortality that was independent of age, gender or underlying disease (17). In contrast to this, another large retrospective cohort study found that after adjusting for baseline diseases, anaemia remained a rather weak independent predictor of postoperative mortality (143). However, *Baron* et al noted that the aforementioned study by *Saager* et al (143) did not grade anaemia as mild, moderate or

severe and that in the Baron study there were a high proportion of patients in the mild group and therefore, it is conceivable that the overall effect of anaemia was similar in both studies (18).

The obvious problem with any retrospective cohort data is that anaemia is associated with co-morbidities such as ischaemic heart disease, heart failure, diabetes, renal impairment, and cirrhosis and therefore all such studies are prone to confounding results.

In a balanced account the authors of the *Baron* paper explain that any large dataset needs to be interpreted with some caution with regard to causality when it is as complex as a large surgical population across multiple European countries. They point out that the severely anaemic patients were more likely to have received blood and with its known adverse outcomes therefore might have compounded any effects of the original anaemia. The authors also eloquently outlined that the associations between preoperative anaemia and postoperative outcomes in this kind of study make no account for changes to microcirculatory function or organ perfusion (18).

If one were to use the Bradford Hill Criteria for the presence of preoperative anaemia and poor postoperative outcomes to try to move from an association to a verdict of causation then it is arguable that all nine criteria are fulfilled (161). However, when originally described, the biological/mechanistic connections between exposure and disease were not as well understood as they are today (162). In an era of epigenetics, and 'omics' it is aspirational to attempt to quantify any potential causal relationship at a molecular level rather than the 'black box' that existed in Hill's time (162).

An important large randomised controlled trial - the PREVENTT study published recently is discussed in detail in section 3.2.2.1.1 (22). We await another randomised controlled trial set up to try to answer the questions relating to the relationship between the presence of perioperative anaemia, the effects of treating said anaemia and the measured outcomes, be they length of stay, reduction in red cell transfusion, change in haemoglobin level, time to get up and walk or simply mortality (163,164) (ITACS- NCT02632760). Section 3.7.3 will specifically look at published studies examining iron therapy and outcome.

3.2.2 Does treating perioperative anaemia improve outcomes?

This section will focus on iron therapy for the treatment of IDA/iron deplete/iron restricted anaemia in the perioperative space. Sections 3.6 & 3.7.1 will focus briefly on red cell transfusion as a therapy for perioperative anaemia although this could be a thesis in itself and is therefore not the main focus of this work. This thesis does not cover the treatment of other forms of anaemia encountered in the perioperative setting such as B12 or folate deficiency for which there are readily available, cheap and simple treatments and where the prevalence is less than 10% (165) and often found concurrently with iron deficiency. There is little debate in the literature as to what should be done if B12 or folate deficiency is found in the perioperative period. Admittedly it is undoubtedly apparent that very often outside of regulated systems to detect this it very often goes unnoticed. However, recent UK perioperative practice is evolving rapidly and many institutions have guidelines for the management of perioperative anaemia that include screening for nutritional causes (137).

There are data comparing oral iron with placebo or usual care and oral iron versus. intravenous iron. *Lidder* et al found a reduction in red cell transfusion in a small RCT in

colorectal cancer patients (166) but the numbers were very small and it is possible that this was simply type 1 error as the proportion of anaemic patients was higher in the non-intervention group meaning that they may have received more blood for this reason alone.

Oral iron, although cheap, is poorly tolerated by patients, with significant gastrointestinal upset amongst the major side effects. Often the perioperative time frame does not allow sufficient time for oral iron to adequately raise haemoglobin levels and therefore some guidance suggests that if less than six weeks are available before surgery then i.v. iron is preferred (137). Whilst oral iron remains an option and research continues into superior, more tolerable preparations (167), until there is a reliable preparation that adequately and promptly restores total body iron then intravenous preparations are likely to be favoured. The remaining focus will be on intravenous iron.

3.2.2.1 Intravenous iron

How does intravenous iron work? Current intravenous iron preparations consist of iron-carbohydrate complexes. Following intravenous injection, the iron-carbohydrate complex is taken up and phagocytosed by the reticuloendothelial system and the remaining iron core is exported out of the cell and transported for erythropoiesis and storage (168). New erythrocytes generated following the correction of iron-restricted erythropoiesis in bone marrow have a longer half-life than transfused erythrocytes (169).

Numerous observational studies (170–174) have suggested improvements in haemoglobin levels, reduction in red cell transfusions and a decrease in hospital stay. However, this has not been borne out in the randomised controlled trials. The short answer is we do not know yet if intravenous iron has any outcome benefits in perioperative anaemic patients. A systematic review of published and currently registered studies examining the effect of iron therapy on perioperative outcomes was updated in December 2019 and is worthy of some discussion (169).

The authors identified six randomised controlled trials with a total of 372 patients. Importantly, this number was far short of the calculated 819 that would be required to detect a 30% reduction in red cell transfusion if this were the primary outcome of the said studies. The authors recognised this as a weakness of their meta-analysis. They included studies that evaluated the use of preoperative iron therapy to correct anaemia. Four studies were prospectively registered in a clinical trial registry (20,21,175,176) and the two older studies do not appear to have been registered (166,177). It is worthy of note that a major drawback of a meta-analysis in this circumstance has limited strength to form the basis of any clinical practise guidelines.

Three studies were in colorectal surgery (21,166,175), one in gynaecological surgery (177), one in orthopaedic surgery (176), and one in major abdominal surgery (20).

Important factors to recognise about the individual studies are summarised in Table 2

Study	*Number of participants*	*Area of medicine*	*Product type and dose used*	*Time period between product and surgery*	*Primary outcome measure*	*Problems with the study*
Edwards 2009 (175)	RCT- i.v. iron vs. placebo- 60 patients (note only 18 anaemic)	Colorectal cancer	i.v. iron sucrose 600mg vs. placebo	Minimum of 2 weeks	Transfusion rates, amount of blood transfused, [Hb]	Very small numbers of anaemic patients. Not adequately powered
Kim 2009 (177)	RCT - 66 patients [Hb] <90g.l^{-1}	Gynaecological surgery for menorrhagia	i.v. iron sucrose (based on total iron deficit) vs. oral iron (80 mg/day iron succinylate)	Starting 3 weeks preceding surgery	Increase in [Hb]	Did not report red cell transfusion rates
Froessler 2016 (20)	RCT- 72 patients, i.v. iron vs. usual care	Major abdominal surgery	i.v. iron - single dose ferric carboxymaltose 1000mg or 15mg/kg. Second dose given post operatively of 50g per 100ml blood loss	(Median 8 days prior)	Primary- red cell transfusion (lower in iron group) Secondary- [Hb], ICU admission, morbidity, mortality, LOS, iron status and QofL	Trial was stopped early due to high rates of red cell transfusion in the usual care group
Keeler 2017 (21)	RCT i.v. iron vs. oral iron - 116 patients	Colorectal cancer	Ferrous sulphate 200 mg twice a day or intravenous ferric carboxymaltose with dose based upon haemoglobin level and weight.	At least 2 weeks before surgery	Primary: red cell transfusion, (no difference). Secondary: Amount of blood per participant, 90-day mortality (no difference), [Hb] levels.	Possibility of a type 11 error due to low overall rates of BT. Very short time period for i.v. iron to act.
Lidder 2007 (166)	Oral vs. usual care- 45 patients	Colorectal cancer	Oral iron versus no iron therapy	2 weeks pre surgery	Primary BT (Lower in iron group)	Very small numbers did not exclude non anaemia patients. Not adequately powered
Serrano-Trenas 2011 (176)	RCT of 200 patients- i.v. iron vs. standard care	Orthopaedic surgery- hip fracture surgery	i.v. iron sucrose 600mg vs. standard treatment	Started day of admission 3 doses at 48-hour intervals.	Primary BT (no difference)	Not adequately powered for the primary outcome

Table 2: Summary of important aspects of the 6 studies included in the meta-analysis of iron therapy for preoperative anaemia by *Ng* et al (169)

For the primary outcome:

> "Five trials reported the primary outcome (proportion of participants who received allogeneic blood transfusions) for 316 people (200 iron versus standard care or placebo, 116 oral iron versus intravenous iron). Meta-analysis of iron therapy versus placebo, no treatment or standard care showed no reduction in the proportion of participants who received a blood transfusion risk ratio (RR) 1.21, 95% confidence interval (CI) 0.87 to 1.70; $I^2 = 54\%$; 4 studies, 200 participants; moderate-quality evidence. Only one study reported transfusion after oral iron or intravenous iron and reported no difference in transfusions (21)." (169)

Regarding secondary outcomes, there was no difference in [Hb] in the studies with oral iron but there was with i.v. iron (it increased). There was not enough data on quality of life (QoL) or morbidity. However, a recent publication (follow up study) of the 'IVICA' study by *Keeler* et al that was not included in the aforementioned meta-analysis did analyse QoL and found that the patients in the intravenous iron group reported higher QoL compared to those in the oral iron group. They used the Short Form 36, EuroQoL 5-dimension 5-level and Functional Assessment of Cancer Therapy- Anaemia questionnaires and found that i.v. iron was statistically superior for improving QoL (178). This is in keeping with some of the large randomised trials in heart failure that demonstrate quality of life improvements after i.v. iron (179).

A recent noteworthy study that was not a pure study of intravenous iron but nonetheless was a well conducted randomised trial examined a combined therapeutic approach. *Spahn* et al gave ferric carboxymaltose, erythropoietin alpha, vitamin B12, and folic acid to elective cardiac surgical patients who were anaemic or iron deficient. They randomly assigned patients in a 1:1 ratio to receive placebo or the combination therapy. The primary outcome was the number of RBC transfusions during the first 7 days.

They recruited 505 patients. The combination treatment significantly reduced RBC transfusions from a median of one unit in the placebo group (inter-quartile range 0-3) to zero units in the treatment group (0-2) during the first 7 post-operative days. Despite fewer RBC units transfused, subjects in the treatment group had a higher haemoglobin concentration, higher reticulocyte count, and higher reticulocyte haemoglobin content (180).

This study is interesting as they showed a significant difference in red cell transfusion in a very short time frame from treatment to surgery of only one day. The doses given are worthy of mention; 20 mg/kg ferric carboxymaltose and 40 000 U subcutaneous erythropoietin alpha as the i.v. iron dose is certainly in keeping with modern perioperative practice whereas many older studies used older preparations in smaller doses. To my knowledge this is the only combination therapy perioperative study. Importantly, the serious adverse event rate was the same in the treatment and the placebo arm. Additionally, there was no difference in thromboembolic events which has historically been a concern with studies delivering erythropoietin alpha (180).

3.2.2.1.1 The PREVENTT study

This study is worthy of discussion here as it is the largest randomised controlled study in abdominal surgery to date. It was a double-blind, parallel-group, placebo-controlled trial of

i.v. iron therapy (ferric carboxymaltose, 1000 mg) versus placebo (saline) in anaemic patients undergoing major open elective abdominal surgery(22). The Co-primary endpoints were the rate of red cell transfusion or death and the number of red cell transfusions from randomisation to 30 days postoperatively.

Among 487 participants randomised, death or red cell transfusion occurred in 67/243 subjects in the placebo group (28.3%) and 69/244 subjects in the i.v. iron group (29.1%). Death (1% vs. 1%), postoperative complications (11% vs. 9%), hospital stay, or days alive and out of the hospital at 30 days did not differ among groups. However, both haemoglobin concentrations at the time of surgery and postoperative haemoglobin concentrations were higher in the i.v. iron treatment group and may have led to improved postoperative recovery.

Of interest, hospital readmissions within 8 weeks after surgery were significantly lower in the i.v. iron group (22% vs. 13%), as these patients had fewer wound infections. These effects merit further investigation. It is important to recognise that this outcome was not adequately powered in PREVENTT, but future perioperative studies should incorporate readmissions into their power calculations.

The study had a number of strengths. It was a multi-centre study (although importantly 25% of the recruits came from a single centre). They had a very high adherence to the intervention with a low loss to follow up and low levels of attrition. The per protocol analysis and the intention to treat analysis showed very similar results. Along with the pre-defined subgroup analysis this meant that any non-adherence with other parts of the protocol was unlikely to have influenced the results.

There were, however, a number of significant problems with the trial.

1. Perhaps most importantly they did not specify iron deficiency as a pre-requisite to recruitment, only that the patient needed to be anaemic. This resulted in only 76% of the cohort being truly iron deficient based on a transferrin saturation level of <20%. This might have meant that they recruited non-informative patients which may have diluted any beneficial effects. This also meant that for the primary outcome it would have been underpowered if one wanted to consider true iron deficiency as the most likely candidate for any improvement seen with i.v. iron therapy.
2. The cohort contained mostly mildly anaemic patients with a median pre-enrolment haemoglobin concertation of 111 $g.l^{-1}$ and only 17% of the study patients had a [Hb] of <100 $g.l^{-1}$. There appears to be a dose response relationship whereby lower levels of [Hb] respond to i.v. iron to a greater degree than higher [Hb]. Additionally, even after treatment 80% of the patients in the treatment arm remained anaemic. The total rise of only 4.7 $g.l^{-1}$ is significantly lower than the other perioperative randomised trials (20,21,180) (see also Chapter 8 of this thesis).
3. The length of time taken to complete the trial and the timing of the release of the NICE guidance midway through the trial encouraging clinicians to treat iron deficiency pre surgery affected recruitment to the trial. Many clinicians not wishing to recruit their most anaemic subjects rather than treating them within established perioperative iron clinics instead.
4. Ferric carboxymaltose at a dose of 1000mg was pragmatic but was certainly not a high enough dose for most patients. Similar studies have used higher doses and seen greater rises in [Hb] (see also Chapter 8).
5. The median time between treatment and surgery of 14 and 15 days respectively (treatment versus. placebo) was possibly too short to see the peak effect (see section 3.2.2.1.2.1).

6. There was no restriction or guidance on who administered blood and why a patient was given blood within the trial. This was a pragmatic choice as it would have been exceptionally difficult to control even within the remit of a RCT. However, as this was a co-primary endpoint it was a weakness of the study.

In summary, the PREVENTT trial showed that 1000 mg of ferric carboxymaltose given shortly before elective major abdominal surgery to a cohort of anaemic patients (but lacking a diagnosis of iron deficiency) may not reduce red cell transfusion rates but may have beneficial effects in the postoperative period leading to a clinically relevant reduction in readmission rates.

3.2.2.1.2 Some of the main problems with this research

3.2.2.1.2.1 Timing of I.V. iron prior to surgery

The administration of iron therapy before surgery ranged from the day of admission in one study to three weeks prior to surgery in another. It is my view that none of the studies included in the meta-analysis (or in PREVENTT) left adequate time for the full effects of iron therapy to have been realised. The more modern intravenous iron preparations such as ferric carboxymaltose and iron isomaltoside 1000 have an effect on [Hb] that can be seen within fourteen days (181), with most seeing a rise at around twenty-one days, but clinical experience still maintains that often their peak effect isn't until twenty-eight or sometimes even more days (see Chapter 8). Previous studies reported longer periods of time (around six weeks) for intravenous iron preparations to have full effect (182,183). Despite this, it is known that a haematopoietic effect is seen at just five days (184). Chapter 8 explores this further.

3.2.2.1.2.2 Product and dosing regimen used

The studies in Table 2 used a wide variety of iron products in a wide variety of dosing regimens. Only two of them used what would be considered a more 'modern' i.v. iron preparation in doses commonly used in clinical practise in 2021 (20,21). It is therefore highly plausible to conclude that any effect on the primary outcome (in this case red cell transfusion reduction) could have been influenced by underdosing. The same principle is applicable to the PREVENTT trial. Interestingly, a recent study in elective cardiac surgery gave a dose of 20mg/kg of ferric carboxymaltose (which for an 80kg patient would have been 1600mg) (180).

It is my opinion that the combination of a short time frame and relatively low iron doses in the context of a meta-analysis that itself was 447 patients shy of the required number to detect a 30% reduction in red cell transfusion, puts any conclusions on fairly weak grounding. I would also suggest that the same possible problems applied in the PREVENTT trial.

3.2.2.1.2.3 Conclusions

'ITACS' also using ferric carboxymaltose (led by Paul Myles) (164) is still recruiting. It may be that this helps to answer questions around perioperative outcomes and intravenous iron.

So, what does this mean? The observational studies show a benefit so why did the RCTs not show one? The results, however, may reflect the small sample sizes in the six included studies and their ability to detect a difference with so few data. As outlined above, there are significant short comings regarding dosing regimens, product type and duration of treatment prior to surgery. The stark differences reported in the IV iron study (60 % reduction in transfusion) versus usual care reported by *Froessler* et al (20) is at odds with a broadly similar cohort of patients studied by *Keeler* et al who showed no reduction in red cell transfusion between IV iron and oral iron (21). I suspect that neither study shows the true effect and unfortunately the meta-analysis discussed in this section goes no further to untangling this.

Does the PREVENTT study change this? Whilst this was a well conducted RCT due to the reasons outlined in the weaknesses section of 3.2.2.1.1. I do not feel that the question has been adequately answered. If anything, it again shows that the safety of i.v. iron was very good with no change in transfusion rates but a possible reduction in infection and readmission rates which warrants further investigation.

Like red cell transfusion, intravenous iron is of course not without potential detrimental effects. Historically, this focused on anaphylactic reactions and certainly some of the older preparations were prone to this. The 'Fishbane' reaction is however much more common, quoted between 1:100-1:200 infusions. However, in my experience it is much less than this if the product is given slowly. A major risk is extravasation from a poorly placed cannula or in a small peripheral vein. Skin staining that results from. This can be permanent and all patients must be consented for this possible significant side effect. Another possible complication is delayed hypophosphatemia and this has been described with multiple currently available intravenous iron products. The mechanism is poorly understood and this is remains an active area of research.

3.2.3 What about blood?

Section 3.4.7 will address the effects of blood manipulation on healthy volunteers and athletes (as there is a wealth of data on them). The sparse data in clinical subjects is evaluated in section 3.7.1. The relationship between performance and blood volume, tHb-mass and haemoglobin concentration will be explored.

Previous research in athletes and clinical subjects has examined the effect of allogenic and autologous red cell transfusion on human performance, some of the research has looked at important patient related outcome measures in the perioperative setting and this will be discussed in sections 3.7. However, there is now a fairly widely shared acceptance due to both cost, risk, and the potential negative immunological consequences of allogenic (homologous) red cell transfusion that it is *probably* on balance detrimental for perioperative patients outside of acute blood loss or extremely low [Hb] chronic states. This is reflected in the studies that are currently in progress exploring patient blood management strategies in the perioperative setting. Whilst I am not arguing that red cell transfusion in any setting or at any particular threshold is definitively detrimental for all perioperative patients, there is a shortage of evidence for its benefit. A wide body of literature in the general medical fields is conclusive that restrictive transfusion thresholds are non-inferior (leaving the somewhat grey area of acute coronary syndromes and cardiac surgery out of the discussion for now). This is a hotly debated subject in the scientific literature and does warrant explanation which is why I have outlined a broad overview in section 3.6.

3.2.4 Non anaemic iron deficiency

Non-anaemic iron deficiency (NAID) is exactly that; the finding of iron deficiency (usually defined using laboratory metrics aside from ferritin alone such as transferrin saturation) without anaemia, as defined by the WHO. It is crucial to realise that there is no single laboratory parameter comprehensively reflecting the iron status of an individual. The state of anaemia is effectively a final stage of iron deficiency. Iron deficiency is broadly separated into two types, actual iron deficiency (AID) and functional iron deficiency (FID) although the terminology can be confusing and other terms are used synonymously in the literature (185).

Absolute iron deficiency: this refers to the absence of sufficient iron stores to maintain effective erythropoiesis (186).

Functional iron deficiency: this exists where, despite an apparently adequate store, iron cannot be effectively mobilised to participate in erythropoiesis (165,187). Our understanding of FID has been greatly enhanced by the discovering and sequencing of the iron regulatory peptide hepcidin. One of the diagnostic problems is the fact that ferritin is an acute phase protein, that is raised in inflammatory states. At the same time, inflammation drives the upregulation of hepcidin which has two principal functions (although this is overly simplifying its effect). Firstly, it prevents iron uptake in the small intestine and secondly it stops pathogens obtaining free iron as part of the innate immune response (188).

NAID is an increasingly recognised phenomenon in both elite athletes, the general 'well' population and in certain disease states. A recent meta-analysis concluded that there were simply insufficient trials to make any meaningful conclusions about the effect of intravenous iron on any outcomes beyond stating that it may result in a small, clinically insignificant increase in haemoglobin concentration (188). Interestingly, guidance exists in the perioperative literature to support treatment for NAID patients despite the lack of evidence for efficacy or outcome benefit (137).

3.2.4.1 Is NAID a problem in perioperative patients?

This truly does remain unknown as the data in this area is simply not available. As stated by *Miles* et al:

> "An overall estimate of non-anaemic iron deficiency across every patient population, and the distribution between absolute and functional iron deficiency cannot be given at this time, although upcoming prospective work in a variety of populations will likely be useful in providing much needed demographic data." (188)

Regarding the effect on perioperative outcomes there is a small amount of retrospective data available but this has only identified possible meaningful outcome measures for further work which is being undertaken (189).

3.2.4.2 Exercise performance in NAID?

To my knowledge there are no clinical studies examining this in the perioperative space. Data from the mid-1970s in animal studies suggested that iron deficient rats showed impaired running ability compared to controls (190). There are many studies examining this in athletes with mixed results (191–193). A meta-analysis in 2013 suggested that iron therapy was beneficial in improving iron status and aerobic performance in athletes (194). From a mechanistic perspective NAID could impair aerobic capacity by reducing the ability to extract and utilise oxygen from haemoglobin. A more recent meta-analysis found 20 studies that had examined the effects of iron therapy on fatigue and exercise performance. They concluded that iron therapy reduced subjective measures of fatigue but without objective improvements in physical capacity (195). Whilst this area certainly requires further research it is not considered further in this thesis.

3.3 Laboratory 'normal' ranges

It is important to recognise the laboratory cut off limits for 'normal' vary. Unfortunately, these are not always rooted in robust evidence. Some laboratories have adopted normal ranges that are based on the 5th to 95th percentile range, consigning 5% of the population to the polycythaemia category, and thereby often mandating, inappropriately, the measurement of RCM (54). Some laboratories have developed their normal ranges based on studies from healthy volunteers. Fascinatingly, and honestly, when the chief haematologist from the Mayo clinic described this in a publication about polycythaemia vera he admitted that:

> "It was later discovered that nearly 70% of the healthy, 'normal' volunteers had been frequent blood donors; half of them were iron deficient, and some were frankly anemic by any criterion.'(54)

This meant that clinicians would underdiagnose anaemia and over diagnose polycythaemia vera. Put together with the deficiencies described with the WHO definition in section 3.1.1 and the fact that that PV variation can dramatically affect [Hb] (section 2.6.4.3), when one considers the cost and time put into investigating polycythaemia vera and anaemia respectively an argument for the measurement of tHb-mass (with reference ranges developed) grows ever stronger. This is of particular relevance when clinicians are using interventions to improve oxygen delivery and is explored in Chapter 8 of this thesis when tHb-mass and [Hb] is measured pre and post intravenous iron in an anaemic/iron deficient cohort awaiting surgery.

3.4 Haemoglobin and tHb-mass manipulation in the context of sports science and elite performance

Much of this section has been published in a review article (1) and in work carried out by myself and *Dr James Otto* (28,29,33,34). This section will include a summary of topics examined in the papers referenced above.

3.4.1 Cardiopulmonary exercise testing and performance

Physical performance is not only important to athletes attempting to win Olympic medals or the Tour de France but can also be correlated to outcomes in the perioperative space. This is where our interest in this subject began. Measures of physical performance are numerous but arguably the most comprehensive/dynamic global functional assessment available to humans is a cardiopulmonary exercise test (CPET) (196). A CPET provides joint data analysis that allows the complete assessment of the cardiovascular, respiratory, muscular and metabolic systems during exertion, being considered gold standard for cardiorespiratory functional assessment (197). Throughout this thesis when CPET is mentioned this refers to a maximal exercise test on a static cycle ergometer, the exact methodology used is outlined in Chapter 8.

$\dot{V}O_{2max}$ is classically defined as 'a plateau in oxygen uptake attained during maximal exercise despite further increases in exercise workload, thereby defining the limits of the cardiorespiratory system' (198). However, many individuals do not reach a plateau in oxygen uptake despite maximum exertion, and the term $\dot{V}O_{2peak}$ is used instead, being the highest measured oxygen consumption during exercise, typically averaged over a 30 second period. $\dot{V}O_{2AT}$ is defined as 'the highest sustained intensity of exercise for which the measurement of oxygen uptake can account for the entire energy requirement'. An alternative definition is 'the exercise intensity at which lactate starts to accumulate in the blood stream' (199). These oxygen uptake variables are in part dependent on the oxygen carrying capacity of the blood, which is in turn dependent on blood haemoglobin levels.

The fixation on $\dot{V}O_{2max}$ and $\dot{V}O_{2peak,}$ both in clinical practice and also historically in elite sports performance has often missed some of the key aspects of a maximal exercise test. A high total aerobic capacity ($\dot{V}O_{2peak}$ /$\dot{V}O_{2max}$) is of course important for success in endurance sports. However, exercise efficiency/economy, may also be critical determinants of performance. For example, two athletes with the same $\dot{V}O_{2max}$ do not necessarily perform to the same level in an endurance performance test or race: the athlete with the higher $\dot{V}O_{2AT}$ is likely to perform better. Furthermore, the efficiency or economy with which work is done relative to energy expenditure may be important. For example, *Lucia* et al showed that a range of $\dot{V}O_{2max}$ levels amongst elite cyclists could be compensated for by differences in efficiency (200). Regarding efficiency, these measurements are not often carried out in routine clinical CPET within hospitals). Intriguingly, the premise that improvement in physiological variables (i.e., aerobic capacity) enhances athletic performance (i.e., races or gold medals won) has not been well investigated.

3.4.2 Haemoglobin concentration [Hb] versus. total haemoglobin mass (tHb-mass)

Haemoglobin [Hb] concentration at extremes evidently influences exercise performance. *Dr Otto* studied this relationship between [Hb] and CPET performance retrospectively. He found that there was a modest relationship between lower [Hb] and lower oxygen uptake variables. [Hb] accounted for 9% and 6% of the variation in $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$ respectively.

This led on to work to compare [Hb] and tHb-mass with CPET variables. Traditionally, the concentration of circulating haemoglobin [Hb] has been used as a clinical measure of the blood's oxygen carrying capacity. However, a low [Hb] may be due to a reduced amount of haemoglobin (absolute mass of circulating haemoglobin; tHb-mass) or an increased volume of dilution (plasma volume). Thus, [Hb] may be stable and tHb-mass low in the context of acute bleeding, [Hb] normal or elevated, but tHb-mass low in the context of dehydration, or [Hb] low but tHb-mass normal or high in the context of excess plasma volume (fluid). Therefore, the use of [Hb] to define blood oxygen carrying capacity may be misleading under some circumstances (1).

Intuitively, an increase in [Hb] should increase oxygen delivery to the tissues. Yet this is not clear cut, with several studies conducted in athletes (35) and diabetic patients (60) suggesting little correlation of [Hb] with oxygen consumption ($\dot{V}O_2$) at peak and at anaerobic threshold (AT) (33). One of the reasons that tHb-mass has a better correlation is due to the dual role it plays in this; on the one hand it determines [Hb] in concert with the total blood volume but it also raises blood volume via erythrocyte volume and this double effect explains the superior correlation described by *Schmidt* and *Otto* (33–35).

> "It appears that tHb-mass is of greater utility in blood manipulation in elite athletes trying to improve sporting performance as it is more stable and predictable over time and also has a more direct correlation with performance."(1)

The question addressed in the above review was whether tHb-mass, in comparison with haemoglobin concentration, was a more precise and accurate variable to guide targeting of haemoglobin manipulation. A central hypothesis being that potentially improving physiological reserve in patients may lead to improved clinical outcomes (1).

3.4.3 Does manipulation of the blood work in sport?

In short, the answer to this question is an undeniable yes. There is a reason that Bjarne Riis was colloquially referred to as 'Mr 60' after his reported haematocrit of 60% during the mid-1990s (201). The exceptional, 'unbelievable' performances of pro cyclists (infamously Lance Armstrong) that occurred during the 'EPO years' (roughly 1993-2008) where it was believed that wide-spread use of EPO was the norm in the professional peloton were indeed 'unbelievable'. The Chinese distance runners during a similar period and more latterly the revelations of systemic doping within Russia's anti-doping agency (Rusada) between 2012 and 2015 (202) put together with anecdotal evidence from the likes of Dr Michale Ferrari all point toward enormous endurance performance gains with blood manipulation. Of course, the details of these abuses are not readily available and the extremes taken by individuals are likely to have exceeded anything that is documented in the scientific literature and there are sadly deaths in young athletes to support this notion (203).

The basic physiological principles of why blood manipulation might improve performance are explained by the component elements of blood that supply oxygen to respiring muscles (see sections 2.2 & 2.3). The aim remains the same that increasing oxygen delivery ($\dot{D}O_2$) through elevating haemoglobin levels will augment maximum oxygen uptake ($\dot{V}O_{2max}$) and perhaps more importantly (for endurance events) increase the workload at which anaerobic threshold (AT) is reached. There is still debate around the factors that limit $\dot{V}O_{2max}$, with candidate mechanisms including central control, cardiac limitation, mitochondrial

utilisation, and total oxygen delivery (the product of cardiac output and blood oxygen content). However, there is some uncertainty about the dominant controlling factor. Many authorities agree that in highly trained athletes, $\dot{D}O_2$ is a factor that contributes to $\dot{V}O_{2max}$ limitation (204–206) and that $\dot{V}O_{2max}$ is also, at least in part, dependent on a number of underlying genetic factors that are not amenable to modification through training (207). Therefore, blood manipulation to augment $\dot{D}O_2$ has been seen as a logical, albeit illegal, approach to augmenting $\dot{V}O_{2max}$ and thereby improving athletic performance (1).

Intriguingly, the premise that improvement in physiological variables (i.e., aerobic capacity) enhances athletic performance (i.e., races or gold medals won) has not been well investigated. Having said that, the effects of blood manipulation on a range of physiological variables, including $\dot{V}O_{2max/peak}$ and $\dot{V}O_{2AT}$, are both of relevance for athletes and may have significance in clinical contexts (1,208).

3.4.4 The mechanisms to increase [Hb] both legal and illegal

The mechanisms outlined below will briefly describe some of the key studies in the field of sports science. For a more detailed review of this subject please see (1). Intravenous iron will be explored in greater depth, as the relationship between exercise capacity, anaemia and haemoglobin content in an elective surgical cohort are specifically explored in Chapter 8. See section 3.2.2.1 for a description of recent perioperative studies that have examined the use of intravenous iron.

3.4.5 Does physical training increase tHb-mass?

Malczewska-Lenczowska and colleagues measured the tHb-mass of 176 National standard athletes encompassing endurance and non-endurance sports in Poland. They found a significant difference in tHb-mass (using the oCOR) in the endurance athletes compared to the non-endurance athletes supporting higher relative tHb-mass in endurance athletes compared to untrained subjects (209).

Elite rowing ergometer performance has also been linked to tHb-mass with an increase of 100 g tHb-mass being associated with an increase of 24 W measured on the ergometer (210).

Schumacher et al studied spinal cord injured (mainly paraplegic) athletes and compared them to untrained controls and able bodied athletes. They concluded that tHb-mass is higher in endurance trained than in untrained spinal cord injured subjects and therefore seems to adapt to chronic endurance exercise (211).

Montero and *Lundby* performed an elegant exercise training study. They took 78 healthy males who did not participate in organised sport. They were then split into groups whereby they undertook a differing number of exercise sessions per week ranging from one to five times a week. They measured CPET and tHb-mass using the modified *Burge* and *Skinner* method described in section 2.6.1. They showed that non-response to an exercise training programme could be eliminated using high intensity training and ensuring that enough minutes per week were undertaken. The improvements correlated with the amount of exercise performed, with a dose response correlation. Of particular interest to this thesis

was the discovery that increases in tHb-mass were the strongest determinants of $\dot{V}O_{2max}$ improvement, which in turn explained most of the variance in maximal incremental power output (212).

In a study examining the effect of exercise training on cardiac output, tHb-mass, and any structural changes to the heart via static echocardiography. *Bonne* and colleagues demonstrated that exercise over 6 weeks improves blood volume and cardiac output. This can then be eliminated by phlebotomy. They showed no changes in the structure of the heart and thus concluded that the exercise training-induced increase in BV is the main mechanism increasing CarO after 6 weeks of endurance training in previously untrained subjects (213).

Further weight to the argument that improvements in exercise capacity induced by exercise training predominantly come from changes to BV and tHb-mass were demonstrated in a study that examined muscle biopsies, tHb-mass (modified *Burge* and *Skinner* method) and CPET from 16 untrained but healthy volunteers. They underwent 6 weeks of 3-4 times a week exercise training. $\dot{V}O_{2peak,}$ CarO, tHb-mass all increased after the 6-week programme. Skeletal muscle biopsies were analysed for mitochondrial volume density (MitoVD), capillarity, fibre types and respiratory capacity (OXPHOS). MitoVD was increased but OXPHOS was not after 6 weeks. Phlebotomy was then performed to achieve the pre intervention red blood cell volume. Using multiple regression peak CarO, RBCV and tHb-mass were found to be independent predictors of $\dot{V}O_{2peak}$. This inferred that after 6 weeks the improvements seen in exercise capacity are primarily BV derived rather than due to muscle adaptation (214).

However, in highly trained athletes there may be a training ceiling. A study of prescribed high intensity training over 3 weeks (11 sessions) did not show any changes to BV (*Schmidt* and *Prommer*; oCOR method) or CPET variables over a matched control group when both maintained their usual training over the study period (215).

3.4.6 Altitude

The effect of altitude training on elite athletic performance has been the central subject of numerous other theses. It is not new either, with interest in the effect of altitude on erythropoiesis dating back to the early 1900s (67) and the effect of altitude on stimulating erythropoiesis being described over a hundred years ago (216).

There are numerous questions within the field regarding the details of how this is actually done, including but not limited to; the optimum height to go to, the ‘live high train low’ (LHTL) philosophy, duration of altitude exposure and what exactly to do at altitude, which are beyond the scope of this thesis.

A comprehensive meta-analysis in 2013 considered studies that had measured tHb-mass using CO. They concluded that camps as short as two weeks of classic (at high altitude for the duration of the camp) and LHTL altitude would likely increase tHb-mass and that most athletes could expect benefit (13). They concluded that tHb-mass was estimated to increase by 1% for every 100 hours of high-altitude exposure. For athletes of course, this is a balance between effective erythropoiesis versus the ability to train at altitude and not decondition. There comes a point, likely above 2500m where training intensity decreases. Muscle atrophy and metabolism are dramatically altered to a detrimental level above this altitude. Therefore 2000-2500m is usually recommended for athletes.

A later study pooled data from ten studies including 145 elite endurance athletes. They examined pre and post altitude training camps measurements including CPET and tHb-mass. They concluded that both $\dot{V}O_{2max}$ and tHb-mass increased after altitude exposure and that the correlation between per cent change in tHb-mass and per cent change in $\dot{V}O_{2max}$ was significant ($p<0.0001$, $r^2=0.15$). tHb-mass and $\dot{V}O_{2max}$ increased by ~3% such that each 1% change in tHb-mass resulted in a 0.6–0.7% change in $\dot{V}O_{2max}$ (217). Of note, it is hypothesised that altitude affects exercise performance in ways other than simply increasing tHb-mass. Recent work has elucidated that adaptations in PV are also present in the Sherpa population of Tibet and Nepal. This means that they possess superior exercise capacity and have greater reproductive success compared to Andean high-altitude natives. This may be due to lower [Hb] due to a greater PV (218).

Many athletes now opt for altitude tents at home simulating the effect of low barometric pressure over night. According to the aforementioned meta-analysis, athletes may expect a mean rise of 3% in their tHb-mass in only two weeks with approximately 97.5% obtaining at least a 1% increase. These values must be digested in the context of the marginal gains and the often miniscule margins that elite sporting performances are won or lost by (13).

There are a number of studies in clinical subjects using altitude tents to stimulate the haemopoietic system (219). As far as I am aware this methodology has not yet been applied to perioperative patients.

3.4.7 Blood manipulation and blood volume measurement

The re-injection of blood to improve performance is not new. As mentioned at the very beginning of this thesis (Chapter 2on page 3). The earliest reports of ‘blood doping’ in the scientific literature date back to 1945–1947 (14,15). As outlined in a review on the subject, the truth about the limits that elite athletes have pushed the erythropoietic system for performance enhancement may never be known (1). There is little doubt that allogenic and autologous (more common in elite sport) red cell transfusions have been proven to enhance performance.

Interestingly, there are few studies directly addressing the question of whether autologous red cell transfusion improves athletic performance. *Berglund* et al demonstrated a significant fall in the race times of cross-country skiers when compared to matched controls pre and post autologous red cell transfusion (220). *Williams* et al demonstrated improved 5-mile treadmill run times (mean improvement of 44 s) with reduced self-reported perceived exertion after autologous red cell transfusion (221). *Brien* et al took 6 well-trained runners and improved their 10 km time by an average of 1 min. Using a double-blind cross-over design, each runner received a 400 ml autologous transfusion of blood or saline repeated 5 days apart with a 10 km race 5 days after each treatment. Five out of the 6 runners had faster race times after transfusion (222).

3.4.7.1 Blood manipulation and measuring blood volume

In 1949 *Sjöstrand* and colleagues measured haemoglobin mass in ‘well trained’ individuals using a modification of the methods described by *Haldane* and *Gréhant* and noted they had a higher tHb-mass: see section 2.6.1.

Early work in this field by *Ekblom* et al examined the relationship between blood volume, tHb-mass, and exercise performance. In 1972 he was able to demonstrate in a small study that performance decreased with venesection and improved again after re-infusion of autologous blood. *Ekblom* used 51Cr labelled red cells- to examine changes in tHb-mass and blood volume. He also noted that there was a marked overnight improvement in exercise capacity and $\dot{V}O_{2\,max}$, the paper discussed in detail the relative possible contributions of total blood volume increases versus the new improved oxygen carrying capacity via increased $\dot{D}O_2$ (223) , a debate that continues and was explored further in work by *Otto* et al (29,34). In 1987 *Berglund* and *Hemmingson* demonstrated that blood doping improved endurance performance in cross country skiers (220). This has subsequently been confirmed a number of times (224).

For reasons (that have been discussed in detail in sections 2.6.1) of ease and reproducibility, CO-rebreathing is the method of choice to measure blood volume in athletes (70,117). It is also reassuringly comparable regardless of which of the current two best described methods are used; namely the oCOR used throughout this thesis (5) and the longer method described by *Burge* and *Skinner* (74). *Keiser* et al compared the two techniques against each other and concluded that CO rebreathing is unequivocally the most superior way to measure tHb-mass (70). See section 2.6.6 for a detailed description of this study.

3.4.7.2 Unanswered questions

The story is not as opaque as it might seem. There are some unanswered and controversial questions that I believe are worthy of consideration.

The main questions in this arena are:

1) Does the injection of whole blood or fractionated red cells improve [Hb]- and with this blood volume and total circulating haemoglobin mass?

2) Is transfused blood useful regardless of what it does to blood volume or oxygen carrying capacity? And, at what point in time does it become useable/useful. On a microcirculatory level and from a mitochondrial perspective I do not think that this has been adequately answered in the literature.

3) Is there a difference between autologous blood and allogenic blood?

4) At what point does it become detrimental? I.e., is there an upper limit of improved performance?

5) Crucially, does transfusion *always* result in measurable performance improvement? And how long does it last?

3.4.8 Recombinant human erythropoietin (rHuEPO)

Recombinant human erythropoietin has been more widely studied than autologous red cell transfusion within the sports science community. As early as 1991 *Ekblom* et al showed an improved $\dot{V}O_{2max}$ post rHuEPO injection in 15 volunteers (225).

Russell et al were the first to characterise the submaximal and maximal exercise adaptations to prolonged use of low dose rHuEPO (226). Birkeland et al showed in a double-blind placebo-controlled trial that injection of 5000 IU of rHuEPO thrice weekly for 4 weeks improved $\dot{V}O_{2max}$ by 7 % (227). In 2007, *Thomsen* et al stated that:

> "Although the positive effect of rHuEPO treatment on $\dot{V}O_{2max}$ is clearly established, it remains unknown as to what its impact is on endurance performance".

They investigated the effect of rHuEPO on $\dot{V}O_{2max}$ and time to exhaustion during cycle ergometry in healthy volunteers. rHuEPO significantly increased $\dot{V}O_{2max}$ by 9.1 and 8.1 % in week 4 and 11, respectively, with no changes in the placebo group (208).

3.4.8.1 Studies examining the effect of rHuEPO on tHb-mass

Lundby et al gave healthy subjects 5000 IU of rHuEPO over 15 weeks at a frequency to maintain a Hct of 50%. Their main aim was to test the theory that EPO leads to a progressive elevation of tHb-mass with a concomitant reduction in plasma volume. The *Burge* and *Skinner* method was used to measure tHb-mass. [Hb] increased from 14.2 ± 0.6 before treatment and reached a peak of 17.1 ± 0.5 $g.dl^{-1}$ after 12 weeks of rHuEPO administration. Red blood cell volume was increased from 2933 ± 402 ml before rHuEPO treatment to 3210 ± 356 (9.8 ± 1.3%, $P < 0.01$), 3117 ± 554 (5.9 ± 2.0%, $P < 0.05$), and 3172 ± 561 ml (7.8 ± 2.0%, $P < 0.01$) after 5, 11 and 13 weeks, respectively. This was accompanied by a decrease in plasma volume which resulted in a relatively unchanged total blood volume.

They found that rHuEpo increased [Hb], and thus arterial oxygen content, by simultaneously increasing tHb-mass and depressing plasma volume. They hypothesised that the reduction in plasma volume might act as a fast mechanism to increase arterial oxygen content rather than the well-known slower adaptations on erythropoiesis. Their mechanistic reasoning described either an effect on mediating vascular tone and MAP, i.e., rHuEPO vasoconstricts and thus is associated with a reduction in plasma volume. The second mechanism proposed that rHuEPO induced a negative-feedback mechanism elicited by an excess of rHuEpo resulting in an attenuation of the activity of the rennin–angiotensin–aldosterone axis, avoiding the development of excessive hypervolaemia, and thereby maintaining total blood volume within a narrow range (228). These findings have subsequently been supported by other authors (229).

As mentioned in section 2.8.3 (on page 43) much of the *'best'* data in this area will never be made available for rigorous scientific analysis due to its illegal nature. It may be that the combination therapies as recently studied by *Spahn* et al (180) have actually been used by elite athletes for some time?

3.4.9 Intravenous iron

In elite sports science the use of and debate around whom might benefit from iron supplementation has a long and complex history that is beyond the scope of this thesis. The question of iron deficiency being a candidate mechanism for impaired athletic ability has been studied since the 1970s with interesting early animal work in rats suggestive of a

plausible biological effect and an improvement in performance after iron dextran treatment (190).

Section 3.2.2.1 details how i.v. iron works. In an iron deficient or deplete individual, it is therefore intuitive to think that there may be performance enhancing features. This becomes even more pertinent when one considers that iron loss is greater in elite athletes and iron turnover is potentially disrupted due to hepcidin upregulation during intense training which distorts absorption from the diet. The prevalence in endurance athletes is therefore high (230).

There are mixed results in athletes with regard to iron improving performance with some studies supporting improved performance (231) (oral iron), (230) (i.v. iron) and others demonstrating no changes in CPET variables (192,232) (i.v. iron). A meta-analysis of iron supplementation in non-anaemic iron deficient athletes (NAID) concluded that exercise performance is increased with iron therapy (194). There is also a signal that iron may have performance enhancing effects apart from its obvious haemopoietic agency (231).

With the advent of newer i.v. iron products (Ferric carboxymaltose and iron isomaltoside 1000) alongside the medical community a renewed interest in the sports science world led to studies examining whom might benefit from these products. Studies started to discover what is now well known in the heart failure literature around the non-haematopoietic effects iron can have on fatigue and subsequently quality of life as researchers noted that despite no changes in [Hb] these effects were seen (233).

3.4.9.1 Intravenous iron studies that have measured tHb-mass using CO re-breathing

Garvican et al studied the effect of oral and i.v. iron (iron carboxymaltose (Ferinject®)) in elite distance runners who were iron deficient (ferritin <35 $\mu g.l^{-1}$ and transferrin saturation <20%, or ferritin <15 $\mu g.l^{-1}$ low (LOW)) or iron replete (ferritin <65 $\mu g.l^{-1}$ sub-optimal (SUB)). Iron status and tHb-mass using the oCOR were assessed at one, two, four, six and eight weeks after treatment. They also tested exercise performance on a treadmill for running economy, $\dot{V}O_{2max}$ and $\dot{V}O_{2AT}$ at baseline and again at 8 weeks. They demonstrated that both forms of iron supplementation increased ferritin. [Hb] did not change significantly in any group, tHb-mass increased in the i.v. iron LOW group and was accompanied by an increase in $\dot{V}O_{2max}$ (mean +3.3%) and an improvement in run time to exhaustion (mean +9.3%). They concluded that i.v. iron may enhance endurance capacity if tHb-mass is compromised. tHb-mass is superior for monitoring changes after i.v. iron (230).

Woods et al studied fatigue, mood disturbance and exercise capacity in elite distance runners without anaemia or clinical iron deficiency. They defined this as ferritin levels of 30-100 $\mu g.l^{-1}$ (which is debateable) (Chapter 8). Athletes were randomly assigned to receive 3 doses of ferric carboxymaltose (100mg, which is a very low dose) or a placebo of normal saline over a 4 week period. Each i.v. injection was closely followed by a 3000 m time trial run and a monitored training session consisting of an all-out 400 m time trial and 10 x 400 m training session, performed on consecutive evenings. Iron status, mood and fatigue were examined throughout, with tHb-mass (using the oCOR method) assessed pre and post the intervention period. Mood disturbance and fatigue both improved in the i.v. iron group without changes in performance, [Hb] or tHb-mass. This study supported the possible non-haematopoietic effects of iron on mood and fatigue. The doses used in this

study were however relatively small, presumably due to concerns about iron overload in subjects that were debatably not iron deficient.

The effect of i.v. iron on performance is the subject of Chapter 8 and is discussed further there.

3.4.10 Other agents/drugs

The range of products both tested and in development is large and beyond the scope of this thesis. They have been reviewed elsewhere (234,235). The mainstay of research has focused on stimulating erythropoietin synthesis via the hypoxia inducible factor alpha pathway (HIF1α). HIF stabilisers/activators are compounds that act by mimicking hypoxia and thereby stimulate EPO synthesis. Xenon and argon are also both HIF activators and have both been reportedly used as performance-enhancing agents in the recent years (236).

Towards the end of the 1990s, interest had grown within clinical medicine and the sporting world in using artificial oxygen carriers and perfluorocarbon emulsions. However, neither has been adopted in either setting, probably due to well-recognised adverse effects and ease of detection (237).

Gene therapy is also theoretically possible, but some early reports highlighting significant safety concerns including life-threatening red cell aplasia and extreme erythrocytosis have probably limited its use (238). There are also EPO-mimetic peptides such as '*Peginesatide'* that are not currently in production but are nevertheless candidates for abuse (235).

3.4.10.1 Studies examining the effect on tHb-mass of other agents

A recent study examined the effect of xenon gas on tHb-mass. Seven subjects breathed 70% Fi_{Xe} for 2 min on 7 consecutive days, and EPO, total blood, and plasma volume were measured. Phase III involved assessment of fourteen subjects for EPO, total blood volume, $\dot{V}O_{2max}$, and 3-km running time before and after random assignment to 4 weeks of xenon or sham gas inhalation.

Seven consecutive days of dosing significantly elevated plasma volume (+491 mL; 95% CI 194-789; $P = 0.002$). Phase III showed no significant effect on EPO, tHb-mass, plasma volume, $\dot{V}O_{2max}$, or 3-km running time. The 7 day acute physiological response appeared to be transient, and 4 weeks of xenon inhalation did not stimulate increases in plasma volume or erythropoiesis, leaving cardiorespiratory fitness and athletic performance unchanged (239).

A similar study examined ten consecutive daily doses of intermittent low dose carbon monoxide (CO) gas on tHb-mass, aerobic performance, and peak power exercise tolerance. Eighteen healthy males were randomly assigned to the intervention or placebo (room air). CO inhalation (1.2ml/kg) did not significantly alter tHb-mass (p=0.99), peak oxygen uptake (p=0.59), peak power output (p=0.10), submaximal oxygen uptake (p=0.91),

submaximal RER (p=0.22), lactate threshold (p=0.65), or peak-power exercise tolerance (p=0.60) (240).

The same research group then modified their methodology to increase the number of CO doses given in a day by performing a similar study but instead of once daily dosing for ten days they opted for 5 daily doses for 3 weeks. The subjects were randomly distributed to the intervention group (n = 11) inhaling CO or to the placebo group (n =11) inhaling air instead. They used an equation to calculate the amount of CO to give each time based on the resulting COHb level after the last inhalation that was delivered 4 hours previously. The results demonstrated that this study was safe, achieving almost identical oscillations in COHb over the course of the day (7% after and 4% before each inhalation). tHb-mass significantly increased over the three-week study period (+43.7 ± 32.0 g) and remained at the higher level for three weeks after the completion of the intervention. Maximum power on the cycle ergometer increased immediately and one week after the intervention. $\dot{V}O_{2max}$ tended towards higher values but was not significant. However, there was a significant relationship ($p< 0.001$) between the individual change in tHb-mass and the individual change in $\dot{V}O_{2max}$. The slope of the regression line of ~4 indicated that a change in tHb-mass by 1g resulted in a change of $\dot{V}O_{2max}$ by 4 $ml \cdot min^{-1}$,which closely agrees with previous studies (35). The tHb-mass changes of 4.3% were similar to those seen after an altitude training camp of approximately ~480 hours at ~2500m (241).

3.5 The Athlete's Biological Passport (ABP)

Much of the research in this field has focused on the fight against doping and as it remains difficult to detect all forms of blood manipulation, other strategies were created. The Athlete's Biological Passport (ABP) was introduced in 2008. It was established to detect blood doping in athletes through longitudinal monitoring of erythropoietic markers. Mathematical algorithms are used to define individual reference ranges for these markers for each athlete. The ABP has made a significant impact on the prevalence of blood manipulations in elite sport (64). However, one of the major criticisms of the ABP is that it does not account for diurnal variations in plasma volume and does not contain a measure of tHb-mass.

tHb-mass would be a superior parameter to measure for the ABP as it cannot be masked by changes in PV and is stable over time. It was argued in 2008 that the measurement of tHb-mass should be incorporated into the ABP (31). In an anti-doping case it is the role of an expert to evaluate and determine the possible role of PV variation (64).

The utility of tHb-mass measurements over [Hb] to assess, track and evaluate any changes in the context of elite sport or more importantly within the clinical setting are a major focus of this thesis.

3.6 The perioperative red cell transfusion story

The plentiful, long, and controversial history of red cell transfusion in the perioperative space is beyond the scope of this thesis. However, the story is important within the context of 'patient blood management' (PBM) and how this relates to blood volume measurement, blood conservation strategies and the quest for alternative therapies.

The perils of allogenic red cell transfusion are discussed in section 3.2.1 with only a single unit transfusion having detrimental impacts upon the full spectrum of perioperative outcomes (157,158). Blood products are costly, scarce and fraught with negative consequences. From the apparent risks of incorrectly labelled samples and acute transfusion reactions to the potential negative immunological consequences.

> "There is a glaring lack of evidence to support the hypothesis that PRBC transfusion has clinically beneficial effects on tissue oxygenation and outcome." (242).

For what is an incredibly common therapy it is perhaps somewhat surprising to find a paucity of any outcome data to support its use. Admittedly, as mentioned in Chapter 2 there is a clear biological gradient demonstrating that mortality increases as [Hb] decreases (17–19) so there becomes a point whereby clinicians probably face no choice but to use allogenic blood as a lifesaving therapy.

Any red cell transfusion story within perioperative or critical care medicine must start with the TRICC trial, the study by *Hérbert* et al in 1999 changed transfusion practice within this community. Prior to TRICC usual practice was to aim for a [Hb] of >100g.l^{-1}. In the study the trigger for transfusion in the restrictive arm was 70g.l^{-1} and in the liberal arm it was <100g.l^{-1}. In this non-inferiority study they found that a restrictive strategy was at least as effective and possibly superior (243). Further randomised trials that followed TRICC supported the fundamental message that lower transfusion triggers were non-inferior see Table 3. The patient blood management movement mentioned at the outset of this thesis (Chapter 2) has gone a long way to establishing alternative strategies and for driving research into red cell transfusion.

Study	***Groups***	***Outcome measures***
TRACS (cardiac surgical patients 2010)	Liberal: maintain a haematocrit ≥ 30% Restrictive: strategy haematocrit ≥ 24%	No difference in a composite endpoint of 30-day mortality and severe comorbidity
FOCUS (hip fracture patients 2011	Liberal: < 10 g.dl^{-1} Restrictive: < 8 g.d l^{-1}	A liberal transfusion strategy did not reduce rates of death, or inability to walk independently on 60-day follow-up, or reduce in-hospital morbidity in elderly patients at high cardiovascular risk
Villanuvea et al. (Upper gastrointestinal bleeding 2013)	Liberal: < 9 g.dl^{-1} Restrictive: < 7 g.dl^{-1}	As compared with a liberal transfusion strategy, a restrictive strategy significantly improved outcomes in patients with acute upper gastrointestinal bleeding
TRISS study (patients with septic shock 2014)	Liberal: < 9 g.dl^{-1} Restrictive: < 7 g.dl^{-1}	Among patients with septic shock, mortality at 90 days, rates of ischaemic events and use of life support were similar among those assigned to red cell transfusion at a higher haemoglobin threshold and those assigned to red cell transfusion at a lower threshold; the latter group received fewer transfusion

Table 3: Comparison of landmark papers comparing liberal and restrictive transfusion triggers in critically unwell patients. Taken from (244). Used with permission from RightsLink®, License number: 4924370388569

Evidence for how to manage patients with acute coronary syndrome remains weak; but many believe that a higher threshold may be beneficial, and certainly practice within the UK has commonly aimed for 90–100 $g.l^{-1}$ in this group. The TITRe2 study was set up to attempt to answer the question in the elective cardiac surgical population (where UK transfusion practise varied markedly from region to region). It concluded that there was no difference in the incidence of the primary outcomes, which were: sepsis; an ischaemic event; myocardial infarction; acute kidney injury; or infarction of the gut within 3 months of randomisation. However, a specified post-hoc analysis did show a worrying increase in mortality at 3 months in the restrictive arm (26).

National bodies including the Association of Anaesthetists of Great Britain and Northern island (AAGBI) in the UK and the American Association of Blood Banks (AABB) in the United states alongside the National Institute for Clinical Excellence (NICE) and a Cochrane review from 2016 all recommend broadly similar transfusion triggers for perioperative patients in line with the restrictive arms of the aforementioned randomised trials (244). This is in line with a national drive, certainly within the UK, to decrease blood product usage from National Health Service Blood and Transplant (NHSBT).

Overall progress in this area has been made with initiatives such as the single unit transfusion policy implemented by NHSBT that encourages clinicians to only order a single unit and then recheck the [Hb] prior to further decision making (245). This does not however mean that the best evidence is always adhered to, and future research might look to incorporate tHb-mass measurements into decision making algorithms around when to transfuse red blood cells.

3.7 Blood manipulation and exercise testing in clinical medicine

3.7.1 Red cell transfusion

There are very few studies that have examined allogenic or autologous red cell transfusion on exercise performance within clinical medicine.

Wright et al measured CPET pre and post (two- six days) allogenic red cell transfusion in twenty stable haematological outpatients. There was a mean [Hb] rise of 8.3 $g.dl^{-1}$ after a median of 3 (range 1-4) units of blood. The $\dot{V}O_{2AT}$ increased from a mean (SD) of 10.4 (2.4) to 11.6 (2.5) $ml{\cdot}kg^{-1}min^{-1}$ (p = 0.018), a change of 1.2 ml kg^{-1}. min^{-1}. After correction for the change in [Hb] the $\dot{V}O_{2AT}$ increased by mean (SD) of 0.39 (0.74) ml kg^{-1}. min^{-1} per g dl^{-1} [Hb]. In this study $\dot{V}O_{2peak}$ and peak work rate also increased. The authors stress that these findings were not unanimous and that the small sample size limited wider extrapolation.

A number of small studies in paediatric/young adult patients suffering with beta thalassaemia major examined the effect of red cell transfusion on CPET demonstrating similar improvements in exercise performance after red cell transfusion. *Villa* et al

demonstrated a 25% increase in $\dot{V}O_{2max}$ after 8.26 (±2.31) ml.kg^{-1} of red blood cells (246). *Marinov* studied eleven children and eleven matched controls and found a significant improvement in $\dot{V}O_{2max}$ 28.5 (± 5.0) vs. 36.2 (± 7.1) ml·kg^{-1}min^{-1}); p<0.05 (247).

Interestingly *Grant* et al tested nine patients after transfusion but did not find any significant changes in exercise. However, the mean starting [Hb] in this study was 11.1 g.dl^{-1} so it is possible that any effect may have been diminished.

Benedetto et al studied eighteen adults with thalassaemia major. They measured CPET pre and post 500ml of red blood cells. Haemoglobin increased from 10.5 ± 0.8 g.dl to 12.1 ± 1.2 (p<0.001), $\dot{V}O_{2peak}$ from 1408 to 1546 ml·min^{-1} (p<0.05), and $\dot{V}O_{2AT}$ from 965 to 1024 ml·min^{-1} (p<0.05) (248). The authors do acknowledge that the increase in exercise capacity may have been in part due to an acute PV expansion as the repeat CPET was performed only two hours after the red cell transfusion.

3.7.2 Human recombinant erythropoietin (rHuEPO)

Mancini et al conducted a randomised, single blinded trial of rHUEPO vs. placebo in twenty-three anaemic patients with heart failure (NYHA III or IV). Patients received 15-30,000 IU of rHuEPO or placebo for three months. CPET was performed at baseline and at the completion of therapy. There were significant increases in haemoglobin (11.0 ± 0.5 to 14.3± 1.0 g/dl.-1, p=0.05), $\dot{V}O_{2peak}$ (11.0 ± 1.8 to 12.7 ± 2.8 ml·kg^{-1}min^{-1}, p=0.05) and exercise duration (590 ± 107 to 657 ± 119 s, p=0.004) in the rHuEPO group but no significant changes in the control group.

Palazzuoli et al combined rHuEPO with oral iron in a double blind randomised controlled trial of forty patients with heart failure and anaemia. The treatment arm received rHuEPO (twice weekly for three months) and daily oral iron versus the placebo group who received normal saline s/c injections and oral iron. Exercise parameters improved in the treatment group but not in the placebo group. $\dot{V}O_{2peak}$ increased from 12.8 ± 2.8 to 15.1 ± 2.8 ml·kg^{-1}min^{-1} (p=<0.05); and the $\dot{V}O_{2AT}$, from 9.2 ± 2.0 to 13.2 ± 3.6 ml·kg^{-1}min^{-1} (p=<0 .01). They also saw an improvement in NYHA classification (249).

In a similar study of 41 patients assigned to placebo or s/c darbepoetin alfa for 27 weeks no significant differences were found in $\dot{V}O_{2peak}$ but quality of life was noted to improve in the intervention group (250).

The effect of rHuEPO in patients with chronic renal failure is unquestionably of clinical benefit. The reduction in chronic red cell transfusions and iron overload with all of the risks associated with this approach were virtually eliminated with its introduction in the 1980s. Interestingly, the data shows mixed results regarding changes to $\dot{V}O_{2peak}$ amongst the studies that have examined the response to rHuEPO with CPET (251–255). Notably, the effect on [Hb] (which is well studied and of proven benefit) does not always correlate with improvement in $\dot{V}O_{2peak}$ lending support to the theories of impairment of O_2 transport to the mitochondria and/or alterations in the regulation of the oxidative phosphorylation in the cell that have been invoked as the two potential explanations of the phenomenon.

3.7.3 Iron therapy

There are a wealth of studies in heart failure (HF) examining the effect of iron on exercise capacity, NYHA classification and quality of life amongst other things. The definitive studies are led by one research group who have published multiple high quality randomised controlled trials.

- Briefly, the FAIR-HR study in the NEJM in 2009 examined iron deficient patients with heart failure with or without anaemia. I.v. iron (ferric carboxymaltose (FCM)) vs placebo. Significant improvements were seen with FCM group in the distance on the 6-minute walk test (6MWD) and quality-of-life assessments (179).
- CONFIRM-HF again used the 6MWD to assess exercise performance. It was a multi-centre, double-blind, placebo-controlled trial that enrolled 304 ambulatory symptomatic HF patients. Patients were randomised 1:1 to treatment with i.v. iron, as FCM (FCM, n = 152) or placebo (saline, n =152) for 52 weeks. Treatment with FCM significantly prolonged 6MWT distance at Week 24 (difference FCM vs. placebo: 33 ± 11 m, p=0.002) (256).
- EFFECT- HF used CPET to quantify changes in exercise performance. In this trial they randomly assigned HF patients to either FCM or standard care for twenty-four weeks. The primary end point was change in $\dot{V}O_{2peak}$. Although quality of life improved, exercise capacity did not. However, this was highly sensitive to the imputation strategy in the control group where four patients died during the study and therefore had a vale of zero for $\dot{V}O_{2peak}$. The authors concluded that that further research was required to answer the question (197).

Earlier smaller studies in HF had shown an improvement in CPET variables (257). The IRONOUT-HF study was a double blind RCT comparing oral iron with placebo. In 225 patients they demonstrated no change in exercise capacity using CPET (258) .

The use of i.v. iron in patients with chronic kidney disease is now standard treatment in combination with ESAs. Until recently the research into i.v. iron had lagged behind the research into rHuEPO in this area. The PIVOTAL trial was a large non-inferiority randomised trial of 2141 patients on haemodialysis who were iron deficient and already on an ESAs (259). Patients were randomised to either high dose i.v. iron sucrose (400mg every month) or reactive low dose (targeting a minimum ferritin of 200 μg/L and TSAT of 20%). Both groups targeted a [Hb] of 10-12$g{\cdot}dl^{-1}$. The groups were very similar at baseline and the two arms were well separated with median iron sucrose doses of 264 mg versus 264 mg in the high and low groups respectively. The high-dose IV iron approach was non-inferior with regard to the primary composite outcome of non-fatal myocardial infarction, non-fatal stroke, hospitalisation, or death (and it was subsequently shown to be superior with adjudicated outcomes rather than investigator-reported outcomes). It is postulated that the dose-sparing effect of IV iron therapy on ESAs might contribute to the cardiovascular safety profile of high-dose IV iron (260).

Research continues into the optimum dosing regimen for i.v. iron in these patients. The effect of i.v. iron on exercise capacity has not been adequately addressed in the current literature (261). There are very few clinical trials that have utilised CPET variables as end points in renal patients. A recent review article outlined why it would be progressive to do so (262).

3.8 CPET and tHb-mass specifically

Changes to tHb-mass by numerous blood manipulation techniques were described in sections 3.4.5-3.4.10, many of these strategies used blood volume measurement techniques and specifically the oCOR to assess these changes. In clinical medicine I was involved in the only study (to my knowledge) to measure tHb-mass using the oCOR and then to objectively assess fitness using CPET in perioperative subjects (34).

Previous retrospective work by *Otto* et al had demonstrated that the relationship between preoperative [Hb] and CPET variables was only modest with [Hb] accounting for 9% of the variation in $\dot{V}O_{2peak}$ and 6% of the variation in $\dot{V}O_{2AT}$.

These findings have recently been backed up in a pre-specified sub-study of the prospective cohort trial - the 'METS' study to examine the associations of preoperative [Hb] with preoperative cardiopulmonary exercise testing performance ($\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$) and postoperative complications (263). In this study [Hb] explained 3.8% of the variation in $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$ (P<0.001). Both studies concluded that [Hb] can only explain a small-moderate proportion of the variation in exercise capacity. This aligned with what has previously been demonstrated in the sports science literature (35).

Our hypothesis was that tHb-mass might be a more useful measure of oxygen-carrying capacity and might correlate better with CPET-derived fitness measures in preoperative patients than [Hb]. We measured [Hb] and tHb-mass using the oCOR in 42 patients awaiting elective surgery who then underwent CPET. We found that [Hb] was unrelated to $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$ (r=0.02, p=0.89 and r=0.04, p=0.80, respectively) and explained none of the variance in either measure. In contrast, tHb-mass was related to both (r=0.661, p<0.0001 and r=0.483, p=0.001 for $\dot{V}O_{2AT}$ and $\dot{V}O_{2peak}$, respectively). The tHb-mass explained 44% of variance in $\dot{V}O_{2AT}$ (p<0.0001) and 23% in $\dot{V}O_{2peak}$ (p=0.001). Figure 7 taken from the paper is shown below.

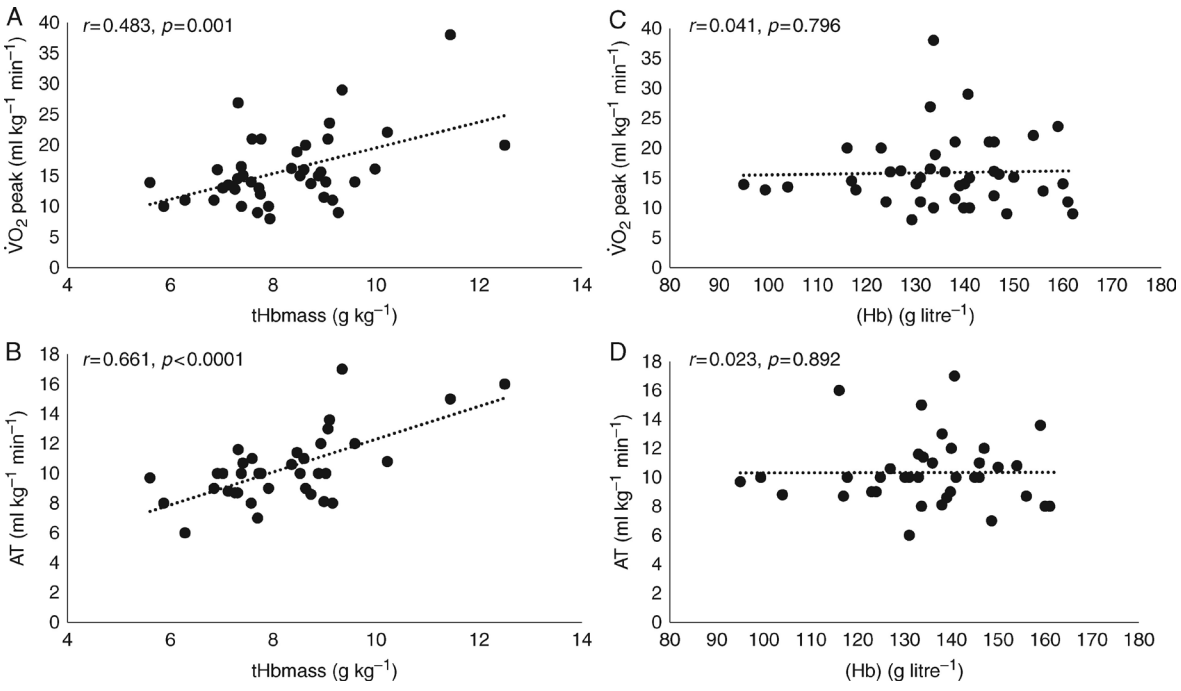

Figure 7: Unadjusted relationship between haematological variables and exertional oxygen consumption. Relationship of $\dot{V}O_{2peak}$ (in millilitres per kilogram per minute) to tHb-mass (in grams per kilogram) (A) and [Hb] (in grams per litre) (C) in 42 patients. Relationship of AT to tHb-mass (in grams per kilogram) (B) and [Hb] (in grams per litre) (D) in 39 patients. AT, anaerobic threshold; [Hb], haemoglobin concentration; tHb-mass, total haemoglobin mass; $\dot{V}O_{2peak}$, peak oxygen consumption. Used with permission from RightsLink® as the author of this work.

The results were in keeping with the only other smaller study to assess tHb-mass and CPET in patients that is discussed in 3.9.2. These findings led towards this thesis and in particular the study presented in Chapter 8.

The only other clinical study examining tHb-mass and CPET was that by *Koponen* et al. They studied 12 men with type 1 diabetes mellitus and 23 (age, anthropometry and self-reported physical activity-matched) controls. They measured [Hb], tHb-mass (using the oCOR), and CPET (using a cycle ergometer) for each subject. Despite similar [Hb] and Hct in both groups, the diabetic group had lower tHb-mass, EV, PV, and BV than the control group. tHb-mass and BV remained lower even after correction of body surface area, and fat free mass. They also found a reduction in $\dot{V}O_{2max}$ in the diabetic group. Of note, they also found a lack of correlation between [Hb] and $\dot{V}O_{2max}$ but a good correlation between $\dot{V}O_{2max}$ and tHb-mass in both groups of r=0.71, p<0.01 and 0.67, p<0.05, respectively. Linear regression demonstrated that the diabetic group were unable to reach similar $\dot{V}O_{2max}$ to the control group for the same tHb-mass (60).

3.9 The measurement of tHb-mass in clinical medicine

When considering the major differences between elite athletes and untrained or significantly physiologically impaired and/or deconditioned clinical subjects it is important to consider the fine balance between oxygen supply and demand. Maximal oxygen consumption $\dot{V}O_{2max}$ or $\dot{V}O_{2peak}$, (the distinction between the two having been explained in section 3.4.1) being the highest measured oxygen consumption during exercise, typically averaged over a 30 second period. In a sense this can represent endurance performance, although as discussed in section 3.4.1 & 3.4.3 there is a lot more to performance than this metric alone. It is according to Fick's equation determined by the oxygen supply of the blood ($\dot{D}O_2$) and the consumption at skeletal muscle:

$$\dot{V}O_2 = Q\,([O_2]_a - [O_2]_v)$$

In words: oxygen consumption $(\dot{V}O_2)$ = blood flow (Q) times the arterio-venous difference. Depending on the individual, one of these factors becomes more important; for elite athletes the rate limiting step is usually oxygen supply whereas in most clinical subjects the ability to use and process the oxygen is the problem.

So, under normoxic conditions maximal exercise capacity relies on [Hb] and CarO. The reason that we believe tHb-mass to be a superior marker to [Hb] has been discussed in section 3.4.2. The inference that the data in healthy subjects or elite athletes can be translated in its entirety to clinical subjects is probably flawed and was one the major reasons behind the original concept of this body of work. In this section studies measuring tHb-mass and blood volume will be briefly reviewed.

3.9.1 Perioperative studies and tHb-mass

We have already discussed our work with elective surgical patient in section 3.8. We could not however find any other studies in the perioperative space that have measured tHb-mass using CO re-breathing. *Slight* et al conducted a study in elective cardiac surgical patients but used a calculation to estimate red cell losses during cardiopulmonary bypass rather than actually measuring red cell mass. Whilst their intentions were noble and their conclusions in line with our study described below in section 3.9.2 the inaccuracies with the calculations of estimated red cell volume in our experience are too great to be clinically useful.

Ivert et all studied postoperative patients who had undergone coronary artery bypass grafting. Blood volume was measured using the alveolar CO method described by *Sjöstrand* (73). They measured BV before surgery, one year after and again at five years. They also measured exercise performance with a symptom limited cycle ergometer. They showed that BV (which was reduced in comparison to subjects without angina), tHb-mass and exercise capacity all increased at one year but exercise capacity declined five years after surgery (264).

A 1993 study using computer modelling and then a modified *Sjöstrand* CO re-breathing method in ventilated intensive care patients is worthy of mention and will be discussed further in Chapter 7.

3.9.2 Other clinical studies measuring tHb-mass

There are a wealth of historical studies measuring BV and tHb-mass in patients dating back to those of Rowntree in the 1900s (105). In 1978 *Bratteby* et al compared the *Sjöstrand* method of CO re-breathing with Cr^{51}. There are numerous studies in pregnancy, renal medicine, endocrinology to name a few that have used Evans blue dye to measure plasma volume (96–100) see section 2.6.2.

In addition to the study of *Otto* et all mentioned in section 3.8 we also studied the relationship between [Hb] and tHb-mass more widely. We studied 109 participants consisting of: healthy volunteers, surgical patients, those with inflammatory bowel disease, chronic liver disease or heart failure. We found a good correlation between [Hb] and tHb-mass in the first three groups (r=0.687–0.871, p<0.001). However, they were poorly related in liver disease (r=0.410, p=0.11) and heart failure patients (r=0.312, p=0.16). Here, haemoglobin mass explained little of the variance in its concentration (adjusted R^2=0.109 and 0.052; p=0.11 and 0.16), whilst plasma volume did (R^2 change 0.724 and 0.805 in heart and liver disease respectively, p<0.0001). This is illustrated in

Figure 8 below.

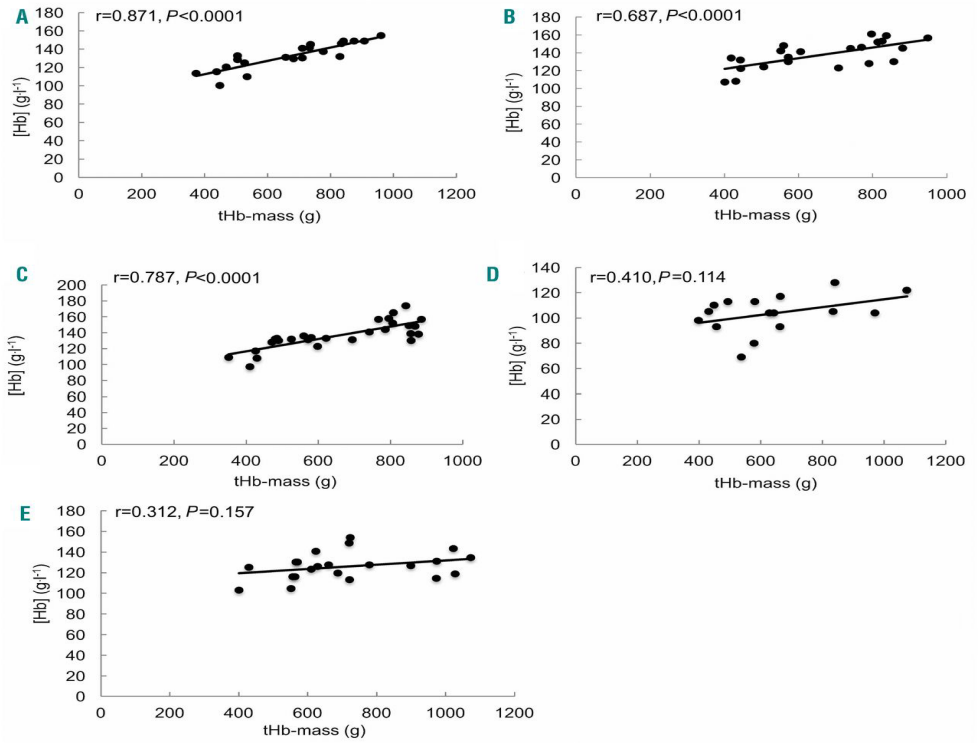

Figure 8: Unadjusted relationship between haemoglobin concentration and total tHb-mass. Healthy controls (A, n=21), patients with IBD (B, n=22), surgical patients (C, n=28), liver disease (D, n=16) and HF (E, n=22). tHb-mass (g): total haemoglobin mass; [Hb] ($g.l^{-1}$): haemoglobin concentration.

These data we believed had wide reaching implications for patients with disorders of plasma volume who may be diagnosed as 'anaemic' whilst having a tHb-mass which is normal or even elevated. Which in certain instances could lead to harm (28).

3.9.2.1 Studies in cardiology measuring tHb-mass using CO re-breathing

Ahlgrim et al attempted to validate the oCOR for use in patients with reduced ejection fraction (EF) heart failure. They found that the standard oCOR resulted in impaired vascular mixing (delayed total body distribution of COHb), they calculated that this resulted in a systematic underestimation of about 1% when calculating tHb-mass. They felt that this error was small and clinically irrelevant. They concluded that the oCOR could safely be used in this patient group (121). Based on our previous work described above we would agree with these conclusions (28).

Due to some concerns around using the oCOR in patients with coronary heart disease due to the added hypoxia and ischaemia from CO a study was performed to ascertain the safety profile in this group. This concluded that cardiovascular function remained normal during exposure to approximately 6% HbCO, indicating that the method is safe to perform on patients with stable coronary artery disease (63).

Diaz-Canestro et al measured BV and tHb-mass in twenty-four subjects with heart failure with preserved ejection fraction (HFpEF) and eighteen healthy age and sex matched control (HC) individuals. Their aim was to assess the relative contribution of PV to other key haematological markers and other blood solutes. They used the modified *Burge* and *Skinner* method that is commonly employed by this group as opposed to the oCOR. They used linear regression to analyse the association of PV with the haematological variables

and other solutes. The main findings demonstrated that PV in HFpEF patients was: (i) negatively associated with Hct and Hb concentration; (ii) negatively associated with blood electrolytes such as K^+ and Ca_2^+, and (iii) positively associated with circulating erythropoietin. None of these associations were detected in HC individuals (265).

The findings concur with our findings in patients with reduced EF heart failure described above on page 58). Interestingly, they discovered a positive linear relationship between tHb-mass and PV in the HFpEF which is contrary to our findings described above. This finding may be due to the fact that the aetiology of anaemia is likely different in HFpEF versus reduced EF heart failure.

3.9.2.2 Other clinical studies using CO re-breathing techniques

There are a wealth of clinical studies examining polycythaemia vera (PolV) many of which used the dye techniques outlined in section 2.6.2. Since the discovery of the JAK2 mutation, measurement of red cell mass has decreased. Whilst some authors still argue for the measurement of red cell mass (266) others argue that a bone marrow biopsy is required. The British Society for Haematology (BSH) guidance from 2018 has the measurement of RCM in its diagnostic algorithm but it is not an essential criterion. They also make a point of noting the variability of access to RCM testing but do not mention using the oCOR (267).

Wrobel et al used the oCOR to track disease monitoring in a single patient with haemochromatosis (61).

Houtman used CO re-breathing (*Sjöstrand* method) to measure tHb-mass and BV in patients with high spinal cord lesions finding that compared to able bodied counterparts they had a lower tHb-mass and BV (268).

3.10 Why measuring blood volume perioperatively might be useful

The problems with [Hb] as a surrogate for the blood oxygen carrying capacity have been outlined in section 2.5 tHb-mass is the principal determinant of total blood O_2-carrying capacity and may provide additional information regarding the clinical status of patients than that provided by [Hb] alone. The major drawback has been the complexity of measuring tHb-mass in the clinic setting and the learning time required to master any of the techniques. As discussed in section 2.6 and specifically in section 2.6.6 the oCOR now offers a technique which is relatively easy to master, cheap to perform, is safe and has a high degree of precision.

Knowledge of the blood volume, tHb-mass, plasma volume and cardiac output are extremely useful in the perioperative and intensive care settings. Often clinicians are left to make decisions around blood, fluid and haemodynamic manipulation with an incomplete picture of these metrics. The dynamic status of these parameters has meant that all monitoring devices used in these settings have drawbacks. One aspiration of Chapter 7 was to collect preliminary data on developing a technique to eventually use in ventilated subjects. At the present time such a method does not exist, and clinicians are often ‘shooting in the dark’ when making assessments of blood volume, plasma volume and any haemoglobin deficit. Transfusion triggers within perioperative medicine and critical care remain controversial (see section 3.6).

The ability to accurately assess perioperative blood loss for example would be extremely useful and it is well known that medical staff are historically inaccurate at doing so (269). Guiding red cell transfusion decisions would be another useful application of the oCOR.

Other clinic-based applications such as the assessment of polycythaemia vera, tracking the response to any haematopoietic therapy such as iron or rHuEPO, would also have wide reaching application. Chapter 5 aimed to assess the utility of the oCOR in patients with chronic liver disease and ascites who are known to have disordered total body water and altered circulatory dynamics. It was hypothesised that they *could* represent a patient group with similarities to critically ill intensive care patients or perioperative patients with gross fluid abnormalities.

In an era of bespoke patient centred treatment plans and shared decision making we need more precise tools to measure parameters that might affect perioperative outcomes or help patients make better informed decisions. Targeting and monitoring blood manipulation therapeutics based on tHb-mass may in the future provide just this.

3.11 Background summary

The background to this thesis has been set out in two parts. In part 1 (in Chapter 2) the composition of blood and its role as an oxygen transporter was explored. The potential superiority of haemoglobin mass measured in grams (tHb-mass) as a metric for measuring the oxygen carrying capacity of the blood compared to haemoglobin concentration ([Hb]) was established (see 2.5.1). The history of blood volume measurement was described (see 2.6) and it was postulated that the modifications made to carbon monoxide re-breathing for the measurement of tHb-mass now make it the superior method for clinical use (see 2.6.6).

Sections 2.7 & 2.8 explored the important features that affect accuracy and precision regarding using the optimised carbon monoxide re-breathing method (oCOR) as described by *Schmidt* and *Prommer*. This method was used throughout the experiments described in the data chapters, five through eight.

In part 2 of the background (in Chapter 3) the importance of anaemia was defined and discussed within perioperative medicine. A major focus of this thesis is around blood volume measurement and then its manipulation potential within the broader area of perioperative medicine. The final data driven chapter (Chapter 8) focuses precisely on this.

In section 3.4 the subject of [Hb] and tHb-mass manipulation amongst athletes and healthy volunteers was explored. The impact of red cell transfusion in clinical medicine has important historical (and current) significance under the remit of ‘patient blood management’ and any other modalities used to manipulate the erythropoietic system must be examined within the context of blood as a therapy. A brief history of perioperative red cell transfusion was outlined in section 3.6.

Blood manipulation studies that objectively measured exercise performance (primarily focused on CPET) were examined in section 3.7. In light of the study questions posed in chapter 8 the literature that had previously examined CPET and the measurement of blood volume was reviewed, this was set out in section 3.8. Section 3.9 then examined studies

that have measured tHb-mass, firstly within perioperative medicine 3.9.1 and then within wider medicine in section 3.9.2.

Section 3.10 explored why perioperative blood volume measurement might be useful in clinical practice.

3.12 Hypotheses

This section will outline how the observations made in Chapter 2 and Chapter 3 led to an overall hypothesis and therefore aim of this thesis. Research aims are set out and finally objectives for each chapter below. I endeavoured to explore the relationship between exercise capacity, anaemia, and haemoglobin content in an elective surgical cohort. The hypothesis being that an improvement in haemoglobin content would increase oxygen delivery and improve exercise performance. A related further hypothesis (not tested in this thesis) is that this improved exercise capacity could lead to an improvement in surgical outcomes.

3.12.1 Overall aim:

To test the hypothesis that increasing tHb-mass before surgery will increase physiological reserve defined using CPET.

3.12.2 Overall objectives:

1. To evaluate the oCOR test under diverse pathological conditions that could represent disorders of plasma volume. In the case of liver disease, carbon monoxide mixing times and hence sampling time periods for the oCOR may be affected.

2. To refine the performance characteristics of the oCOR in healthy volunteers, particularly in relation to interval testing and its safety within short time frames.

3. To test the hypothesis that valid measurements of Hb mass could be made using a "single breath" modification of the oCOR technique.

4. To test the hypothesis that intravenous iron will augment tHb-mass (measured by oCOR) and thereby improve physical fitness (measured using CPET) in patients preparing for surgery.

These objectives are explored in each study below and they each have their own objectives as outlined

3.12.3 Study 1- Chapter 5

The first observation was that the oCOR technique has had very little use in clinical subjects and the methodology applied to healthy volunteers and elite athletes may not hold true for patients; especially with those with significant pathology. Published data had

already brought into question the suitability of the carboxyhaemoglobin distribution time and hence sampling times for the oCOR in patients with poor ejection fraction as described in section 3.9.2.1. This was important for any resultant studies with clinical subjects and would form the basis for future work in a planned interventional study that forms the final data chapter of this thesis.

The hypothesis was that in patients with chronic liver disease (CLD) due to changes in regional (e.g. enteric) and global blood flow dynamics (270–275), and the presence of expanded arteriovenous shunts (276). Patients with CLD may have delayed admixture of CO gas leading to an inadequate rise in carboxyhaemoglobin within the standard sampling time frame of 6-8 minutes described in the classical technique. Another hypothesis was that venous sampling (chosen throughout this thesis due to ease of getting blood from subjects, local expertise, and reliability within the time frame) as opposed to capillary sampling would not unduly change the sampling time period.

The aims were therefore as such:

Primary aim

1. To ascertain if the original oCOR technique resulted in adequate mixing of carboxyhaemoglobin throughout the circulation and could therefore be used to measure tHb-mass in patients with CLD.

    i. Objectives

    - To recruit patients with chronic liver disease and ascites requiring paracentesis electively.
    - To teach them the oCOR technique and carry it out using the standard method and then to prolong the blood sampling time and plot the resultant carboxyhaemoglobin values against time to ascertain if the peak carboxyhaemoglobin level occurred after the traditional 6-8 minutes.

Secondary aim

1. To report the safety and efficacy of the standard carbon monoxide dosing regimen in chronic liver disease (CLD), such that it can be used (and improved) in confidence by others.

    i. Objectives

    - To use the dosing regimen as described in 5.4.3.
    - To analyse the safety and efficacy of the CO dose used.

3.12.4 Study 2- Chapter 6

The second observation was that the oCOR test as described by *Schmidt* and *Prommer* and used widely within the athletic community has a safety stipulation that a period of 12 hours should be left between tests to allow carboxyhaemoglobin levels to return to baseline. I found one study that had attempted to challenge this but their protocol involved activity in all cases to increase pulmonary ventilation and blood flow (126). If the oCOR were to be

used more widely within medicine and particularly within any acute setting, then repeatability within a short time frame would be essential.

The hypothesis was therefore that carboxyhaemoglobin levels would adequately return to a safe enough level to allow repeat testing using supplemental oxygen alone within a 3-hour period. This would comfortably allow repeat testing within a single day.

Primary aim

1. To ascertain whether tHb-mass testing could be safely repeated within 3 hours if carboxyhaemoglobin levels were actively reduced by breathing supplemental oxygen alone ($PROC_A$).
    i. Objectives

    - To recruit healthy volunteers and teach them the oCOR technique.
    - To establish a safe environment to administer high flow oxygen and to monitor patients for any adverse signs of CO toxicity.
    - To ensure that no exercise occurred within the 3-hour time period between testing.

Secondary aims

2. To compare two other carboxyhaemoglobin clearance procedures (gentle cycling alone, or gentle cycling in combination with oxygen administration-procedure B and C ($PROC_B$ & $PROC_C$) respectively.

    i. Objectives
    - As above, to ensure no additional activity outside of the study protocol.
    - To ensure that cycling whilst on high flow oxygen was feasible within the clinical research facility.

2. The final aim was to evaluate the reliability of duplicate tHb-mass measurements in healthy volunteers, who were not the elite or 'sub elite' athletes largely studied by others before.

    i. Objectives
    - To ensure that no recruits to the study were highly trained athletes
    - To apply meticulous technique to each experiment to ensure that both researchers involved had personal competence with the oCOR technique prior to the start of the study.

3.12.5 Study 3- Chapter 7

The third observation was that the oCOR is reliant upon an awake, compliant, spontaneously breathing subject who is able to obey simple commands. This limits its use somewhat in the critical care and perioperative space. We wished to explore if the method

could be adapted to a 'single breath' technique that *may* allow the measurement of tHb-mass in subjects on a mechanical ventilator in the future.

The hypothesis was that delivery of a single carbon monoxide bolus into the breathing circuit of a participant without the need for re-breathing could be used to reliably measure tHb-mass, so long as exhaled gas could be analysed. We thus sought to develop such a technique.

Primary aim

1. To evaluate a method of measuring total haemoglobin mass using a single breath of carbon monoxide gas.

    i. Objectives
    - To build a prototype gas collection system with an engineer.
    - To test this new technique against the established oCOR.

Secondary aims

1. To assess the reliability of this new methodology compared to the standard optimized carbon monoxide (CO) re-breathing method (oCOR).

    i. Objectives
    - To perform the standard oCOR and analyse the typical error within the same subject.

2. Assess the test-retest reliability of the oCOR in this cohort.

    i. Objectives
    - To use the cohort to ensure that researcher quality assurance is up to standard with regard to test re-test reliability.

3.12.6 Study 4- Chapter 8

The final observation was that anaemia is associated with adverse perioperative outcomes and impaired physical fitness. Correction of anaemia before surgery through intravenous iron therapy is becoming common practice.

I set out to explore the relationship between exercise capacity, anaemia, and haemoglobin content in an elective surgical cohort. Specifically, I aimed to test the hypothesis that augmenting haemoglobin through intravenous iron therapy would improve preoperative physical fitness (cardiopulmonary exercise testing (CPET) variables).

Primary aim

1. To evaluate the feasibility of performing a CPET, measuring tHb-mass and then administering intravenous iron and repeating a CPET prior to surgery in patients being considered for elective surgery at University Hospital Southampton (UHS).

    i. Objectives

- To recruit perioperative patients via the clinical perioperative anaemia and CPET services with enough time to administer i.v. iron and repeat a CPET test with a minimum of 10 days between tests and before their planned surgery date.
- To safely administer i.v. iron after ensuring that they met the clinical criteria for the UHS perioperative anaemia service.

Secondary aims

1. To compare CPET parameters, oxygen consumption at the anaerobic threshold ($\dot{V}O2_{AT}$) and peak oxygen consumption ($\dot{V}O_{2\ peak}$) before and after the administration of intravenous iron.

    i. Objectives
    - To perform a standardised CPET protocol with the same ramp each time.
    - To blindly analyse CPET by 2 independent researchers.

2. To evaluate the relationship between [Hb] and tHb-mass and CPET derived exercise variables and to assess any incremental change in haemoglobin concentration [Hb] and total haemoglobin mass using the optimised carbon monoxide rebreathing technique (oCOR).

    i. Objectives
    - To teach patients the oCOR technique and then perform it after CPET test so that CO does not affect the ability to perform a maximal CPET.
    - To draw blood twice more prior to the final visit to analyse changes in [Hb] after i.v. iron.

Chapter 4 - Methods

This chapter outlines some of the common methodology found throughout the four data driven chapters (5-8). Each individual data chapter will have its own methods section but will refer back to this chapter for any common methodology. There will also be an explanation of some of the common analytical approaches taken throughout the thesis including a brief description of the Bland Altman technique (277) and Hopkins' (83) reliability method for the measurement of 'typical error' when comparing test re-test reliability in the context of tHb-mass measurement using the oCOR.

4.1 Measurement of tHb-mass using the Optimized Carbon Monoxide Re-Breathing Method (oCOR)

The use of carbon monoxide (CO) to determine tHb-mass was first proposed in the late 1800s, with refined techniques being published 100 years later (278) (see 2.4.2). In 2005, *Schmidt* and *Prommer* reported a simpler and faster technique (described in detail below), which also required less blood sampling (5). It was applied almost exclusively, however, in the fields of athletic physiology, and thus failed to come to the attention of the bulk of the broader clinical/medical community. In recent times this has changed (section 2.9).

The following description is adapted from the original description by *Schmidt* and *Prommer* and unless otherwise stated is the method used to measure total haemoglobin mass throughout this thesis (5). The author of this thesis spent a week in the laboratory of Professor *Schmidt* at the University of Bayreuth, Germany to learn the techniques as described in detail below. The description below is in a stepwise fashion, it includes the modifications that we used in our laboratory.

COHb concentration in blood was measured before and after- two minutes re-breathing a known CO volume (dose depending on sex, weight, body habitus, and if the subject was 'trained' or 'untrained' (see discussion in Chapter 5) and [Hb]). In the main, doses for healthy volunteers were between 0.7 $ml.kg^{-1}$ and 1.0 $ml.kg^{-1}$. In patients, the doses were from 0.5 $ml.kg^{-1}$ - 1.0 $ml.kg^{-1}$. Individual chapters will describe the exact calculations and the rationale for using different dosing regimens.

1. Each participant was seated for 15 min to allow stabilisation of plasma volume (PV).

2. During this time the glass spirometer was flushed with 100% oxygen and the 3-litre gas reservoir bag (Intersurgical, Berkshire, UK) was filled with 100% oxygen (see Figure 6). The glass spirometer was checked for any residual water from the cleaning process. The stopcocks were all checked manually for leaks in the system. The spirometer used was a bespoke spirometer specifically designed for haemoglobin mass measurement using the optimized carbon monoxide re-breathing method (Spico-CO Respirations-Applicator, Blood Tec, Germany).

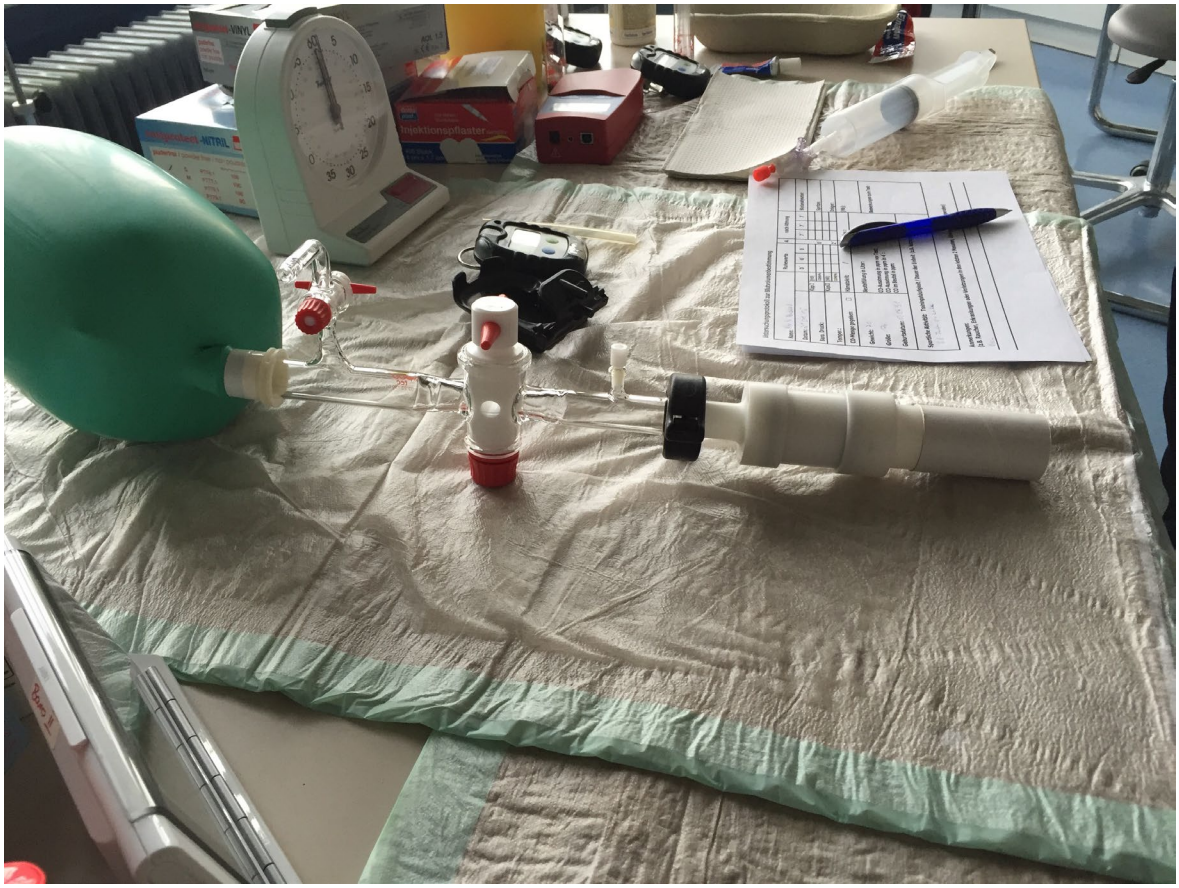

Figure 9: The Spico-CO Respirations-Applicator, Blood Tec, Germany. The green 3-litre reservoir bag is filled with 100% oxygen. The 2 stopcocks are both shown in the closed position. The cardboard mouthpiece is visible distal to the soda lime canister.

3. The plastic receptacle that the mouthpiece attached to was filled with a new filter and medical grade 'soda lime' - 'Spherasorb'® (Intersurgical, Berkshire, UK). Approximately 10 grams filled it completely with room to screw it securely shut and have a compact arrangement of granules. For each experiment the investigator consistently filled to the edge of the bottom screw thread line. The black external clamps were placed firmly onto the system and once again it was flushed with 100% oxygen to check for any leaks.

4. An intravenous canula (20G BD Venflon™, NJ USA) was placed in a large antecubital fossa vein. A Catheter extension tube (PUR) with 'Autoflush' and clamp ('Octopus 1' Vygon, Brussels, Belgium) was placed and it was established that it easily bled back prior to starting the procedure. Laboratory haemoglobin if not known was checked at this point.

5. A sample of venous blood was taken to measure haemoglobin concentration using a Haemocue™ machine (Radiometer Copenhagen, Denmark).

6. The dose was calculated and measured out using a Braun 100ml B BRAUN OMNIFIX solo syringe (Melsungen, Germany). Two syringes were prepared (in case one was spilt en-route to the laboratory) with a sealed 3 way tap on the end and an adapter built to fit into the bespoke spirometer made for the procedure by Blood Tec (Spico-CO Respirations-Applicator, Blood Tec, Germany). They were prepared outside in the gas cage at University Hospital Southampton NHS

Foundation Trust. A piece of silicone tubing was used to draw gas from the regulator into the syringe, each syringe was filled to 100ml 3 times and passed back through the tubing, on the 4th time 100mls of CO gas was collected. The exact dose was then prepared immediately prior to delivery to the patient to avoid any gas leakage. The small amount of excess CO gas was deposited out of a laboratory window.

7. Baseline lung end tidal concentration of carbon monoxide was measured prior to the start of the test using a CO gas analyser (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany). Participants fully exhaled to residual volume, blowing directly onto the CO analyser using a mouthpiece supplied by Dräger. This was repeated 3 times and the mean value was used.

8. Baseline venous blood samples were drawn via Na-heparinized syringes (RAPIDLyte, Siemens Healthcare Diagnostics Inc, USA). The samples were analysed using a laboratory blood gas analyser (Radiometer, ABL800 FLEX) for carboxyhaemoglobin percentage values. Each sample was analysed three times within 1 hour of collection. The analyser used in all studies was subjected to regular maintenance and quality control checks; the accuracy of which has been evaluated elsewhere (122). The mean of the 3 values was used in the calculation of tHb-mass.

9. The carbon monoxide re-breathing procedure was started immediately after blood was drawn. A plastic nose clip with foam supports on the nose bridge was placed on the subject's nose (Vacumetrics 'Snuffer' foam padded disposable nose clips, Vacu Med, California, USA). The patient then took a maximal breath in and exhaled to residual volume. They attached their mouth to a cardboard mouthpiece (Vacu Med, California, USA) and maximally inhaled, as this occurred the investigator flushed the CO gas into the spirometer and an assistant started the stopwatch (2 timers started). The spirometer contained 10 grams of 'soda lime' carbon monoxide scrubber (Intersurgical, Berkshire, UK).

10. The oxygen/carbon monoxide mixture was then held in for 10 seconds then exhaled into the spirometer where it was re-breathed with normal tidal breathing for 1 minute and 50 seconds into the closed circuit *via* the spirometer. During this time the CO detector was held closely to the spirometer to detect any leaks in the system.

11. At the end of the 2-minute period (10 seconds breath holding and 1 minute 50 seconds re-breathing) the subject took a maximal inhalation and then maximally exhaled into the gas collection bag attached to the spirometer (Intersurgical, Berkshire, UK).

12. At 4 minutes post inhalation the subject once again breathed out into the CO detection device (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) in order to determine the end tidal CO concentration and thereby the amount of CO exhaled after disconnecting the patient from the spirometer, that will not have been absorbed into the blood.

13. At 6 minutes blood was drawn from the indwelling cannula and processed 3 times in the same way as above.

14. At 8 minutes blood was drawn from the indwelling cannula and processed 3 times in the same way as above.

15. At the end of the procedure the cannula was removed and the subject left the laboratory.

- [Hb] and haematocrit values were measured using HemoCue (HemoCue AB, Radiometer, Sweden) and blood gas analyser (Radiometer, ABL800 FLEX, Copenhagen) respectively.

4.1.1 Cleaning of the equipment for the measurement of total haemoglobin mass using the Optimized Carbon Monoxide Re-Breathing Method (oCOR)

After each use the single use items were disposed of safely in clinical waste bins (mouthpiece, soda lime, filter). The spirometers were dismantled and rinsed using warm water. It was then soaked for 30 minutes in Milton™ tablets (Laboratoire Rivadis, France). The spirometers were then left overnight to dry. The following day they were reassembled flushing out any excess water using high flow oxygen. Filters were replaced for each experiment (Blood Tec, Germany|). Soda lime was not inserted until immediately before a new experiment to prevent it drying out.

4.2 Calculation of tHb-mass

Total haemoglobin mass (tHb-mass) was calculated using a specifically designed excel spreadsheet (Microsoft Excel 2011 for Apple Macintosh) using the formula:

- tHb-mass (g) = K x MCO (ml) x 100 x (Δ%COHb x 1.39)-1.
- K = barometric pressure x 760^{-1} x [1(0.003661 x temperature)].
- MCO = COadm – (COsystem + lung (after disconnection) + COexhaled (after disconnection).
- COadm = CO volume administered into the system.
- COsystem + lung (after disconnection) = CO concentration in spirometer x (spirometer volume + remaining volume in the lung after disconnection).
- COexhaled (after disconnection) = end-tidal CO concentration x alveolar ventilation x time.
- Δ%COHb = difference between baseline %COHb and %COHb post CO administration (average of 6- and 8-min %COHb values).
- 1.39 = Hufner's number (constant) (ml CO x g Hb-1).

Residual volume and alveolar ventilation were calculated according to "Standardized lung function testing. Official statement of the European Respiratory Society" (279). CO concentration is in parts per million (ppm).

Blood volume (BV), plasma volume (PV) and red cell volume (RCV) were calculated from mean corpuscular haemoglobin concentration (MCHC), [Hb] and tHb-mass, as below:

- BV (ml) = tHb-mass (g)/[Hb] (g. dl-1) • 100
- RCV (ml) = tHb-mass (g)/MCHC • 100
- PV (ml) = BV – RCV

The volume of exhaled CO gas was not measured routinely. This was in keeping with the original description (5) and updated description by *Schmidt* and *Prommer* (6). Their theoretical calculation of an exhalation of 0.16ml min^{-1} for CO makes very little difference to calculated tHb-mass values. Due to practical difficulties, we therefore calculated CO exhalation by measuring end - expiratory CO concentration and then estimated minute ventilation.

To account for CO loss to myoglobin we used the calculation as described in the original paper in 2005 by *Schmidt* and *Prommer* (5) of multiplying the dose administered by 0.014. The modification as described in their 2007 paper has subsequently been found to overestimate loss to myoglobin (personal communication with Professor *Schmidt* 2019) (6). As the calculation was applied consistently and the actual difference is extremely small it was felt to be of little significance to much of the work of this thesis. It is mentioned here for completeness and for transparency about how the calculations were performed.

4.3 Important statistical principles used throughout the thesis

4.3.1 The Hopkins method of assessing reliability

Reliability is the reproducibility of values of a test, assay or other measurement in repeated trials on the same individuals (83). Reliability is important in any experiment. When measuring total haemoglobin mass and attempting to modify experimental techniques, assessing measures of reliability are vital. Reliability becomes important when one wishes to ascertain trends over time within the same individual. For example, assessing blood loss in an intensive care patient over the course of minutes to hours or assessing athletes for possible blood doping using the athlete's biological passport (ABP) (section 2.3.1).

The main measures of reliability are within-subject random variation, systematic change in the mean, and retest correlation.

4.3.1.1 Within subject variation

A statistic which captures the random variability of within subject variation is easily explained by considering the weight of an individual. Suppose a weight is taken in kg 10 times and the values are 72.0, 72.3, 72.8, 73.1, 73.0, 72.9, 72.8, 72.3, 72.2 and 73.2. The within subject standard deviation of these values is also known as the standard error of measurement. The main noise in this case likely comes from the weighing scales but there is also biological variation. This standard error of measurement is akin to the 'typical error'.

It is important to have an idea of typical error that is unaffected by changes in the mean when looking at the difference between 2 trials on the same participant. The values for the change score yield such an estimate. Simply divide the difference score between the 2 measurements by √2.

An example would be as follows:

Participant	Trial 1 - tHb-mass	Trial 2 – tHb-mass	Difference scores
1	968	952	-16
2	887	880	-7
3	679	685	6
4	900	906	6
5	784	769	-15

Table 4: Example of the Hopkins method for calculating test re-test reliability

The SD of these difference scores = 10.8 and the standard error of the mean is 4.831.

10.8/√2 = 7.64. But as a coefficient of variation equates to 0.92. Which would be highly reasonable for the measurement of tHb-mass using the oCOR and well within the standards reported from the leading laboratories performing this test.

For many measurements in science the typical error gets bigger as the value of the measure gets bigger. For example, several trials might yield a tHb-mass with a mean and typical error in a healthy male of 1000 ± 10g whereas an elderly female performing several tests might have a mean and typical error of 450g ± 5g. Although the absolute values of the typical error are different, the values expressed as a percentage of their respective means are similar: 1.0% and 1.1%. This form of the typical error is a coefficient of variation.

It is sometimes more applicable to every participant than the raw typical error. As a dimensionless measure, it also allows direct comparison of reliability of measures irrespective of calibration or scaling. This is the typical percentage error.

Hopkins argues that the 'typical error' ought to be adopted as the standard measure of reliability and much of the literature that has compared different measurement techniques for measuring blood volume using carbon monoxide re-breathing have used the methodology that Hopkins described in 2000. It is therefore worthy of basic explanation (83).

The Hopkins method for typical error calculation is described below:

"A statistic that captures the notion of random variability of a single individual's values on repeated testing is the standard deviation of the individual's values. This within-subject standard deviation is also known as the standard error of measurement. In plain language, it represents the typical error in a measurement." (83)

Following this approach, the test-retest typical error is computed as follows:

1. Computed difference in total haemoglobin mass assessed in repeated tests.
2. Computed standard deviation of the difference in total haemoglobin mass assessed during the two tests.
3. Mean standard deviation divided by the square root of 2.

This is the method used by Professor *Schmidt's* group *(personal communication, 2019)*. A test-retest typical error of around 2% is acceptable in the difference in tHb-mass between repeat tests as described in the tables available at www.sportsci.org, written by Will G Hopkins (83). It should be noted that the confidence limits for the typical error are derived from a chi-squared distribution. For small degrees of freedom, the upper limit tends to be skewed out relative to the lower limit. *Tate* and *Klett* provided an adjustment that reduces the skewness by minimising the width of the confidence interval, although it is then not an equal-probability interval. With only slight adjustment the *Tate* & *Klett* limits can be represented conveniently by a single factor (see Table II in (280)).

The Hopkins description of using the coefficient of variation in this way has been used in a number of the data chapters in this thesis when comparing test-retest measurements. This is in keeping with the majority of research that has used the oCOR test.

In 1983 *Bland* and *Altman* described in detail a new measure of reliability - 'limits of agreement' which has become eponymously known as the 'Bland-Altman method' and is visually displayed using 'Bland-Altman plots'. Their method is briefly described below.

4.3.2 Bland-Altman

In their seminal work they outlined how previously scientists had often incorrectly used correlation coefficients to compare measurement techniques; be these 2 trials on the same individual using the same equipment or the comparison of a new measurement technique using different equipment or assay process. In the paper (281) and a subsequent one (277) they outline the problems with using correlation coefficients. By plotting the differences in values between the tests against the mean values of each test it is possible to investigate the relationship between the true value and the measurement error.

Bias can be calculated by this and is estimated by the mean difference and the standard deviation of the differences. If there is consistent bias, then the value can be added or subtracted; hence a test can be consistently biased, and this allows one to mathematically account for this. Neither method however tells you what the true value actually is.

4.4 Comparison of the Hopkins and Bland-Altman methods.

There are reasons one or other method is preferable. Differing studies within this thesis have used both methods and both have value. Visually Bland-Altman plots are useful to simply explain reliability between trials. There are, however, some advantages to using the Hopkins method for certain aspects of reliability studies such as those contained within this thesis.

One pertinent issue with using limits of agreement for smaller studies is that the values of the limits of agreement are dependent upon the sample size of the reliability study from which they are estimated. So, when comparing the magnitude of the limits of agreement between two studies one must account for the numbers of degrees of freedom between the studies. This does not occur when one uses the typical error as the sample size does not influence the expected value.

When looking for changes between trials within a single subject, limits of agreement also have some disadvantages. Using 95% confidence limits to represent the precision of the estimate of a population is not necessarily the basis for using 95% confidence limits for the difference in an individual's score. For analysing subtle changes of an intervention between two trials; for example, a change in tHb-mass after an altitude camp or after a red cell transfusion one might reasonably look for a rise of around 50-80g which in a man might be only a 5-7% change (based on 1000g of tHb-mass). If the limits of agreement are 95% ± 7% then this magnitude of change might be ignored when actually it makes a clinically significant impact. However, limits of agreement are useful for test-retest scenarios if appropriate 95% confidence limits are used for that individual. This is relevant in both clinical medicine and elite sporting performance where winning or losing often comes down to a fraction of a percentage and the concept of the aggregation of marginal gains that gained popularity around 2010 comes into play. For example, a 3% improvement in peak oxygen consumption may be the difference between being able to fight off a serious post-operative complication like a chest infection.

4.5 Summary

Chapter 4 has outlined the common methods applied throughout the following data driven chapters of this thesis. Section 4.1 provided a step-by-step guide to measuring blood volume and tHb-mass using the oCOR. The calculation of tHb-mass is outlined in section 4.2. The common statistical methodology is explored in section 4.3 with a comparison of the two methods outlined in section 4.4.

Chapter 5 - Application of the optimized carbon monoxide rebreathing method for the measurement of total haemoglobin mass in chronic liver disease

This chapter has been published in the journal ***Physiological Reports*** (2) (Appendix E)

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5.1 Chapter in context

This chapter documents a methodological study measuring blood volume and total haemoglobin mass using the oCOR in a group of patients with chronic liver disease and ascites. As a group known to have disordered total body water and altered circulatory dynamics it was hypothesised that they *could* represent a patient group with similarities to critically ill intensive care patients or perioperative patients with gross fluid abnormalities. As an outpatient group attending hospital for elective paracentesis, it was felt that they would represent a more stable group to study prior to using the technique in the perioperative or intensive care setting.

Using this cohort also allowed us a consistent and reliable way to recruit patients to this novel clinical study. Two particularly important overarching themes of this thesis were tested in this study. Firstly the applicability of the oCOR in clinical subjects is still in its infancy with only a handful of clinical studies having been performed, many of which have been done by myself and *Dr James Otto* (3,28,34). Other clinical studies are referenced in this chapter and in section 3.9 . Section 2.8.3 discusses the importance of circulatory mixing times after CO inhalation and the effect this has on calculated tHb-mass in different circumstances. In this chapter I have explored this and referenced some recent complimentary work in this area (120). Individual cohorts with differences in cardiorespiratory function ought to undergo similar experiments to ensure that the methodology originally described by *Burge* and *Skinner* (74) and modified by *Schmidt* and *Prommer* (5) is applicable in that particular cohort. As ever, a balance needs to be struck between precision, safety, and practicality for any novel clinical test.

5.2 Abstract

Background: Anaemia is common in liver cirrhosis. This generally infers a fall in total haemoglobin mass (tHb-mass). However, haemoglobin concentration ([Hb]) may fall due to an expansion in plasma volume (PV). The ‘optimized carbon monoxide rebreathing method’ (oCOR) measures tHb-mass directly and PV (indirectly using haematocrit). It relies upon carboxyhaemoglobin (COHb) distribution throughout the entire circulation. In healthy subjects, such distribution is complete within 6-8 minutes. Given the altered circulatory dynamics in cirrhosis, we sought in this pilot study, to assess whether this was true in cirrhosis. The primary aim was to ascertain if the standard timings for the oCOR

were applicable to patients with chronic liver disease and cirrhosis. The secondary aim was to explore the applicability of standard CO dosing methodologies to this patient population.

Methods: Sixteen patients with chronic liver parenchymal disease were studied. tHb-mass was determined using the standard oCOR technique before elective paracentesis. Three subjects had an inadequate COHb% rise. In the remaining 13 (11 male), mean ± standard deviation (SD) age was 52 ± 13.8 years, body mass 79.1kg ± 11.4 kg, height 175 ± 6.8 cm. To these, mean ± SD dose of carbon monoxide (CO) gas administered was 0.73ml·kg^{-1} ± 0.13ml·kg^{-1}COHb values at baseline, 6 and 8 minutes (and '7-minute value') were compared to those at 10, 12, 15 and 20 minutes after CO re-breathing.

Results: The '7-minute value' for median COHb% (IQR) of 6.30% (6.21%-7.47%) did not differ significantly from those at subsequent time points (8 mins: 6.30% (6.21%-7.47%), 10 mins: 6.33% (6.00%-7.50%), 12 mins: 6.33% (5.90%- 7.40%), 15 mins: 6.37% (5.80%-7.33%), 20 mins: 6.27% (5.70%-7.20%)). Mean difference in calculated tHb-mass between minute 7 and minute 20 was only 4.1g, or 0.6%, p=0.68. No subjects reported any adverse effects.

Conclusions: The oCOR method can be safely used to measure tHb-mass in patients with chronic liver disease and ascites, without adjustment of blood sample timings. Further work might refine and validate appropriate dosing regimens.

5.3 Introduction

The concentration of haemoglobin in the circulation ([Hb]) is determined by its total circulating mass (tHb-mass) and the plasma volume (PV) in which it is suspended. Anaemia, defined as a reduction in [Hb] to <120 g·l^{-1} in non-pregnant females and <130 g·l^{-1} in males (152), is common in patients with chronic liver disease (CLD), with a reported prevalence of 75% (282). To this, a fall in tHb-mass (due to gastrointestinal blood loss, haematinic deficiency, haemolysis and bone marrow suppression) may contribute (283). Equally, a rise in PV could contribute to anaemia. However, this element is often ignored in clinical practice, largely due to difficulties in measuring PV (28).

tHb-mass can be measured directly by the methods of *Burge* and *Skinner* (74), modified by *Schmidt* and *Prommer* (the optimized carbon monoxide (CO) re-breathing method (oCOR)) (5) (see Chapter 4). Here, binding of a known volume of CO to Hb, and measurement of carboxyhaemoglobin concentration (COHb) allows tHb-mass to be measured and, using this and knowing haematocrit, PV to be derived. Predominantly applied to monitor athletes' responses to altitude training (13), it has more recently been used in clinical medicine (28,34,60,61,121,265). Data regarding oCOR application to CLD patients were limited (61), but we demonstrated a poor relationship between [Hb] and tHb-mass in CLD (r=0.410, p=0.11) with PV explaining much of the variance in [Hb] (28).

The accuracy of the oCOR technique depends on the completeness of COHb mixing in the circulation after CO inhalation (8,113) and this appears complete by 8 minutes (5,13). As such, measurement of COHb% at 6 and 8 minutes (averaged to yield the '7 minute' value) is recommended (5), and was used in our study. However, mixing is delayed in disease states such as cardiac failure (121) and polycythaemia (120). Admixture might be slowed in CLD due to changes in regional (e.g. enteric) and global blood flow dynamics (270–275), and to the presence of expanded arteriovenous shunts (276).

Meanwhile, the applicability of the standard oCOR dosing regimen has not been reported in CLD patients. The desired absolute rise in COHb from baseline- 4.0% ~ 6.5% (ΔCOHb%) with peak levels <10% (3,125,126) represents a tradeoff between precision and safety. Cirrhosis leads to higher baseline COHb% due to elevated levels of inducible heme oxygenase (HO-1) driving increased heme conversion to biliverdin, with associated breakdown of CO production (272). Because most haemoximeters estimate COHb% to only a single decimal place, resulting smaller increments in COHb% could result in lower precision of measurement (122,125). Meanwhile, COHb >15% can be associated with headaches and changes in visual evoked potentials (127).

Traditional oCOR methodology distinguishes between 'trained' (athlete) and 'untrained' (non-athlete, but healthy) subjects with 1.0 $ml \cdot kg^{-1}$ and 0.8 $ml \cdot kg^{-1}$ doses of CO respectively being safely used for men and 0.7 $ml \cdot kg^{-1}$ and 0.6 $ml \cdot kg^{-1}$ doses for women (5). Of note, CLD patients are likely to be significantly more deconditioned than the 'untrained' group in the original paper (5) and, as such, further dose reduction might be indicated. 'Appropriate dose reduction' has also been commended 'for patients with significant anaemia and or/morbid obesity' although these states were not clearly defined (8,125). Further, when body mass index is $>30 kg \cdot m^2$, the use of ideal body weight (IBW) is advocated when calculating the CO dose to be administered (120). Such elements pose problems for the study of CLD patients. Firstly, up to three quarters may be anaemic (282) which, if due to low tHb-mass, could suggest the need for a lower CO dose. The fact that [Hb] relates poorly to tHb-mass in CLD (28) further confounds CO dose-estimation. Finally, ascites and/or oedema may distort measured body weight and prevalent sarcopenic and/or low body fat mass confound 'ideal body weight'. The potential for delivering COHb% values beyond those desirable is therefore high.

In this prospective, pilot observational clinical study, we sought to address both these issues. We sought primarily to ascertain if adequate circulatory mixing of CO occurs within the standard time frames (5). Our secondary aim was to explore the applicability of standard CO dosing methodologies to this patient population.

5.4 Materials and Methods

The study was conducted at University Hospital Southampton NHS Foundation Trust between May 2015 and October 2017. Ethical approval was granted by the London - Camden and Kings Cross Research Ethics Committee (REC reference: 13/LO/1902), with an amendment allowing venous sampling for up to 20 minutes after CO inhalation being likewise approved. This multi-site ethics application and amendment was requested by myself as well as local R&D and clinical research facility approvals. Written informed consent was obtained from all participants.

5.4.1 Subjects

Sixteen patients with CLD and diuretic-refractory ascites were recruited by the clinical team from those undergoing elective day-case paracentesis. Potential participants were approached directly in outpatients or at a visit for elective paracenteses to be given a patient information leaflet about the study. This was achieved via the liver failure consultants, speciality registrars and specialist nursing teams. We have previously

published data derived from venous samples at 6 and 8 minutes after CO inhalation in 16 subjects (28). In 5 of these, there was no venous sampling after 8 minutes. We thus obtained full data (venous sampling at 6 & 8 minutes then additionally at 10,12,15 and 20 minutes after CO inhalation) in an additional 5 patients and report these data for the total of 13 subjects (Table 5) Three subjects were excluded due to inadequate increases in COHb (see Table 6). The aim was to recruit enough subjects for a minimum of 10 complete datasets, this was based on previous work (6,113). As this was a pilot observational study we wanted to recruit enough subjects to show proof of concept but also to align with previous experiments performed by professor Schmidt's group who have previously used around 10 subjects when checking mixing times in a certain population groups. We therefore wished to recruit slightly above this to account for any issues with testing and incomplete data sets.

The rationale for selecting patients with chronic liver disease and ascites was both scientific and pragmatic. We had demonstrated that we could recruit this group of outpatients during a previous study (28) and we had a good working relationship with the clinical team, as such the approach and access was relatively straight forward. We also had a plausible biological mechanism as to why they may not have the same mixing times as a fit healthy subject. The nursing staff had a long time with these patients to discuss any aspects of the research study as they had to stay in for hours whilst their ascites drained. From a scientific perspective another research group had already investigated measuring tHb-mass in patients with low ejection fraction heart failure (the other obvious choice to study based on our previous research) and we did not want to repeat this work.

Patient number	**Age**	**Gender**	**Height (cm)**	**Weight (kg)**	**BMI**	**[Hb] ($g \cdot l^{-1}$)**	**tHb-mass (g)**	**CO dose in ml**	**CO dose (ml CO/kg body weight)**	**ΔCOHb%**
1*	76	F	165	81	29.8	98	396	50	0.6	7.2
2	48	M	182.88	96.3	28.8	104	628	48	0.5	4.5
3	47	M	180.3	73.5	22.6	105	431	44	0.6	5.5
4	51	M	176.2	80.2	25.8	117	664	64	0.8	5.8
5*	52	F	161	58.7	22.6	113	581	40	0.7	4.0
6	45	M	182	91.5	27.6	93	662	64	0.7	5.9
7	45	M	175	66.9	21.8	132	590	52	0.8	4.5
8	70	M	170	76	26.3	101	518	46	0.6	5.0
9	43	M	177	83.3	26.6	144	883	60	0.7	4.2
10$	24	M	175.3	63.2	20.6	184	799	64	1.0	4.9
11	47	M	183.5	90	26.7	100	648	72	0.8	6.7
12	58	M	170	78	27.0	118	787	70	0.9	5.5
13	70	M	176.5	90.2	29.0	121	828	70	0.8	4.7
Mean ± SD. Aside from [Hb] Median [IQR]						113.0 [100.5-126.5]	647.0 ± 148.9	57.23 ± 11.00	0.73 ± 0.14	5.20% ± 0.96%

Table 5: Haemoglobin Carbon Monoxide Measures * Female patient, $ Patient had polycythaemia rubra vera (PCV) and requires regular bloodletting for treatment. CO (carbon monoxide); [Hb] (concentration of haemoglobin in venous blood); COHb% (percentage of haemoglobin in the form of carboxyhaemoglobin).

5.4.2 Optimized Carbon Monoxide Re-Breathing Method (oCOR)

tHb-mass was determined by oCOR on the morning immediately prior to abdominal paracentesis (5) (see Chapter 4) Venous COHb% was measured before and after 2 min rebreathing a known CO volume (0.5 to 1.0 $ml \cdot kg^{-1}$, with dose determined according to fitness, frailty, gender and [Hb] as described below). We sought a minimum ΔCOHb% of 4.0% to avoid inaccuracies in tHb-mass measurements (see introduction).

tHb-mass was calculated using the difference in COHb% in blood samples obtained before and (averaged) 6 and 8 minutes after the inhalation of the CO bolus, and using blood samples drawn between minutes 8 and 20. For these calculations, CO diffusion to myoglobin and CO exhaled until the respective time point were considered as previously published (6) (e.g. in the patients studied for min 20, CO volume diffused to myoglobin ranged from 1.4 to 2.9 ml, and exhaled CO volume between 1.0 and 5.1 ml). For detailed calculation of tHb-mass and blood volume derivatives see Chapter 4 and for further explanation see (6).

5.4.3 Dosing of Carbon Monoxide

Carbon monoxide dosing aims to achieve an absolute ΔCOHb% of between 4.0 and 6.5%, between 6 and 8 minutes (the mean being referred to as the '7-minute value') after CO administration. We assumed that all the CLD patients were 'untrained' (see above), and thus used doses of 0.8 $ml \cdot kg^{-1}$ and 0.6 $ml \cdot kg^{-1}$ CO for men and women respectively. In 2 non-anaemic patients dosing was increased to 0.9 $ml \cdot kg^{-1}$ in one patient (World Health Organization (WHO) performance status zero) (284) and further increased (to 1.0 $ml \cdot kg^{-1}$) in another patient (also with performance status zero) with polycythemia rubra vera (PRV), who had a [Hb] of $184 g \cdot l^{-1}$.

It is advocated that consideration be given to further dose reduction in anaemic subjects ($<130 g \cdot l^{-1}$ in men, and $<120\ g \cdot l^{-1}$ in women (152)), and we followed this approach. In total, CO dose was reduced from the 0.8 $ml \cdot kg^{-1}$ or 0.6 $ml \cdot kg^{-1}$ recommended for 'untrained subjects' in 5 individuals (subjects 2, 3, 6, 8 & 9, Table 4) due to them being anaemic (5 subjects, see Table 4) and then further reduced if their performance status was low (high number on the Zubrod scale) at the discretion of the investigators. This occurred in 3 subjects (2,3 & 8 Table 5). If a subject's BMI is $>30\ kg \cdot m^2$, it is suggested that CO dosing is based on IBW (see introduction). However, this did not apply to any of our subjects.

5.4.4 Statistical Analysis

Statistical analysis was performed using GraphPad prism version 7.0c for Apple Macintosh. The Shapiro-Wilk test for normal distribution was used. Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). Paired t-tests were used to calculate differences in tHb-mass at different time points. The Mann Whitney U test was used to calculate differences in COHb% at different time points. Repeated measures ANOVA was used to compare calculated tHb-mass data for each time point.

5.5 Results

5.5.1 Carbon monoxide dosing

The mean ± SD CO dose administered was 0.73ml·kg^{-1} ± 0.13 ml·kg^{-1} (range 42-76ml), with weight-indexed doses ranging from 0.5-1.0 ml.kg^{-1}. Mean dose to males was 0.75 ml·kg^{-1} ± 0.14 ml·kg^{-1}, and to females 0.65 ml·kg^{-1} ± 0.07 ml·kg^{-1}.

In three subjects, ΔCOHb% was <4% (2.6%, 2.7% and 3.2%). Their demographics are shown in Table 5. Two were women, being reflected in their somewhat lower heights and weights. However, no variables differed significantly from the 13 subjects in whom ΔCOHb% was sufficient to allow valid calculation of tHb-mass (p=0.08 for BMI, p= 0.46 for [Hb]). The doses received were in accordance with the description in the methods, with the two females receiving 0.6ml·kg^{-1}, and the one male (who was anaemic [Hb] 105 g·l^{-1}), receiving 0.7 ml·kg^{-1}). In the remaining 13 subjects, venous COHb% increased from a mean of 1.62% ± 0.77% at baseline to a median value of 6.3 (IQR 6.21-7.47) at '7 minutes' giving a mean ΔCOHb% of 5.26% ± 096% (range 4.0%-7.2%). Table 5 shows the doses administered and the ΔCOHb% for each patient, the latter being adequate for all 13 subjects (Table 5).

One patient had a peak COHb% of 10.1% after six minutes, exceeding that which most investigators would aim for. They suffered no ill effects, however, and their COHb% level was below 10% at twelve minutes.

tHb-mass could not be reliably calculated for the 3 patients in whom ΔCOHb% was <4%. This left 13 patients (11 male; mean 52 ± 13.8 years, body mass 79.1 kg ± 11.4 kg, height 175 ± 6.8cm, body mass index 25.8 ± 3.0 kg·m^2). All had established CLD: nine alcoholic cirrhosis (one of whom also suffered hepatocellular carcinoma), one cryptogenic cirrhosis, one with chronic hepatitis C, one Budd-Chiari Syndrome and one non-alcoholic steatohepatitis.

In the 13 in whom COHb rise was >4% (absolute value), median [Hb] was 113 g·l^{-1} (IQR 101-127g·l^{-1}), mean ± SD tHb-mass 647.3 g ± 148.9g, PV 4221ml ± 104 and blood volume 6101ml ± 1194ml. Ten patients (77%) were anaemic (mean ± SD [Hb] 107 g·l^{-1} ± 9.6 g·l^{-1} versus. 153.3 g·l^{-1} ± 27.2 g·l^{-1} for those not anaemic) (Table 5 & Chapter 4).

Patient	Age (years)	Gender	Height (cm)	Weight (kg)	BMI (kg·m^2)	[Hb] (g·litre)	CO dose in ml	ΔCOHb%
1	49	M	176	65.7	21.2	105	46	3.2
2	50	F	162	57.8	22.0	128	36	2.6
3	50	F	165.5	66.3	24.2	129	40	2.3

Table 6: The demographic details of the three excluded subjects in whom the rise in COHb% was <4%.

5.5.2 Carbon monoxide wash-in curve

Figure 10 shows the COHb% values from baseline to twenty minutes after CO inhalation. The peak occurred at either six or eight minutes after inhalation (median 6.33% (IQR

6.25%-7.46%) and 6.30% (IQR 6.20%-7.47%) respectively. The values were 6.30% (6.21-7.47) at seven minutes, 6.33% (6.00%- 7.50%) at ten minutes, 6.33% (5.90%- 7.40%) at twelve minutes, and 6.37% (5.80%- 7.33%) at fifteen minutes, The value at seven minutes did not differ from that at twenty minutes after CO inhalation (6.27% (5.70%-7.20%), p=0.20). In keeping, calculated tHb-mass was also near-constant over this time period (mean $\pm$ SD being 647.3 ± 149.1g at 6 minutes, 648.6 ± 149.1g at eight minutes, and 653.4 ± 162.0g, 659.7 ± 163.2g, 653.5 ±164.9g and 651.4 ± 167.9g at ten, twelve, fifteen and twenty minutes respectively (Figure 11)). The calculated tHb-mass at minutes seven and twenty were similar (p= 0.68) and differed by only 4.1g, or 0.6%, whilst repeated measures ANOVA confirmed no difference in tHb-mass between any other timepoints (p= 0.48).

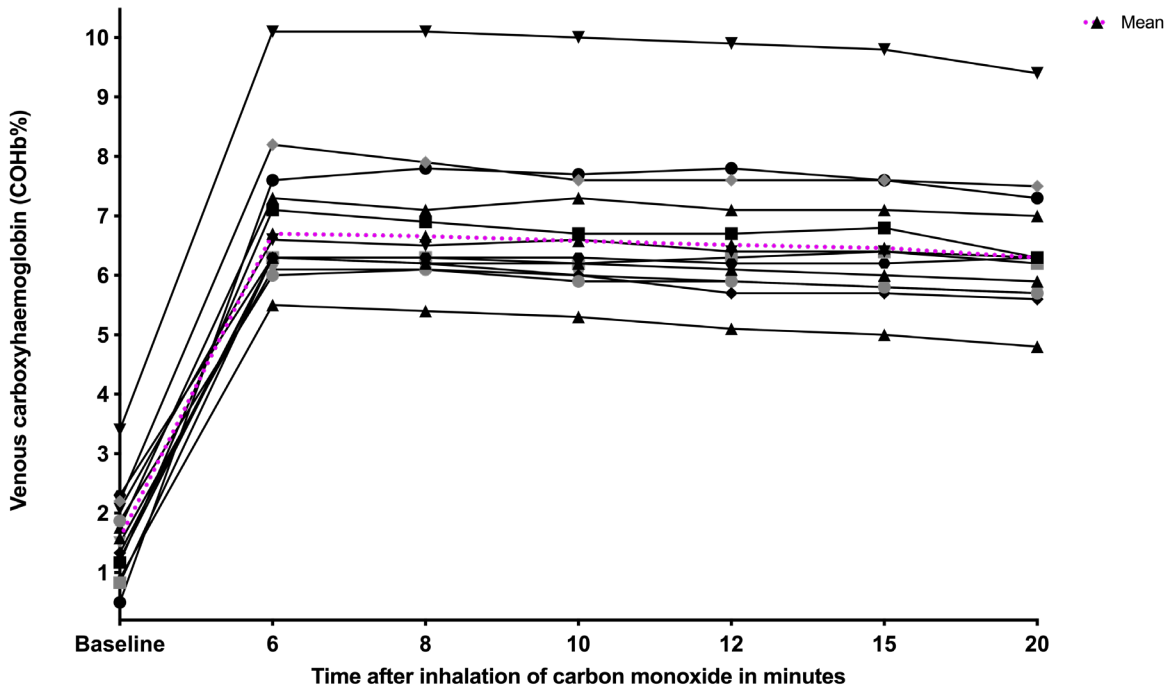


Figure 10: Carboxyhaemoglobin (COHb%) wash in curves from baseline to 20 minutes after CO re-breathing. Legend- Each line represents one individual. Venous carboxyhaemoglobin samples taken at baseline to 20 minutes post inhalation of carbon monoxide gas.

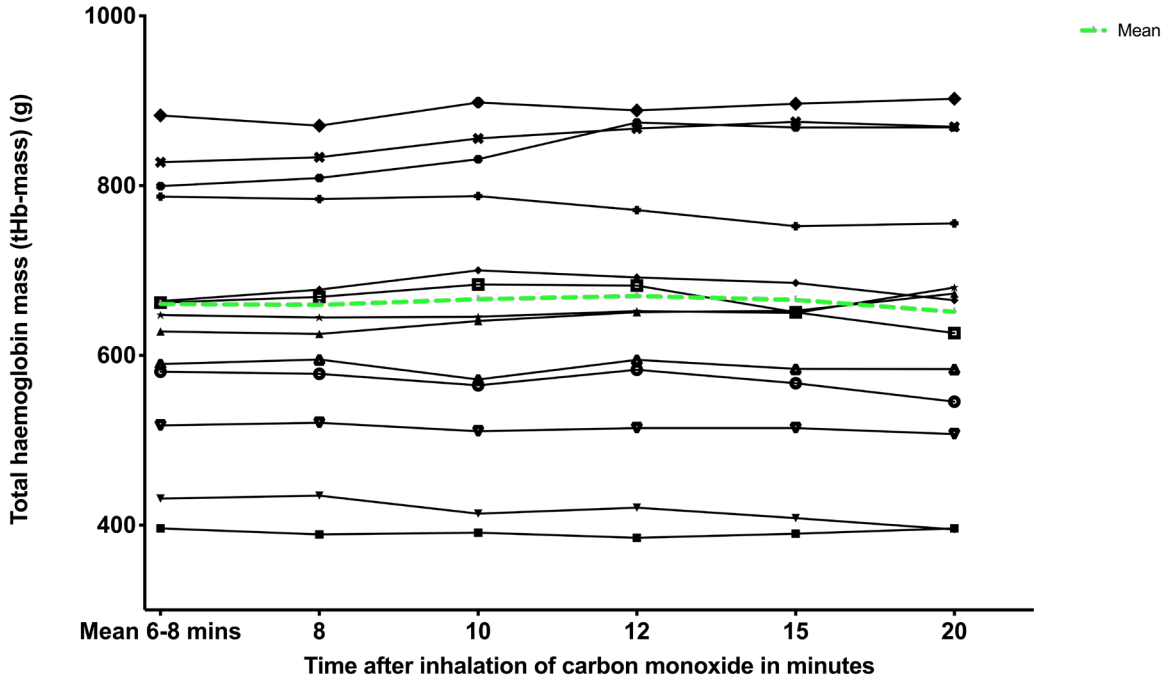


Figure 11: tHb-mass data calculated for different COHb% sampling time points. Legend- Each line represents one individual. tHb-mass was calculated using COHb% values in blood drawn from 6 different time points after inhalation of the carbon monoxide bolus.

5.6 Discussion

We have clarified, for the first time, that the optimized carbon monoxide re-breathing method can be used to calculate tHb-mass in patients with chronic liver disease without modification of the timing of venous sampling. Thus, COHb% values at 6 and 8 minutes giving a '7 minute' (the mean of minutes 6 & 8) do not fall significantly over the next 12 minutes, and calculated tHb-mass thus does not differ significantly (p= 0.68). This suggests that the mixing of CO in the blood is complete by '7 minutes' after inhaling the CO-bolus, and that any blood sample between min 7 and min 20 can be used for the calculation of tHb-mass (Figures 7 & 8). Whilst some COHb% values appear to still be rising between minutes 6 and 8, this difference has little impact on calculated tHb-mass. For example, calculated tHb-mass in patient 2 (see Table 4) is 396.1 g versus. 389 g for 'minute 7' and 8 respectively.

These data suggest that any changes in COHb circulatory mixing kinetics in CLD do not necessitate adjustment of standard sample timings (5), perhaps in contrast to the situation in heart failure (left ventricular ejection fraction <30%) patients (121) and polycythaemia (120).

For precision in tHb-mass measurement by oCOR, the rise in carboxyhaemoglobin (ΔCOHb%) is variably required to exceed an absolute value of 5% (typically 0.5–1.5% at rest, rising to 5–7% post CO rebreathing) (5,122), or to reach values of 5.5-6% (8). Our own unpublished data suggest that values over 4.0% are essential for adequate reliability. Low doses (0.6 ml·kg^{-1}) result in a ΔCOHb% of only 3.4 ± 0.4%, but yield similar tHb-mass values to those when using 1.0 ml·kg^{-1} (791 vs. 788 grams respectively)- suggesting that a ΔCOHb% of <4% may be methodologically acceptable (125). In healthy subjects, a dose of 1 ml·kg^{-1} has been shown to be adequate and safe (125). We have safely and

effectively used 0.4-1ml·kg^{-1} in patients (28,34), and the only other clinical study to provide data (in patients with coronary artery disease) used doses of 0.7ml·kg^{-1} for women and 0.8 ml·kg^{-1} for men (63) and yielded a mean ΔCOHb% of 4.5%. Recent work in polycythaemic subjects used 1.7ml·kg^{-1} and yielded an approximate ΔCOHb% of 3.7% (120). Unfortunately, other clinical studies did not report the doses used (60,61). We accept that we cannot comment on accuracy based on these data as multiple measures at differing doses have not been taken. Further clinical research might address this question.

We show, however, that CO-dosing to achieve ΔCOHb% values >4% (with peaks <10%) can be problematic in CLD patients. Mean CO doses were 0.75 and 0.65ml·kg^{-1} for males and females respectively but ranged from 0.5-1 ml·kg^{-1} (Table 4). Primary dosing was based on gender with the basic assumption that all participants were 'untrained' by nature of their CLD. The original recommended doses of 0.8 ml or 0.6 ml for men and women respectively were used for dosing. However, only 36% (4 subjects) of the male participants in this study received 0.8 ml·kg^{-1}. In five subjects, the dose was reduced as described above (Table 4). In the two male patients with a [Hb] ≤100g·l^{-1} one received a CO dose of 0.7 ml·kg^{-1} and the other a dose of 0.6 ml·kg^{-1} (yielding a ΔCOHb% of 5.9% and 7.2% respectively). Two male patients received higher doses, one patient with JAK2 positive polycythaemia rubra vera (PRV) receiving 1 ml·kg^{-1}, the other was young, unusually had no other comorbidities, had a very good exercise tolerance and was deemed 'relatively fit' compared to the other subjects.

Using the recommended dosing for 'untrained subjects' led to the exclusion of three patients due to 'under-dosing' (rise in COHb% of 2.6%, 2.7% and 3.2% respectively). Of note, two of these cases were women; one of whom had a body mass (57.8kg) lower than many other subjects, and who therefore only received 36mls of CO gas. The remaining subject was a male who received 0.7ml/kg CO dosing. It is possible that the calculated CO bolus will be too high or too low due to [Hb] not always correlating with tHb-mass. By way of example, in the three most anaemic patients ([Hb] 100, 98 and 93 g/l), tHb-mass' were 647.6g for the person with [Hb] 100 g·l^{-1} but 396 g·l^{-1} 1g in the person with [Hb] only 2 g·l^{-1} lower ([Hb] 98 g·l^{-1} - with the highest tHb-mass of the three (662.3g) being in the most anaemic patient ([Hb] 93 g·l^{-1}). In the three patients excluded from the original 16, this was the case: absolute ΔCOHb% values were 3.2%, 2.7% and 2.3% respectively, making calculation of tHb-mass less reliable. They did not have any documented CO gas leaks or other analytical errors that could have resulted in a low ΔCOHb%. In the case of a dilutional anaemia resulting in a falsely low calculated CO bolus and subsequent low ΔCOHb% (<4%), it is possible to repeat the test within only a few hours (3,126). In our previously published work relating to CLD patients, one subject with a [Hb] of only 69 g·l^{-1} was given a dose of 0.4 ml·kg^{-1} producing a ΔCOHb% of 4.7% (28). This highlights the unpredictability of response in severely anaemic patients to bolus CO administration-perhaps because [Hb] and tHb-mass values are divorced when plasma volume is known to be deranged (28). It is of course possible that we had minor leaks that were undetected or that the operator and/or CO detection device did not function perfectly in the 3 excluded patients. Other investigators have reported leak rates of around 14% when performing the CO rebreathing technique, however, this is not my experience of the test (121).

Strengths of this study include the fact that the same two operators performed all oCOR tests in the same laboratory using the same equipment thus reducing measurement error. Conditions were identical for all the patients and all measurements were made prior to paracentesis. Additionally, we did not perform the entire experiment a second time which would have added to the reproducibility of our findings, this could have been in another cohort of patients with CLD or in another similar cohort.

Limitations include that fact that we did not objectively quantify 'fitness'. Nor did we evaluate inter-subject reliability by performing multiple tests on each patient. One of the major reasons for this was down to the length of time it took to perform and teach the technique to each patient who was often quite frail and unwell with their condition; hence they were attending for paracentesis. Whilst theoretically possible that areas with very poor perfusion may not have equilibrated by twenty minutes (our last sample time), there was no appreciable rise in COHb% after the initial peak at either six or eight minutes, making this unlikely, and any possible impact small. Whilst accuracy might have been improved, we were reluctant to use higher CO doses due to the high prevalence of anaemia (77%), and because no other reports existed of the use of the oCOR method in such patients. Due to the pilot nature of this work, we cannot comment specifically on safety, but it is possible that, in future, doses could be adjusted according to baseline values, such that excessively high COHb% levels are avoided. However, it is important to recognise that 'safe levels' of COHb are not exclusively about peak COHb%, and that the duration of raised COHb% is also important. Carboxyhaemoglobin levels have been elevated to 20% in healthy volunteer studies without reported ill effects (285).

Additionally, we did not compare the oCOR method to other established CO re-breathing techniques such as those described by *Lundby*, which is based more closely on the traditional method of *Burge* and *Skinner* (74). Nor did we compare with radiolabelled blood volume measurement techniques which limits our ability to comment on accuracy. However, as previously discussed this is challenging due to there being no defined 'normal' range for tHb-mass and the modern CO techniques described being superior in regard of ease of use.

5.6.1 Future Research

We did not measure tHb-mass and PV after paracentesis. We would advocate that future studies do so, plotting the change in PV (and weight) which occur over time. Future studies might also address the relationship between circulating albumin concentrations/oncotic pressure, ascitic volume, and PV.

There is no clinical cut off which defines 'pathophysiologically low' tHb-mass in the same way in which the WHO has defined a lower limit of [Hb] to define 'anaemia'. Further research should define normative data for PV and tHb-mass for healthy subjects across genders and races, and over wide age ranges. It is hoped that the data within this thesis may aid this work in the future.

Further work is required in order to generate and validate appropriate CO dosing regimens in CLD patients. These might consider baseline COHb%; actual body mass, ideal body mass, the contribution ascitic/oedema mass to overall body mass and true body composition; more precise measures of frailty and fitness; and indicators that a low [Hb] might in fact be due to PV expansion. Studies might focus on repeat measures using a variation of doses in the same subject to measure both accuracy and reliability.

Studies should also be extended across different disease groups- and most especially in those amongst whom plasma volume may be subject to pathological variation. In those with liver disease, trials might address the use of this methodology in guiding clinical intervention (such as diuretic treatment or packed red cell transfusion).

5.7 Conclusions

The oCOR method can be safely used to measure total haemoglobin mass in patients with chronic liver disease and ascites, without adjustment of blood sample timings. Further work might refine and validate appropriate dosing regimens.

Chapter 6 - Replicating measurements of total haemoglobin mass (tHb-mass) within a single day: Precision of measurement; feasibility and safety of using oxygen to expediate carbon monoxide clearance

REplicating MeAsurements of Total Haemoglobin- The 'REMATcH' study

This chapter has been published in the journal ***Physiological Reports*** (3) (Appendix E)

Plumb JOM, Kumar S, Otto J, Schmidt W, Richards T, Montgomery HE, et al. Replicating measurements of total hemoglobin mass (tHb-mass) within a single day: precision of measurement; feasibility and safety of using oxygen to expedite carbon monoxide clearance. Physiol Rep. 2018 Sep;6(17):e13829.

6.1 Chapter in context

At the outset of this thesis the safety of using carbon monoxide gas was of course thoroughly considered. After all, a toxic gas with a high affinity for haemoglobin being used in any context requires careful consideration. The safety record of the oCOR within the athletic and sports medicine community is now well established (13). As stated in Chapter 5:

> "It is important to recognize that 'safe levels' of COHb are not exclusively about peak COHb%, and that the duration of raised COHb% is also important. Carboxyhaemoglobin levels have been elevated to 20% in healthy volunteer studies without reported ill effects (285)."

Despite this, most laboratories and research groups had (until 2015) used a minimum washout period of twelve hours between oCOR tests. In the main, for healthy athletes this did not represent a large problem as repeat testing could happen on consecutive days, perhaps prior to an altitude camp for example.

The reason that most authors recommended that the oCOR can only be repeated after a twelve-hour interval (when breathing room air) (5), was due to the concern that physiological clearance of administered carbon monoxide for the purposes of tHb-mass measurement would, under normal conditions, be expected to take approximately nine hours (based on a post-test COHb level of 7% and a half-life of 4.8 hours) (286).

Despite this, *Naef* and colleagues clearly felt that this level of inconvenience to athletes was not necessary and performed an elegant study to challenge this (126). They showed that multiple tests could be safely performed within a single day in eighteen healthy males. They used exercise and supplemental oxygen to achieve this but did not experiment with oxygen or exercise alone. As reflected in the primary aim of this chapter we wished to explore the effect of oxygen alone on the reduction in carboxyhaemoglobin levels after oCOR testing. The reason for pursuing this was to see if a methodology could be evaluated that *might* be suitable for clinical subjects who were unable to exercise in between oCOR tests due to life limiting conditions, frailty, critical illness, or deconditioning. In the longer

term for precision, disease monitoring and evaluation we felt it important to perform this study to allow future research with the oCOR to allow multiple tests within a working day.

6.2 Abstract

Background

Haemoglobin concentration ([Hb]) is a function of total haemoglobin mass (tHb-mass) and plasma volume. [Hb] may fall by dilution due to plasma volume expansion and changes in the perioperative period may therefore correlate poorly with blood loss. A simple, reliable, repeatable way to measure plasma volume and tHb-mass would have substantial clinical utility. The 'optimized carbon monoxide re-breathing method' (oCOR) meets these criteria. However, it is recommended that a minimum of 12 hours (when breathing room air) is left between repeat measurements.

Methods

Twenty-four subjects underwent three days of testing. Two oCOR tests were performed (T1 and T2), three hours apart, with a different CO clearance method employed between tests aiming to keep the carboxyhaemoglobin level below 10%.

The primary aim was to ascertain whether tHb-mass testing could be safely repeated within three hours if carboxyhaemoglobin levels were actively reduced by breathing supplemental oxygen ($PROC_A$) (Figure 12). Secondary aims were to compare two other clearance methods: moderate exercise ($PROC_B$), or a combination of the two ($PROC_C$). Finally, the reliability of the oCOR method was assessed.

Results

Mean (SD) tHb-mass was 807.9 ± (189.7g) (for T1 on day 1). $PROC_A$ lowered the carboxyhaemoglobin level from the end of T1 (mean 6.64%) to the start of T2 (mean 2.95%) by a mean absolute value of 3.69%. For $PROC_B$ and $PROC_C$ the mean absolute decreases in carboxyhaemoglobin were 4.00% and 4.31% respectively. The fall in carboxyhaemoglobin between T1 and T2 was greatest in $PROC_C$; this was statistically significantly lower than $PROC_A$ (p=0.0039) and $PROC_B$ (p=0.0289). The test-retest reliability for the measurement of total haemoglobin mass was good with a mean typical error (TE) of 2.0%.

Conclusions

The oCOR method is safe and can be repeated within three hours when carbon monoxide is suitably cleared between tests. Oxygen therapy alone adequately achieves this.

Keywords: *blood volume, optimized carbon monoxide re-breathing, plasma volume, red cell volume, total haemoglobin mass (tHb-mass)*

New and Noteworthy

The 'optimized carbon monoxide re-breathing method' is a safe technique that can be repeated within three hours when carbon monoxide is suitably cleared between tests in healthy non-athletic individuals. Using oxygen therapy alone adequately achieves this.

This is the first time that oxygen alone has been used to clear carbon monoxide to facilitate repeat tHb-mass testing within a short time period using the oCOR technique. This would allow repeat testing and quality assurance for elite endurance athletes and if adopted in a clinical setting has the potential to dynamically affect clinical decision making at the bedside.

6.3 Introduction

Humans are obligate aerobes, the oxygen necessary for metabolism being carried by circulating haemoglobin, whose concentration [Hb] has traditionally, been used as a measure of the blood's oxygen carrying capacity.

In 1968, the World Health Organization (WHO) defined anaemia as a [Hb] <130 $g.l^{-1}$ for men and <120$g.l^{-1}$ for women (152). Despite evidence that these levels may actually be inappropriately low they are still widely accepted within clinical practice [2,18]. Perioperative anaemia is common and is associated with greater post-operative morbidity and mortality (17). This may in part be due to impaired oxygen delivery. Measures of preoperative cardiopulmonary fitness measured using cardiopulmonary exercise testing (CPET) such as peak oxygen consumption ($\dot{V}O_{2peak}$) and oxygen consumption at the anaerobic threshold ($\dot{V}O_{2AT}$) are inversely related to postoperative outcome. Both $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$ fall when tHb-mass is reduced by venesection (287) and improve with red cell transfusion (288). However, haemoglobin concentration [Hb] is a function of the total mass of circulating haemoglobin (tHb-mass) and the volume of plasma within which it is carried (PV) and, as such, tHb-mass might be better correlated with patients' CPET performance than [Hb]. In keeping, we have shown that [Hb] correlates modestly (29) or not at all (34) with peak oxygen consumption ($\dot{V}O_{2peak}$) or oxygen consumption at the anaerobic threshold ($\dot{V}O_{2AT}$).

In disease states in which PV expansion is common such as chronic heart or liver failure, [Hb] correlates poorly with tHb-mass (28). tHb-mass is also more stable than [Hb] over time, being unaffected by shifts in PV (which may be exaggerated when intravenous fluids are administered perioperatively) (30,107). Thus, in the perioperative setting changes in tHb-mass may be a better guide to true blood loss than are changes in [Hb]. There is an increasing recognition of the unsuitability of using [Hb] as a reliable guide to perioperative transfusion (1,58).

tHb-mass can be measured directly using the so-called optimized carbon monoxide (CO) re-breathing method (oCOR) (see Chapter 4 for further details). In use since 2005, it has predominantly been applied to athletes in order to monitor responses to altitude training (13), but more recently has been used in clinical medicine (28,34,60,61). There is no 'normal range' for tHb-mass described for healthy subjects. Endurance athletes would hope to have upwards of 13g/kg (males) and 11g/kg (females).

In assessing changes in PV and tHb-mass over the perioperative period, it would be useful to make repeated measurements over relatively short timescales. The reasons behind this are that any test used in an acute hospital or even sports physiology laboratory setting needs to be repeatable over short time frames for precision, training, and quality control purposes. The potential beneficial consequences of this for the clinician are multifactorial but include the ability to assess blood volume dynamically, measure blood loss more

accurately; for example at different time points in a long operation, to guide red cell transfusion in patients with unknown fluid status or to monitor response to therapies (such as diuretics).

However, to date, most authors recommend that the oCOR can only be repeated after a twelve-hour interval (when breathing room air) (5), due to the concern that physiological clearance of administered carbon monoxide for the purposes of tHb-mass measurement would, under normal conditions, be expected to take approximately nine hours (based on a post-test COHb level of 7% and a half-life of 4.8 hours) (286).

To address this, various methods have been proposed which might enhance carbon monoxide clearance rates (126,286) such that tHb-mass assessment could be more rapidly repeated whilst still maintaining carboxyhaemoglobin levels below the proposed recommended maximum value of 10% (28,34,125,126). With carbon monoxide toxicity exposure concentration and time need to be considered. Higher levels for a very short duration possibly being less dangerous than lower levels for prolonged periods. In this regard, mild exercise (inducing a higher rate of pulmonary ventilation) and normobaric 'hyperoxia' (supplementing oxygen at FiO_2 >0.21) are two potentially effective candidate interventions to facilitate clearance of serum carboxyhaemoglobin (31). An important study by *Zavorksky* et al demonstrated that mild exercise, hyperoxia and increased pulmonary ventilation the so called 'triple therapy' was most effective at reducing the half-life of CO administered to healthy subjects. They also found that moderate exercise (in room air) was as effective as breathing 100% oxygen at rest at clearing CO (286). To our knowledge, only one study (in healthy athletic males), using administration of supplemental inhaled oxygen during physical activity, has demonstrated the feasibility of doing this in the context of tHb-mass assessment: here the efficacy of oxygen therapy alone, or of exercise alone was not assessed (126).

The primary aim of this study was to ascertain whether tHb-mass testing could be safely repeated within three hours if carboxyhaemoglobin levels were actively reduced by breathing supplemental oxygen alone ($PROC_A$). The secondary aims were to compare two other carboxyhaemoglobin clearance procedures (gentle cycling alone, or gentle cycling in combination with oxygen administration- procedure B and C ($PROC_B$ & $PROC_C$) respectively. The final aim was to evaluate the reliability of duplicate tHb-mass measurements in healthy volunteers, who were not the elite or 'sub-elite' athletes largely studied by others previously (126).

6.4 Methods

The study took place at University Hospital Southampton NHS Foundation Trust between August 2016 and March 2017.

Ethical approval was granted by the West Midlands (Edgbaston) Research Ethics Committee and NHS Health Research Authority (REC reference: 16/WM/0274). Local permissions were received from the University of Southampton (ERGO ID: 19642), University Hospital Southampton NHS Foundation Trust (R&D CRI 0329) and Southampton Centre for Biomedical Research Clinical Research Facility. The study was performed in accordance with the ethical standard set by the Declaration of Helsinki. Written informed consent was obtained from all participants.

We set out to recruit at least 20 subjects and therefore slightly over recruited to account for possible dropouts or unusable data. The rationale for this number was 2 fold. Firstly, we

based this study on that by Naef and colleagues who recruited 18 subjects, however these were all healthy athletic males (126). Additionally we wanted at least 10 subjects for inter researcher reliability based on numerous previous studies and subsequent direct advice from professor Schimdt's research group who use this number for this their test re-test reliability trails, 20 therefore accounted for 2 separate researchers carrying out the experiments in this study.

Healthy adults aged over 16 years who were physically able to perform the testing protocol were eligible for recruitment. They were recruited via an email to medical students, a poster and word of mouth. The clinical research facility at University Hospital Southampton also has a healthy volunteer registry and an email was sent via this to seek participants for this study. Excluded were adults lacking mental capacity to consent, pregnant women, smokers, prisoners, subjects with a baseline carboxyhaemoglobin level > 5%, or patients with haemoglobinopathies. One subject was a smoker (this was not clear until after the first experiments had taken place and their data were removed). No subjects had stayed at an altitude higher than 1500m for any time in the preceding year. This left data from twenty-four subjects for analysis. In total, 24 x 6 (144) measurements of tHb-mass were planned but 136 were carried out; the missing 8 experiments were due to CO gas running out mid-experiment in three subjects and a blood sample clotting in one subject. Of these 136 measurements, 122 were suitable for analysis. Values being discarded if the rise in carboxyhaemoglobin (ΔCOHb%) was <4.5% (standard practice in our laboratory), if significant leaks were detected (CO ppm>5 using the Dräger Pac 7000), if the breath holding technique was sub-optimal (determined either by a leak using the Dräger Pac 7000 or if the subject failed to breath hold for 10 seconds), or if the resting carboxyhaemoglobin values were greater than 5% (Appendix B). Fourteen subjects underwent three consecutive days of measurement, the remaining ten underwent three separate days within a ten-day period.

tHb-mass was measured twice (a period of three hours separating the first (T1) and second (T2) measurements) using the optimized carbon monoxide rebreathing (oCOR) method. This protocol was repeated on each of three separate occasions, with a different protocol to enhance CO clearance being applied between tests on each day. Figure 12 outlines the experimental design and study activity. The three experimental days had to occur within a ten-day period.

Day 1- Procedure A ($PROC_A$)- Immediately after the first oCOR test, 60% oxygen was administered to seated subjects via a Venturi mask (Intersurgical Ltd, Berkshire, UK) for exactly one hour. An identical oCOR test was repeated exactly two hours later (three hours in total from the end of the first oCOR test).

Day 2- Procedure B ($PROC_B$)- Immediately after the first oCOR test, subjects exercised at low intensity (50 watts and 60-70 revolutions per minute) for forty-five minutes on a static exercise cycle ergometer (Ergoline Ontibike 200, Germany). An identical oCOR test was repeated exactly two hours and fifteen minutes later (3 hours in total from the end of the first oCOR test).

Day 3- Procedure C ($PROC_C$)- Immediately after the first oCOR test, subjects exercised as for $PROC_B$ were positioned on a static exercise cycle ergometer (Ergoline Ontibike 200, Germany) whilst breathing 60% oxygen via a Venturi mask (Intersurgical Ltd, Berkshire). An identical oCOR test was repeated exactly two hours and fifteen minutes later (three hours in total from the end of the first oCOR test).

The fraction of inspired oxygen chosen was 0.6, this was for pragmatic and safety reasons. Firstly, there is a convenient venturi delivery device that consistently delivers 0.6 available. Higher inspired fractions of oxygen can be detrimental if breathed for long periods and in a small subset of patients can be very detrimental if they are known to retain carbon dioxide (289,290). We wanted to balance safety with expedient expulsion of CO gas.

In all cases, subjects were allowed to leave the lab between tests, remaining in the hospital and avoiding formal or strenuous physical activity. Finally, triplicate measurements of tHb-mass taken on separate days (T1 of each procedure) were compared. We deliberately did not include a control procedure for clearance (i.e., no exercise and no inspired additional oxygen). It has previously been demonstrated that clearance of CO under these normal conditions is prolonged and the study by *Naef et al* demonstrated this precisely (126).

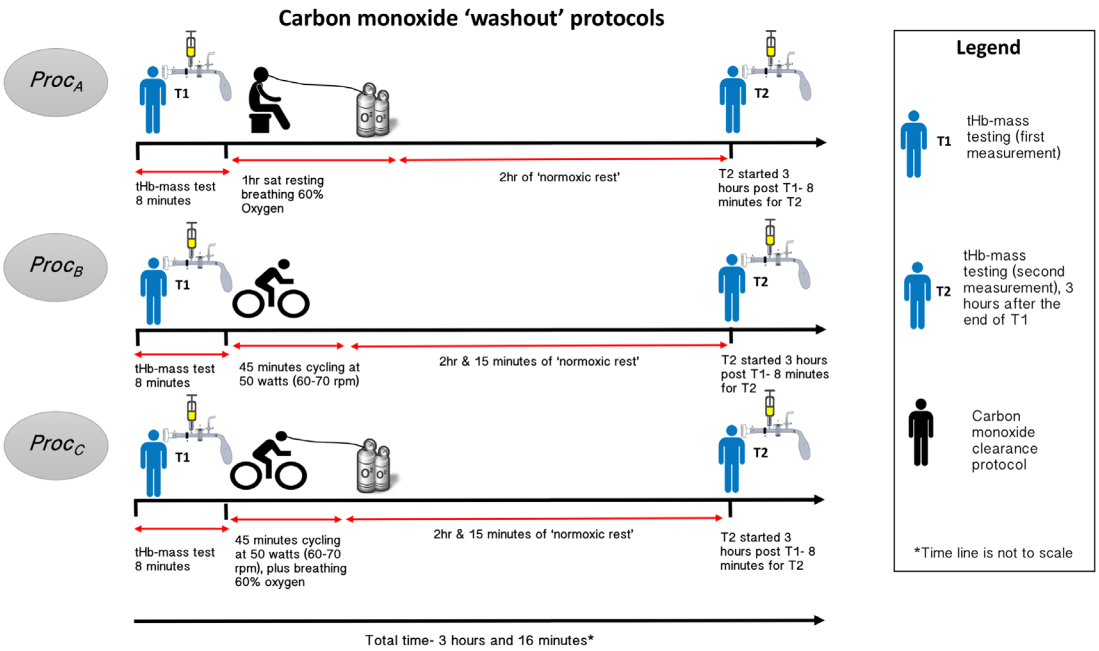


Figure 12: Schematic to describe the experimental sequence

6.4.1 Optimized Carbon Monoxide Rebreathing Method

Subjects completed a baseline carbon monoxide rebreathing test (oCOR) (details of this method can be found in 4.1). Briefly, subjects were seated and inactive for fifteen minutes prior to sampling. Subjects inhaled 0.8-1 $ml \cdot kg^{-1}$ of CO mixed with three litres of 100% oxygen via a glass spirometer (BloodTec, Bayreuth, Germany) and then rebreathed (via a CO_2 scrubber) for two minutes whilst wearing a nose clip. A portable CO gas detector (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) was used during the rebreathing period to check for possible CO leakage at the nose, mouthpiece, and spirometer.

Prior to commencing the rebreathing technique, the investigators inserted an intravenous cannula into the subject's upper limb. Venous blood samples were taken via Na-heparinized syringes (RAPIDLyte, Siemens Healthcare Diagnostics Inc, USA) before (at

baseline) and at six and eight minutes after administration of CO gas. All three blood samples (0, 6 and 8 minutes) were analyzed using a laboratory blood gas analyzer (Radiometer, ABL800 FLEX) for carboxyhaemoglobin percentage values. Each sample was analyzed three times within one hour of collection. The analyzer used in this study was subjected to regular maintenance and quality control checks; the accuracy of which has been evaluated elsewhere (122). [Hb] and hematocrit values were measured using HemoCue (HemoCue AB, Radiometer, Sweden) and blood gas analyzer (Radiometer, ABL800 FLEX, Copenhagen) respectively.

The desired rise in carboxyhaemoglobin is 5% ~ 6.5% after administration of carbon monoxide gas aiming to achieve levels below 10% at the end of the test. This represents a tradeoff between precision and safety. However, 'safe levels' of COHb are not exclusively about peak COHb% but duration of high blood levels is equally, if not more important. Most haemoximeters estimate carboxyhaemoglobin only to a single decimal place meaning that smaller changes in carboxyhaemoglobin could result in lower precision of measurement. We use a minimum Δ%COHb of 4.5% to avoid overestimating tHb-mass. The safety limit of 10% is based on previous work by our group and Professor *Schmidt* who developed the oCOR test. This level allows a significant margin of safety before subjects are likely to experience any significant level of CO toxicity. Previous researchers have also used this level (28,34,125,126). Levels greater than 5% can produce symptoms such as mild headaches and when levels start to exceed 15% most individuals begin to experience headaches and visual evoked potential start to change (127).

6.4.2 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c for Apple Macintosh OSX) and SPSS Statistics (version 25 for Apple Macintosh Chicago, IL, USA). The Shapiro-Wilk test for normal distribution was used. Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). Differences in carboxyhaemoglobin were assessed using the Student's paired t-test.

This was a feasibility study, so a formal power calculation was not required. The aim was to recruit 25 subjects on the advice of Professor *Schmidt* (personal communication) based on the work of their group having performed thousands of tHb-mass measurements. They use a number of ten test/retests to ensure personal operator quality assurance. The statistical principles of test/retest reliability measures as described by *Hopkins* et al (83) would also suggest that ten patients are sufficient to get a meaningful estimate of typical error (TE) for an individual tester.

Test-retest data (repeated measures in one and the same patient) are presented using Bland-Altman plots with limits of agreement (277). Additionally, a specific approach to compute reliability statistics to compare test-retest performance was used (see 4.3.1 for further detail) (83). Typical error of measurement (TE) for tHb-mass was calculated and expressed as coefficient of variation with 95% confidence limits (CL), derived from χ^2 distributions. All tests were two-sided and statistical significance was set at $p<0.05$. TE includes random error (analytic error arising from using the method-specific apparatus and intra-individual biological variation) but not systematic error (bias) (8,13,126). Reported studies using the oCOR have commonly reported this method (70,113,126,291,292).

First measurement (T1)				Second measurement (T2)				Fall in COHb% from the end of T1 to the start of T2	Reliability statistic- Typical Error (TE) (95% CL)
Baseline COHb (%)	**COHb 7-minute value (%)**	**Delta change in COHb (%)**	**tHb-mass (grams)**	**Baseline COHb (%)**	**COHb 7-minute value (%)**	**Delta change in COHb (%)**	**tHb-mass (grams)**		**Overall, 2.0 (1.67-2.59)**
1.09 ± 0.63	6.64 ± 0.90	5.55 ± 0.60	807.9 ± 189.7	2.95 ± 0.55	8.45 ± 0.83	5.50 ± 0.55	789.4 ± 171.2	3.69 ± 0.72	1.61 (1.21 - 2.41)
1.39 ± 0.59	7.10 ± 0.80	5.67 ± 0.60	788.1 ± 180.1	3.10 ± 0.75	8.77 ± 0.96	5.67 ± 0.52	786.2 ± 172.5	4.00 ± 0.83	2.02 (1.53 - 2.99
1.33 ± 0.46	7.10 ± 0.43	5.75 ± 0.34	775.1 ± 188.5	2.72 ± 0.51	8.27 ± 0.72	5.63 ± 0.43	775.7 ± 181.2	4.31*$^{\#\$}$ ± 0.61	2.37 (1.76 - 3.6)

	Procedure	$Proc_A$	$Proc_B$	$Proc_C$

Table 7: The three clearance protocols for washing out carbon monoxide after total haemoglobin mass measurement.

Mean carboxyhaemoglobin (%) values at baseline and 7 min (the mean of min 6 and 8) during test 1 and 2 of each procedure. The Delta change from baseline to minute 7 and the T1–T2 fall in COHb% are shown. The resulting mean total haemoglobin mass is also shown. Typical error of measurement (TE) of tHb-mass for the three carbon monoxide clearance procedures. COHb, carboxyhaemoglobin; PROCA/B/C, procedures A/B/C; TE, typical error of measurement; CL- confidence limits. [1]The reason for slight discrepancy in the value generated by (COHb% 7-min value from T1 – COHb% baseline value from T2) is that not all participants had values for both tests in each experiment. There were two participants who did not have a valid test in T1 but did have a value in baseline value in T2. [2]Significantly different compared to the fall in COHb in PROCA & B (P = 0.0039 and P = 0.0289, respectively). [3]Statistically significant using one-way ANOVA with Bonferroni multiple comparison test.

	Male	**Female**
Age (years)	Median 22, range 20-32	Median 22, range 22-23
Height (cm)	179.4 ± 8.9	161.0 ± 8.3
Weight (kg)	81.6 ± 11.4	63.0 ± 3.40
BMI ($kg.m^2$)	25 ± 3.4	24 ± 2.0
Haemoglobin concentration [Hb] ($g.l^{-1}$)	154.5 ± 13.8	141 ± 2.60
tHb-mass (grams)	863.3 ± 147.0	541.3 ± 27.0
Blood volume (BV) (ml)	6188 ± 962.0	4220 ± 265.0
Plasma volume (PV)(ml)	3565 ± 664.3	2569 ± 182.2
Erythrocyte volume (EV) (ml)	2577 ± 524.0	1651 ± 83.20
Procedure A ($PROC_A$) fall in COHb% from the end of T1 to the beginning of T2	3.54 ± 0.72	4.25 ± 0.38
Procedure B ($PROC_B$) fall in COHb% from the end of T1 to the beginning of T2	3.90 ± 0.77	4.32 ± 1.10
Procedure C ($PROC_C$) fall in COHb% from the end of T1 to the beginning of T2	4.20 ± 0.63	4.72 ± 0.34
$PROC_A$ vs. $PROC_B$- statistically significant?	No p= 0.6543	No p=0.8690
$PROC_A$ vs. $PROC_C$- statistically significant?	Yes p=0.0458	No p=0.1119
$PROC_B$ vs. $PROC_C$- statistically significant?	Yes p=0.0072	No p= 0.5388

Table 8: Male and female differences between the three clearance procedures and resulting tHb-mass. All values are expressed and mean ± standard deviation unless otherwise stated.

6.5 Results

6.5.1 Baseline characteristics

Twenty-four subjects were included in the study (twenty male), aged (median [range]) 23 [20 – 32] years, height 176 ± 11 cm, and weight 78.4 ± 12.8 kg. On day one, test 1, the average results were [Hb] 152.4 ± 13.6 $g.l^{-1}$, hematocrit (Hct) 46.47 ± 4.3% and tHb-mass 807.9 ± 189.7g.

Comparing the 3 carboxyhaemoglobin clearance procedures ($PROC_C$, $PROC_B$ and $PROC_C$)

Table 7 shows the changes in carboxyhaemoglobin for each procedure and the reliability statistics. All three carbon monoxide clearance methods achieved a reduction in carboxyhaemoglobin from the end of T1 to the beginning of T2 on each of the days studied.

Baseline carboxyhemoglobin levels were higher at the start of T2 than T1 for all three procedures (median 1.15% (start of T1) vs. 2.87 % (start of T2) respectively, $p<0.0001$). T1 baseline carboxyhaemoglobin was lower in $PROC_A$ than in $PROC_B$ and $PROC_C$ ($p=$ 0.0010 and 0.0013 respectively). T2 baseline carboxyhaemoglobin was lowest in $PROC_C$ and it differed significantly from $PROC_A$ & $PROC_B$ ($p=0.0039$ & 0.0289 respectively). There were no significant differences between $PROC_A$ and $PROC_B$.

The fall in carboxyhaemoglobin between T1 and T2 (4.31%- absolute mean value for $PROC_C$) was greater in $PROC_C$ than $PROC_A$ (absolute mean value 3.69%) ($p=0.0039$, (the difference between $PROC_C$ and $PROC_A$ being 0.62%) and this result was robust to analysis using one-way ANOVA with Bonferroni multiple comparison test ($p=$ 0.037) (See Table 7). Although $PROC_B$ and $PROC_C$ were initially significantly different this was not the case after applying the Bonferroni test p=0.617 (Figure 13). The absolute mean fall between tests 1&2 for $PROC_B$ was 4.00%. Cycling with supplemental oxygen ($PROC_C$) was therefore statistically more efficient than oxygen alone ($PROC_A$) but not statistically more efficient than cycling alone ($PROC_B$) at lowering carbon monoxide levels in the blood. There was no statistical difference between oxygen alone ($PROC_A$) and cycling alone ($PROC_B$) at lowering carbon monoxide levels in the blood.

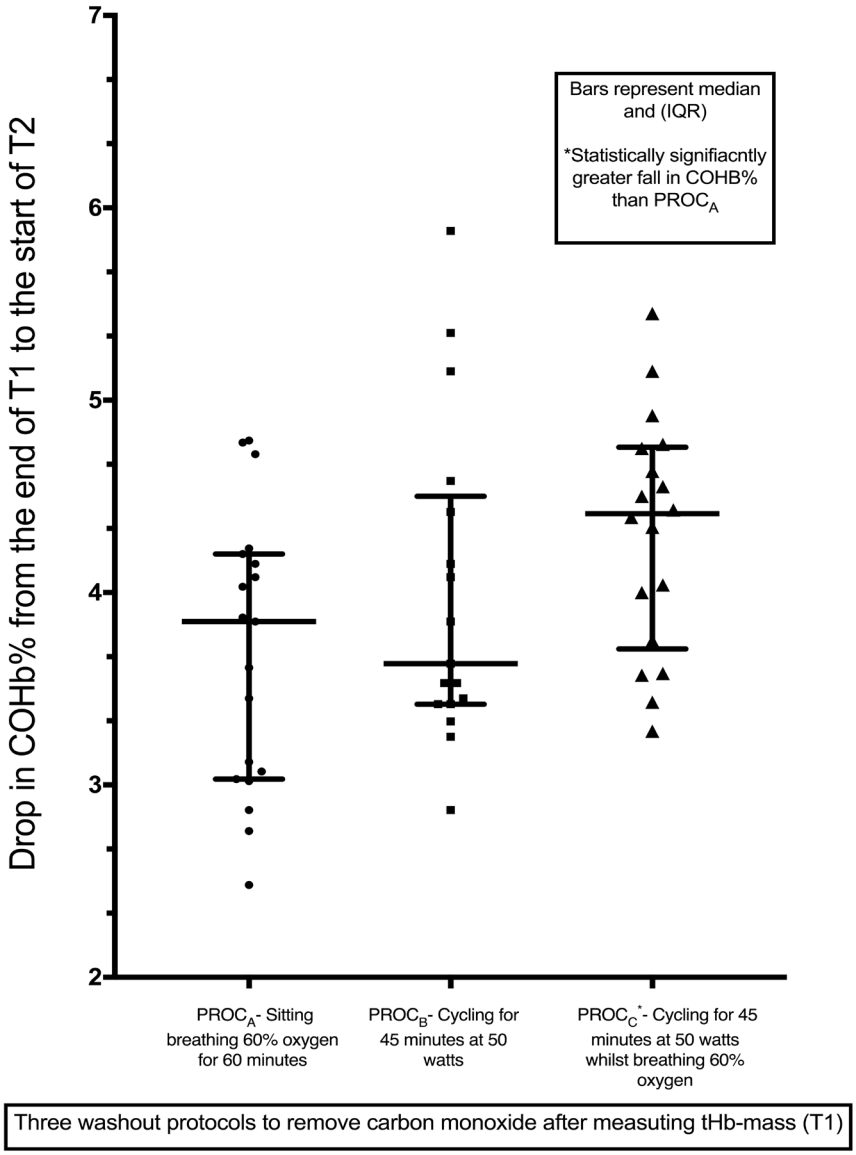


Figure 13: Comparing the fall in carboxyhaemoglobin (COHb%) between test 1 and test 2 of each procedure (T1 & T2). *Median with IQR shown for each procedure. *Denotes the significant difference between $PROC_C$* and *$PROC_A$ (after multiple comparison correction using the Bonferroni method) Abbreviations: COHb, carboxyhaemoglobin; $PROC_{A/B/C}$ procedures A/B/C.*

Differences between males and females revealed a mean ± SD tHb-mass of 863.3 ± 147.0 and 541.3 ± 27.0 respectively. Further breakdown of male and female differences is tabulated in Table 7. All three procedures were relatively more efficient at clearing CO in females (see Table 7). Comparing $PROC_C$ with $PROC_A$ *and* $PROC_B$ they differed statistically as above in males (p= 0.0458 & p= 0.0072) but this did not hold true for females (p= 0.8690). There were no other statistically significant differences between the procedures when males and females were separated.

6.5.2 Test re-test reliability

The overall typical error (TE) for the study was 2.0%, 95% CI (1.67- 2.59). Neither the procedure nor the baseline carboxyhaemoglobin level influenced the calculated tHb-mass. The TE of $PROC_{A}$-$_{C}$ are shown in Table 7 and Figure 14 shows the Bland Altman plots for $PROC_{A-C}$ (277) with limits of agreement. The bias is similar for $PROC_{A-C}$ (0.7, 4.5 & -1.1 respectively), but increased when T1 was compared across the three days (8.4). There were no significant differences between subjects that had the measurements over three consecutive days (fourteen subjects) versus within ten days (eleven subjects) with regard to the precision of repeat measures of tHb-mass.

Serum carboxyhaemoglobin during $PROC_{A}$ and $PROC_{C}$ remained below 10% and ranged from 0.5 – 9.9% and 0.7 – 9.3% respectively. Carboxyhaemoglobin during $PROC_{B}$ ranged from 0.7 – 10.7% (Figure 15). One subject had a serum carboxyhaemoglobin above 10% (10.7% at 6 mins & 10.4% at 8 minutes) after carbon monoxide administration during T2.

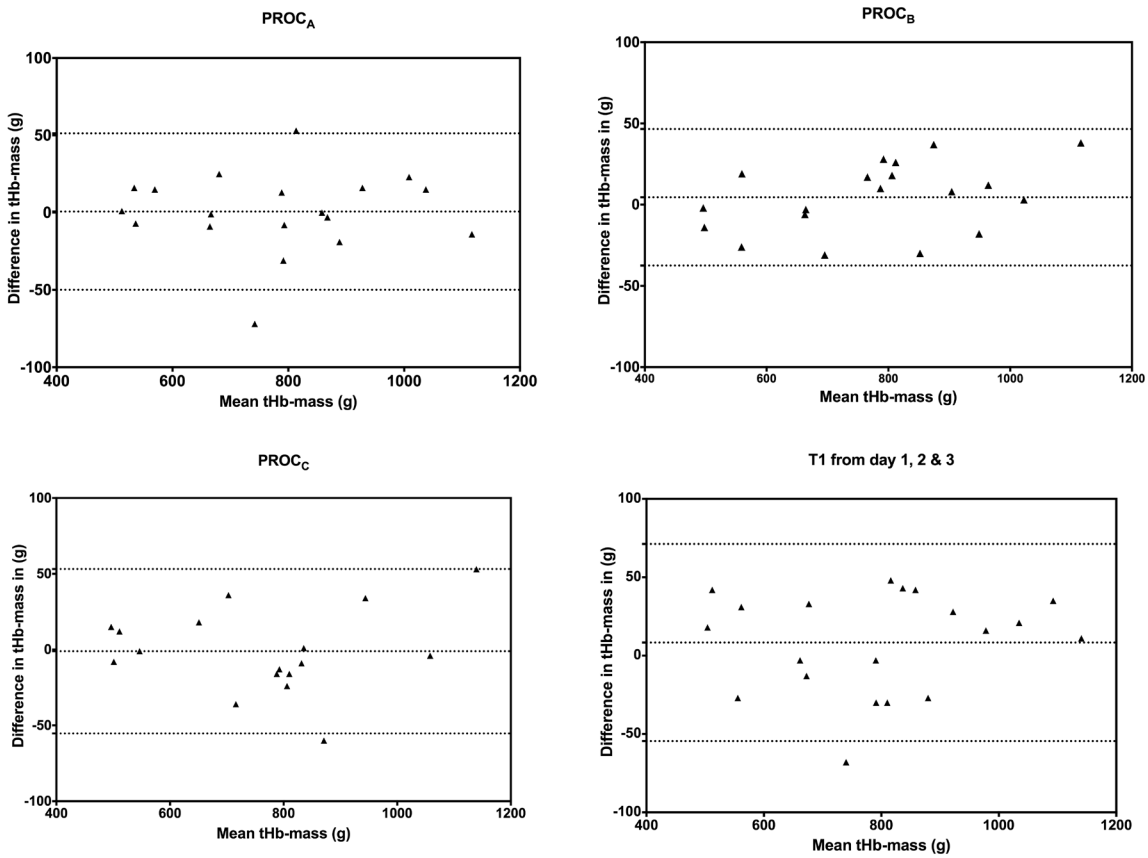


Figure 14: Bland-Altman plots comparing tHb-mass measurements between T1 & T2 for each procedure

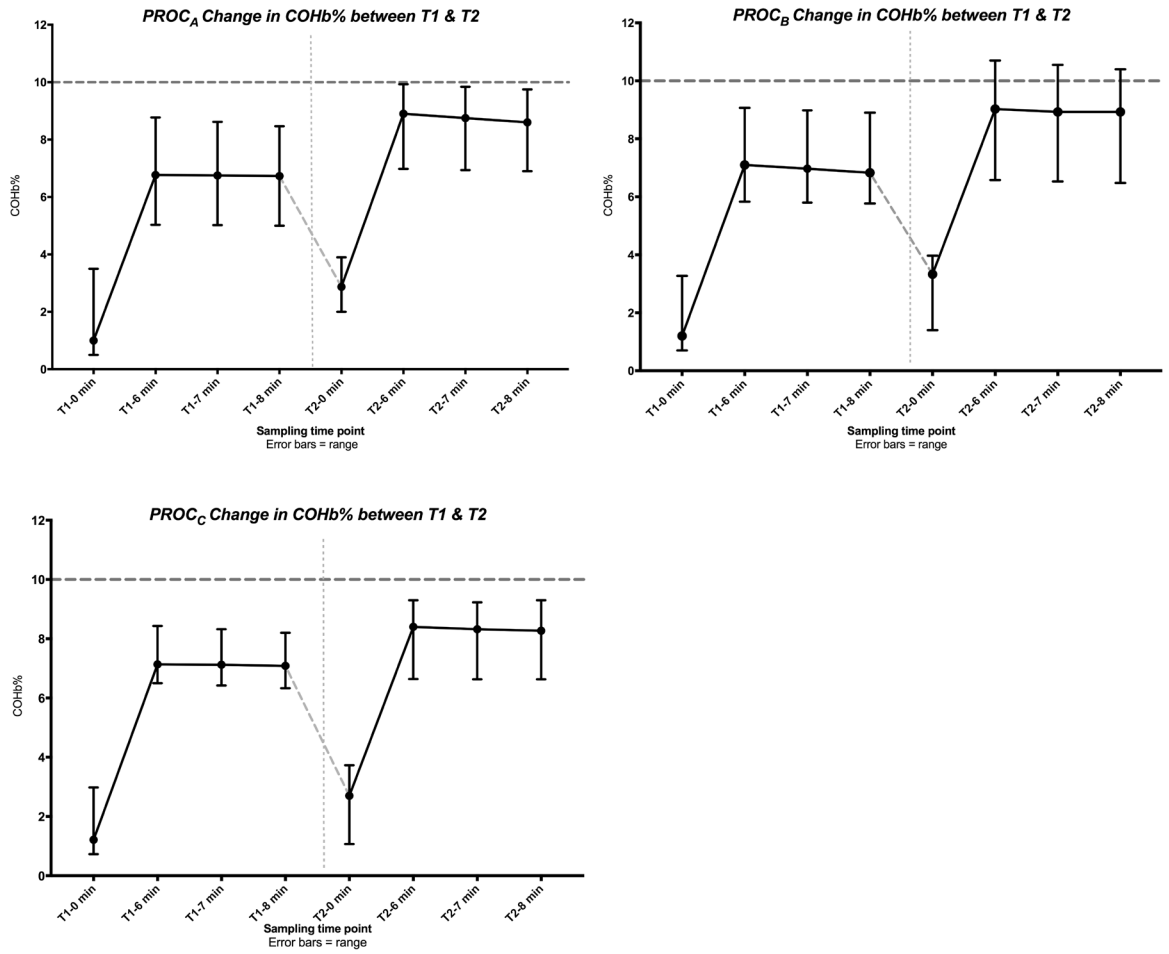


Figure 15: Comparing the fall in carboxyhaemoglobin (COHb%) between test 1 and test 2 of each procedure (T1 & T2) Caption: *Median with IQR shown for each procedure. *Denotes the significant difference between PROC$_C$* and *PROC$_A$ (after multiple comparison correction using the Bonferroni method). Abbreviations: COHb, carboxyhaemoglobin; PROC$_{A/B/C}$ procedures A/B/C.*

6.6 Discussion

In this study repeated measurements of tHb-mass can be safely made within three hours if an adequate carbon monoxide clearance procedure is performed between tests. Administering oxygen alone (without exercise) is sufficient to achieve this and to avoid excess (>10%) carboxyhaemoglobin levels being reached in healthy subjects in this relatively small study. It should be noted that this may not apply to older patients with significant chronic disease.

Co-administering oxygen with mild exercise lowered the carboxyhaemoglobin the most (Figure 14/Table 7). This was statistically different from oxygen alone but not from cycling alone (after application of Bonferroni's test). These data are in keeping with those from a study which evaluated the effectiveness of measures to treat carbon monoxide poisoning (293) and of a study which showed 'hyperoxic exercise' to be superior to six hours of normal daily activity in reducing carboxyhaemoglobin (126). They are also in keeping with the study by *Zavorksky* et al which demonstrated that the 'triple low' of exercise, supplemental oxygen and increased pulmonary ventilation was the most effective way to clear CO from the blood (286). We found no statistical difference between $PROC_A$ and $PROC_B$ but did find that $PROC_C$ was significantly better than $PROC_A$ at lowering carboxyhaemoglobin levels. The fact that there was no significant difference between the other procedures may have been a consequence of the study being underpowered to detect any real difference or may of course represent a lack of any difference between these techniques.

When males and females were compared all three procedures lowered COHb% to a greater extent in female subjects. This is in keeping with previous research and the fact that females have a lower tHb-mass. In a study examining this, gender differences in CO $t_{1/2}$ disappeared when tHb-mass was normalized, suggesting that CO storage explained much of the difference (294). Due to the low number of female subjects within the study it is impossible to conclude further than this.

Strengths of this study include the fact that the same two operators performed all the experiments in the same laboratory with the same equipment. Weaknesses include the fact that one subject performed the carbon monoxide clearance procedures in a different order. Also, a relatively high number of experiments were not completed (14/136) for diverse reasons see Appendix B. These findings do highlight that the oCOR technique is susceptible to technical difficulties, even when performed by experienced well-trained technicians. It is possible that further refinement and the development of an automated oCOR technique in the future could improve this.

Fourteen subjects were studied on consecutive days, and ten over a period of up to ten days. This is unlikely to have been of significance, given that tHb-mass has been shown to be stable over days to months in contrast to haemoglobin concentration and hematocrit which are not (30,107). We did not measure minute ventilation (MV) after CO inhalation and are aware that this does limit our findings due to variances in MV affecting CO removal, with ventilation playing a significant role (286). Another weakness was the period of rest post each procedure for additional washout. Pragmatically this was performed for safety reasons due to the concern that the baseline levels might be too high to test immediately post the washout procedure.

The median age of 22 means that the results of this study may be limited when considering older, potentially frail patients and the generalizability of these findings may be limited.

One subject had a carboxyhaemoglobin level exceeding the accepted safety threshold of 10% Figure 15 ($PROC_B$). They had no symptoms, and the carboxyhaemoglobin level 1 hour later was safely below 10%. It should be noted that the safety limit used in this study (serum carboxyhaemoglobin level of 10%) was a recommendation (125,127) also followed by the earlier study conducted by *Naef* and colleagues (126). We have used this level in all our previous work and if a subject has exceeded 10% have treated them with supplemental oxygen until the level has reduced to below 10%. In fact, serum carboxyhaemoglobin can be raised to 18% in healthy individuals without symptoms of toxicity developing, however the levels are lower in patients with heart disease (as one example) (127). As such, increasing serum carboxyhaemoglobin to even 15% may also be acceptable. As most subjects had a serum carboxyhaemoglobin less than 10% during all procedures, it is reasonable to believe that each of the three clearance methods was safe to use in this population. Much of this safety data is based on historical experiments and animal work (127,295,296). Our group alone has now conducted multiple hundreds of oCOR tests in healthy volunteer and clinical subjects without COHb levels exceeding 11% and without any adverse events being reported (28,34). We have tested smokers in some of our previous work but do not have extensive data on levels and symptoms associated with carboxyhaemoglobin levels in excess of 10%. The question of whether a top level of 10% is indeed a limit of 'safety' for the oCOR method in healthy volunteers or clinical subjects was not a focus of this study, however we recognise that further controlled experiments examining this would be of use. This becomes relevant if multiple testing occurs within short time periods. Further work would be required to examine if the threshold of 10% could be safely exceeded.

6.6.1 Reliability of duplicate total haemoglobin mass measurements

The overall typical error of 2% of repeat measurements within the same subject in this study (Table 7) is in keeping with the only published meta-analysis on the subject, which reported a value of ~2.2% (13). This meta-analysis is important as it groups together a number of small studies from different laboratories and totals 328 participants. If an individual has a tHb-mass of 1000g, a TE of 2.0% equates to a 20g difference between tests. As such, the study by *Naef* et al had a TE of 1.4% that would equate to only a 14g difference. Our results were not quite as precise (126). The median total haemoglobin mass over all 6 oCOR tests was lower in our study than in that of *Naef* et al (126) (797g vs. 914g respectively. This equated to 16g Hb vs.13g Hb based on a TE of 2.0 vs. 1.4) in keeping with our inclusion of four females and a less well-trained population. It is conceivable that part of the difference is also due to the technical precision of the operators and/or the accuracy of the equipment. We used a rise in carboxyhaemoglobin (ΔCOHb%) of >4.5%, some authors argue that higher values are required to improve precision (74).

Bland-Altman plots (Figure 14**)** suggest reasonable precision. The biases were low (0.7, 4.5 & -1.1 for $PROC_{A-C}$ respectively) and higher when the first test of each day was compared (8.4).

6.6.2 The future of the optimized carbon monoxide rebreathing method in anaesthesia and perioperative care

The most promising discovery was that clearing carboxyhaemoglobin through administration of oxygen alone was effective enough to ensure safe carboxyhaemoglobin levels in non-trained individuals and to allow repeat testing within three hours. This clearance method is likely to be the only option for many patients, whose co-morbidities might preclude them from performing exercise. This may allow clinically useful serial measurements to assess blood volume, plasma volume and tHb-mass over the perioperative period, and thus the accurate quantification of blood loss. This extends the potential application of tHb-mass testing in the clinical environment. In the future it may be possible to assess rapid changes in plasma or blood volume and tHb-mass, such as might occur in surgery.

Future studies should seek to investigate the feasibility of duplicate tHb-mass testing in a single day in specific patient groups, and in the perioperative period in particular. A range of ages and comorbidities should be sought. Modification of the oCOR for use in ventilated subjects in the theatre or intensive care environment would also be of great value.

This study represents a step towards successfully integrating the use of the oCOR method as a point-of-care clinical technique. It has become increasingly apparent that [Hb] (and thus the definition of anaemia) is strongly influenced by plasma volume, which can be inappropriately expanded in certain disease states (28). Therefore, efforts to readily measure tHb-mass, as a means of deriving plasma content, are required in clinical medicine. The oCOR method is portable, minimally invasive, quick, and user-friendly.

6.7 Conclusions

The oCOR method is a safe technique that can be repeated within three hours when carbon monoxide is suitably cleared between tests in healthy non-athletic individuals. Using oxygen therapy alone adequately achieves this. This method has minimal bias and good precision making it attractive for regular monitoring of blood volume derivatives. Clearance using oxygen alone can safely be used in healthy volunteers with a high degree of precision and safety and it is possible that this could translate to clinical subjects.

Chapter 7 - A carbon monoxide 'single breath' method to measure total haemoglobin mass- a feasibility study

This data included in this chapter has been published in the journal ***Experimental Physiology*** (4) (Appendix E)

Krehl LM, Plumb JOM, Wachsmuth NB, Haupt S, Kumar SB, Otto JM, et al. A carbon monoxide 'single breath' method to measure total haemoglobin mass: a feasibility study. Exp Physiol. 2021;106(2):567–75.

7.1 Chapter in context

A long-term aspiration of this research was to develop a modification of the oCOR that can be readily applied to patients on a mechanical ventilator, either in the operating theatre or critical care unit. The reasoning behind this was to allow clinicians at the bedside a reliable, accurate assessment of blood volume and tHb-mass. At the present time such a method does not exist, and clinicians are often 'shooting in the dark' when making assessments of blood volume, plasma volume and any haemoglobin deficit. Transfusion triggers within perioperative medicine and critical care remain controversial (see section 3.6).

We set out with some preliminary experiments in healthy volunteers to explore how this might be achieved. Part of this process involved adapting the technique and the other part involved the design and building of a novel piece of equipment to make the measurements. It was felt that this pilot work was an essential first step in the process of potentially developing not only a new test but also a new piece of equipment to carry out the test.

7.2 Abstract

Background

Assessing changes in plasma volume (PV) and haemoglobin mass (tHb-mass) may have substantial clinical utility in guiding red cell transfusion and cardiovascular management. In spontaneously breathing patients, these can be measured through the re-breathing of carbon monoxide (the 'optimized carbon monoxide re-breathing method', (*oCOR*)). Surgical or intensive care unit (ICU) patients may benefit from such measurements, but many are mechanically ventilated. We thus sought to develop a method of measuring tHb-mass in such patients. Here, we describe it, and offer preliminary data relating to its feasibility and agreeability compared to the *oCOR*.

Methods

We measured tHb-mass in thirteen healthy volunteers using the original standard oCOR method (*oCOR1*). We then applied our adapted methodology (*PROCnew*) in a situation simulating mechanical ventilation. Briefly this included a single breath in of CO gas with

collection of all exhaled gas via a one-way valve to simulate mechanical ventilation (with no re-breathing) until the final blood sample.

Ten participants also had a second *oCOR* (oCOR2) to assess the reliability of the original procedure (oCOR1). Meaning that ten subjects had *oCOR1*, *oCOR2* and *PROCnew* (three separate tests). Testing occurred on three separate days with each test a minimum of twenty-four hours apart and all three occurring within a nine-day period. One participant's data was excluded due to technical problems.

Results

The typical error of measurement (TE) for the 2 *oCOR* measurements was 2.1%. However, for *PROCnew* vs. *oCOR1 & 2* it was 8.05% and 9.23% respectively. However, for six of the twelve participants *oCOR1* vs. PROCnew the TE was 2.29%.

Conclusions

Although it is feasible to measure tHb-mass using a 'single breath technique' it is not agreeable with the standard oCOR method. Further refinement of the technique will be required if it is to be used to measure tHb-mass in mechanically ventilated patients.

7.3 Introduction

Haemoglobin is the oxygen-carrying pigment of the circulation. Its circulating concentration ([Hb]) is routinely measured in clinical practice, and low values used to define 'anaemia' (136). However, [Hb] is determined by the total circulating mass of Hb (tHb-mass) and the volume of plasma (PV) in which it is carried. The measurement of such independent variables has distinct advantages given that PV can change substantially with disease, thus altering haemoglobin concentration ([Hb]) when tHb-mass itself has not changed. The importance of total haemoglobin mass measurement (tHb-mass) in the clinical setting and the advantages over haemoglobin concentration [Hb] have been discussed previously (1,3,28,34). Indeed, [Hb] correlates poorly with tHb-mass in patients with chronic liver disease or heart failure, in whom PV may be expanded (28). Despite this, [Hb] is generally used as a trigger for the transfusion of red blood cells.

Likewise, perioperative changes in tHb-mass and PV are common (297,298) due to blood loss, administration of red blood cells, haemodilution or salt/water retention due to the 'surgical stress response' (299). Fluid distribution between physiological compartments and the impact of hypo/hypervolemia on the glycocalyx, and therefore the functional integrity of the intravascular space also influence PV (300). However, decision to transfuse blood to a patient in clinical practice in general, and the perioperative and critical care settings in particular, hinges on a variety of factors. There is a growing recognition that [Hb] may not be the best clinical indicator to guide such decisions (1,58).

Attempts to measure tHb-mass and blood volume in this setting have been made before. In the early 1990s *Christensen* et al published two separate papers. In the first experiment they used the modified method as described by *Sjöstrand* in nine healthy subjects and nine subjects in an intensive care unit using a modified Water's circuit. The COHb rise was 2.1-3.9% which as discussed in Chapter 5 may well not have been high enough for accurate or precise results. Repeat measures in the two groups had coefficients of variation of 6.2 and

4.7% which are significantly greater than we would hope for using the standard oCOR (81). In a second publication they compared their modified method with radiolabelled red cells (using C^{51}) and radiolabelled albumin (using ^{125}I). In 12 mechanically ventilated patients on their ICU who were on <0.8 inspired FiO_2 they employed both methods. The study did not describe the time period between the two methods. Importantly they used a computer model to estimate the handling of CO in the lung which made assumptions about the amount of CO that passed into the pulmonary capillaries and that which may have been left in the alveoli. The mean rise in the CO study was only 3.6%, again lower than what we would usually aim for. The time taken for equilibration in the ventilated subjects was longer than in the healthy volunteers. The blood volume variation compared using Bland Altman stated the 95% limits of agreement were 540ml (± 2SD). This could represent ~ 10% of the blood volume, which would be a large degree of variation between the two methods. The study represented a very interesting concept but acknowledged that further refinements would be required to ascertain reproducibility (82)a.

Using the inhalation of a known volume of carbon monoxide (thus labelling Hb as carboxyhaemoglobin (COHb)) allows measurement of tHb-mass and thus calculation of plasma volume. In self-ventilating subjects, this is achieved using the so-called 'optimized carbon monoxide (CO) re-breathing method (oCOR)' (see 4.1 for details). However, this relies upon the participant being alert, able to follow instructions, and crucially being able to re-breathe the CO in a closed circuit. This precludes the use of oCOR in subjects who are receiving mandatory ventilation from a mechanical ventilator, either under anaesthesia or when sedated on the intensive care unit.

We hypothesised that delivery of a single carbon monoxide bolus into the breathing circuit of a participant without the need for re-breathing could be used to reliably measure tHb-mass, so long as exhaled gas could be analysed. We thus sought to develop such a technique. We here describe this development, and early data relating to its likely reliability.

The primary aim of this study was to develop a new method for measuring tHb-mass in healthy participants simulating a procedure that *might* be used in participants on a mechanical ventilator (*PROCnew*) and to evaluate the feasibility and reliability of this novel method. We aimed to assess reliability compared to the standard optimized carbon monoxide (CO) re-breathing method (*oCOR*). We also repeated the oCOR test to quantify reliability of the standard method within this experiment.

7.4 Methods

This feasibility study took place at University Hospital Southampton NHS Foundation Trust between May 2017 and July 2017. Ethical approval was granted by the South-Central Hampshire B Research Ethics Committee (REC reference: 15/SC/0496). Local permissions were received from the University of Southampton (ERGO ID: 31245), University Hospital Southampton NHS Foundation Trust (R&D CRI 0327) and Southampton Centre for Biomedical Research NIHR Clinical Research Facility. The study was performed in accordance with the ethical standard set by the Declaration of Helsinki. Written informed consent was obtained from all participants.

Healthy adults aged over sixteen years who were physically able to perform the testing protocol were eligible for recruitment. Excluded were adults lacking mental capacity to

consent, pregnant women, smokers, prisoners, participants with a baseline carboxyhaemoglobin level > 5%, or participants with haemoglobinopathies. No participants had stayed at an altitude higher than 1500m for any time in the preceding year. tHb-mass was measured in those recruited using the original oCOR method, and our new methodology (*PROCnew*).

As this was a feasibility study we aimed to recruit 10 subjects for the full study but started with just 3 in a preliminary study to ensure that the newly established equipment worked safely and it was actually possible to carry out the experiments. It was felt that 10 subjects would give an adequate estimation of reliability of the new method based on data from professor Schmidt's and our own previous research examining reliability when measuring tHb-mass. We had already demonstrated very precise measurements in healthy subjects and in subjects with chronic liver disease in our laboratory prior to commencing this study. It was felt that 10 subjects would be enough to ascertain if the methodology worked at all or if indeed it required significant modification prior to any further studies being undertaken.

7.4.1 Optimized Carbon Monoxide Rebreathing Method

All participants completed a baseline carbon monoxide rebreathing test (oCOR) (detailed in 4.1) and described previously (3). Briefly, an intravenous cannula was inserted into the participant's upper limb. Participants were seated and inactive for fifteen minutes before inhaling 0.8-1 $ml \cdot kg^{-1}$ of CO in three litres of 100% oxygen via a glass spirometer (BloodTec, Bayreuth, Germany), and rebreathing (via a CO_2 scrubber) for two minutes whilst wearing a nose clip. A portable CO gas detector (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) was used to identify CO leakage at the nose, mouthpiece, and spirometer. Carboxyhaemoglobin percentage values (Radiometer, ABL800 FLEX) were determined from venous blood samples (Na-heparinized syringes: RAPIDLyte, Siemens Healthcare Diagnostics Inc, USA) taken before (at baseline) and at six and eight minutes after administration of CO gas. Each sample was analysed three times within thirty minutes of collection. The analyser was subjected to regular maintenance and quality control checks, and its accuracy validated (122). [Hb] and haematocrit values were measured (Sysmex XN10, Sysmex, Milton Keynes UK).

7.4.2 'Single breath technique' to measure tHb-mass (PROCnew)

Participants were cannulated and rested as above. As before, participants inhaled 0.8-1 $ml \cdot kg^{-1}$ of CO in 3 litres of 100% oxygen and held their breath for ten seconds with a nose clip on. They then exhaled, this gas passing via a Hans Rudolph one-way valve (Hans Rudolph, Kansas, USA) and anaesthetic breathing tubing (Intersurgical UK Ltd) through a gas flow meter (to measure exhaled volume) and into an airtight gas collection bag where CO concentration was measured by a gas analyser every 30 seconds (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany). They continued this process via the one-way valve so that all exhaled gas was collected for the duration of the test (eight minutes after inhalational in this case). None of the inhaled CO gas was at any stage re-breathed. Blood sampling occurred at baseline, four, six, eight, ten, twelve and fifteen minutes for the first three participants (see below). For the remaining ten participants sampling was as for the oCOR with the exception of an additional sample at four minutes.

In a pilot evaluation, three participants underwent oCOR, followed twenty-four hours later by *PROCnew*. Following full data analysis, a further ten participants were enrolled into the main study. They undertook three separate days of tHb-mass testing within a nine-day period: oCOR on day one (*oCOR1*), and a second oCOR (*oCOR2)* and *PROCnew* on two other days. The order of *oCOR2* and *PROCnew* was varied in a pseudo-random fashion by the investigators. Of the ten participants, three had the three tests on consecutive days, and seven had them over a nine-day period.

7.4.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c for Apple Macintosh OSX). The Shapiro-Wilk test for normal distribution was used. Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%).

This was a feasibility study, so a formal power calculation was not required. The aim was to recruit ten participants (for the full pilot study) on the advice of Professor *Schmidt* (personal communication) based on the work of their group having performed thousands of tHb-mass measurements. They use a number of ten test/retests to ensure personal operator quality assurance. The statistical principles of test/retest reliability measures as described by *Hopkins* et al (83) would also suggest that ten patients are sufficient to get a meaningful estimate of typical error (TE) for an individual tester. The lead author had performed over 100 oCOR tests prior to this study and had a TE in keeping with the published literature (3,28,34)

Test-retest data (repeated measures from the same patient) are presented using Bland-Altman plots with limits of agreement (277). Additionally, a specific approach to compute reliability statistics to compare test-retest performance was used see (4.3.1 for further detail) (83). Typical error of measurement (TE) for tHb-mass was calculated and expressed as coefficient of variation with 95% confidence limits (CL), derived from χ^2 distributions. All tests were two-sided and statistical significance was set at $p<0.05$. TE includes random error (analytic error arising from using the method-specific apparatus and intra-individual biological variation) but not systematic error (bias) (8,13,126). Reported studies using the oCOR have commonly reported this method (70,113,126,291,292).

7.5 Results

In total thirteen participants (seven female, six male) were included in the study, with age (median [range]) 32 [23 – 39] years, height (±SD) 173.4 ± 10.6 cm, and body mass 78.5 ± 22.6 kg. Three participants underwent a pilot evaluation (with full analysis) prior to ten subjects undergoing the full three separate OCOR tests (Table 8). One participant (number 7) *oCOR1* and *oCOR 2* differed by more than 100g and the results have been removed due to unexplained unacceptable variation. This far exceeds the variance of 2.0% for the oCOR published by our group and others (3,8,69). This was either due to an undetected leak or a relatively low ΔCOHb% of 4.7% in *oCOR1*.

The following analysis is based on the remaining twelve participants, nine of whom underwent *oCOR1*, *oCOR2* and *PROCnew* and three of whom underwent *oCOR 1* and *PROCnew* only.

Participant	*oCOR1*	*oCOR2*	*PROCnew*
1	577.8		581.7
2	983.9		921.4
3	1008.6		1019.1
4	845.2	844	683.6
5	463.6	467.2	440.6
6	446	455.9	440.2
7	*Data excluded 1225.6*	*Data excluded 1130.1*	*Data excluded 1201.8*
8	427.5	447.1	550.6
9	876.6	908.3	836.8
10	730.3	689	787
11	863.6	867.3	755.1
12	748.6	756.7	700.4
13	878.6	903.0	826.1
Mean or median	737.5g ± 209.5g	756.7g [461.4g-885.2g]	711.9g ± 182.3g
Typical error % (95% CL)	oCOR1 vs. oCOR2 TE= 2.1 (1.5-3.6)	oCOR1 vs. PROCnew TE= 8.05 (6.02-12.48)	oCOR2 vs. PROCnew TE= 9.23 (6.63-15.79)

Table 9: Raw data tHb-mass in grams from each of the three procedures. Participants 1-3 pre-pilot data did not have a second oCOR test. Participant's 7 values differed by over 100g between oCOR 1 and oCOR 2, so their data was not analysed due to clear technical error with the measurement. In our laboratory a 50g difference in an average participant would warrant a repeat test. Data has been included in the table for completeness.

7.5.1 oCOR1 versus oCOR2

Test re-test reproducibility as demonstrated by these data was in keeping with our previously published work and is in keeping with widely reported typical errors of measurement as described by Hopkins (83). The typical error (CL) for *oCOR1* vs. *oCOR 2* was 2.1 (1.5-3.6). tHb-mass was 737.5g ± 209.5g for *oCOR1*, and 756.7g [461.4g-885.2g] for *oCOR2*.

Table 9 shows the raw data for tHb-mass and the typical error (TE) (See 4.3.1). The mean rise in carboxyhaemoglobin (ΔCOHb%) was 5.8% for *oCOR1*, 6.1% *oCOR2*.

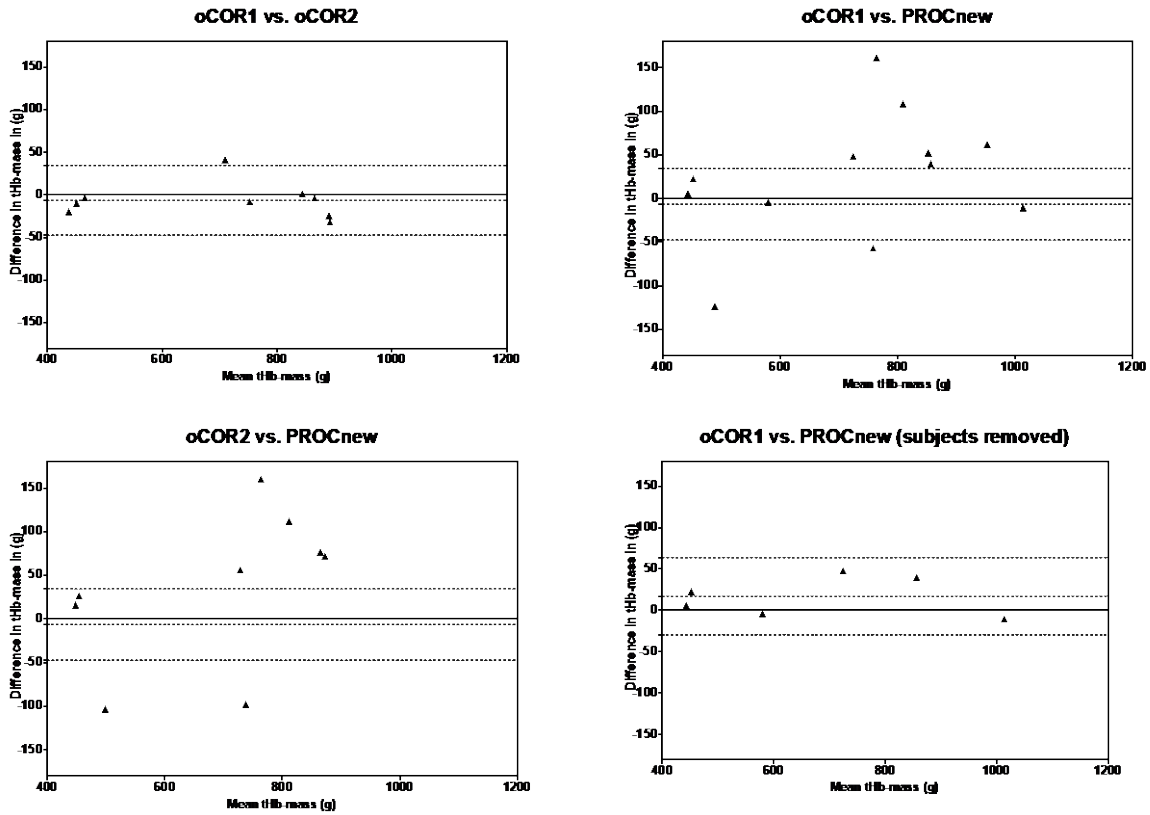


Figure 16: Bland-Altman plots with limits of agreement and bias

7.5.2 oCOR1 & oCOR2 versus. PROCnew

tHb-mass for *PROCnew* was 711.9g ± 182.3g with a (ΔCOHb%) of 4.8%. The agreeability of *oCOR1&2* and *PROCnew* had the flowing typical errors; *oCOR1* vs. *PROCnew* TE= 8.05 (6.02-12.48), *oCOR2* vs. *PROCnew* TE= 9.23 (6.63-15.79).

Figure 16 shows the Bland Altman plots for the three procedures *oCOR1*, *oCOR2* and *PROCnew* with limits of agreement (277). Bland Altman plots biases for *oCOR1* vs. *oCOR2* were -6.5, for *oCOR1* vs. *PROCnew* 25.63 and for *oCOR2* vs. *PROCnew* 35.33.

In a post-hoc sub-analysis of the new method (*PROCnew*) vs. the standard method (*oCOR1*) we excluded all values where there was a greater than 50g difference in tHb-mass (a difference that we would usually aspire to be below in our laboratory, in keeping with our published reproducibility (3)). This left six participants from twelve. Participants 1, 3, 5, 6, 9 &12 (Table 9) of these results the TE was 2.29% (CL- 1.54-4.78%) (Figure 16).

7.6 Discussion

In this study we have for the first time demonstrated that it is feasible to measure tHb-mass with a modified 'single breath technique' (*PROCnew*). We wished to identify a way to measure tHb-mass in mechanically ventilated patients. We thus explored whether it is possible to measure tHb-mass using a single-breath inhalation (and ten-second breath-hold) of carbon monoxide. Exhaled gas was collected and measured for the duration of the testing period (eight minutes). Our data suggests that (*PROCnew*) was only sufficiently agreeable with the standard oCOR method in half of the subjects studied.

Modifications to the carbon monoxide re-breathing technique for the measurement of tHb-mass have been made many times since the technique was revived in 1990 by Fogh-Anderson (301), notably in 1995 when *Burge* and *Skinner* achieved improved precision of the measurement (74). The current technique described by *Schmidt* and *Prommer* reduced the re-breathing period to only two minutes to improve convenience for participants (5). The finding that a bolus of CO gas inhaled with a single breath and only re-breathed for two minutes could produce an adequate rise in carboxyhaemoglobin COHb% was unknown prior to that study.

When compared to the standard oCOR method, a single breath CO-inhalation did not produce agreeable (compared to the standard oCOR method) results across all subjects. Overall, six subjects (participants 1, 3, 5, 6, 9 & 12,Table 9) exhibited a level of agreeability consistent with validity criteria for the index test (3,5,8) (oCOR) when comparing *oCOR1* to *PROCnew* with a TE of 2.29% (See Table 9 and Figure 16 D). However, tHb-mass data were discordant in the other 6- TE- *oCOR1* vs. *PROCnew* = 8.05% & *oCOR2* vs. *PROCnew* = 9.23% (See Table 9 and Figure 16) Possible reasons for this are explored below. In 8/12, participants the tHb-mass measurement was lower in *PROCnew* than *oCOR1* suggesting that a possible inadequate rise in COHb% occurred between baseline and the 7-minute mean measurement (ΔCOHb%). Analysing this in more detail, the participants with the largest discrepancies between *oCOR1* and *PROCnew* had lower rises in ΔCOHb% during *PROCnew*. However, this was not the case in every subject with a large discrepancy and due to the relatively low numbers in this study, firm conclusions on this matter are not possible. In our laboratory we aim for a rise in COHb% (ΔCOHb%) of ~5-6%. There has been extensive debate in the literature about the merits of having a higher (ΔCOHb%) versus the increased toxicity risk (113,123,125). Complete CO mixing throughout the entire blood compartment prior to blood sampling is crucial, and it is conceivable that different individuals in this study took differing initial breaths in, both in terms of volume and speed of airflow. It is possible that a higher CO bolus aiming for ΔCOHb% of ~5-6% during *PROCnew* may have improved precision and should certainly form part of further studies to refine *PROCnew.* Alternatively, a ten second initial breath hold may be inadequate to achieve sufficient CO uptake without subsequent re-breathing as per the standard oCOR. Finally, the accuracy of the CO analyser becomes increasingly important when exhaled volumes are larger: a change as small as 20 ppm in measured concentration could result in 25g difference in tHb-mass, and the quoted detection error for the device we used is ± 2 ppm (direct communication with Dräger).

Strengths of this study include novelty (to our knowledge, this is the first attempt to achieve this goal), the fact that the same operators performed all of the experiments in the same laboratory with the same equipment and that the test-retest reliability of 2.1% was good and in keeping with our previous work and that reported in the wider literature (3,13,69). Weaknesses include the relatively modest sample size, the simulated "mandatory" ventilation using self-ventilating healthy volunteers. The fact that we did not

repeat *PROCnew* also does not help answer the precision versus accuracy question of this new method which might be precise even if not accurate, although accuracy is difficult to quantify when speaking to tHb-mass due to there being no true gold standard and no published 'normal' ranges. We deliberately chose not to repeat *PROCnew* in this initial study as we wished to establish the feasibility of the method rather than assess precision and reproducibility.

Future studies should thus examine the use of larger CO boluses, aiming for at least a 5% ΔCOHb%. Experimenting with longer initial breath holding may also be beneficial. Comparison alongside refinement of multiple *PROCnew* tests to assess test re-test reliability would be the next logical step.

7.7 Conclusions

Although it is feasible to measure tHb-mass using a 'single breath technique' it cannot yet be reliably assessed using a single CO breath. Further refinement of the technique will be required if it is to be used to measure tHb-mass in mechanically ventilated patients.

Chapter 8 - Cardiopulmonary exercise testing before and after intravenous iron: a prospective clinical study

Short title: CAPOEIRA-I STUDY

CArdio **P**ulm**O**nary **E**xercise testing and **I**nt**RA**venous **I**ron

8.1 Chapter in context

The CAPOEIRA-I study was the final study of this thesis and sought to bring together many of the themes explored within the earlier chapters and indeed from previous work building on our published data on tHb-mass and cardiopulmonary exercise testing.

This pilot study looked to first establish the feasibility of recruiting and measuring tHb-mass and CPET in a cohort of pre-surgical patients within a typical NHS perioperative pathway. One of the major barriers to large perioperative trials within the UK NHS has been the difficulty of recruiting patients to an interventional trial with enough lead time between their pre-assessment and surgery date. This is particularly pertinent in cancer trials.

After some consultation with patients, I was able to build into the ethics application and protocol design a system whereby we could recruit patients quickly at the time of their planned CPET appointment via the clinical integration of our preoperative anaemia and CPET services.

8.2 Abstract

Introduction

Anaemia is associated with adverse perioperative outcomes and impaired physical fitness. Correction of anaemia before surgery through intravenous iron therapy is becoming common practice. We set out to explore the relationship between exercise capacity, anaemia, and haemoglobin content in an elective surgical cohort. Specifically, we aimed to test the hypothesis that augmenting haemoglobin through intravenous iron therapy would improve preoperative physical fitness (cardiopulmonary exercise testing (CPET) variables).

Methods

We performed a pilot feasibility study in adult surgical patients having CPET as part of routine care and who were referred to the perioperative anaemia service with a haemoglobin concentration ([Hb]) <130 $g \cdot l^{-1}$ and found to be iron deficient/replete. Patients

underwent CPET and tHb-mass measurements before and a minimum of 13 days after receiving intravenous (i.v.) Iron Isomaltoside 1000 (Monofer®).

Results

Twenty-six subjects being investigated and worked up for potential surgery were recruited, of which 6 withdrew prior to study completion. There was a (mean ± SD) of 25 ± 7 days between baseline visit (where patients received intravenous iron) and the final visit. Haemoglobin concentration ([Hb]) and tHb-mass increased following i.v. iron: ([Hb] (mean ± SD) 109 ± 14 $g.l^{-1}$ to 116 ±12 $g.l^{-1}$, a rise of 6.2%, with a mean difference of 7.3$g.l^{-1}$, p= <0.0001; tHb-mass 497 ± 134.0 g to 546. ± 139g, a rise of 9.3%, mean difference 49g (CI 29.36 to 69.16) p= <0.0001). CPET variables increased (Oxygen consumption at anaerobic threshold ($\dot{V}O_{2\ AT}$): 9.14 ± 1.7 $ml{\cdot}kg^{-1}min^{-1}$ at baseline and 9.77 ± 2.5 $ml{\cdot}kg^{-1}min^{-1}$, p= 0.0964; Peak oxygen consumption ($\dot{V}O_{2\ peak}$): 15.2 ± 4.1 $ml{\cdot}kg^{-1}min^{-1}$ to 16.3 ± 4.4 $ml{\cdot}kg^{-1}min^{-1}$, p= 0.0161.

Conclusions

Performing a baseline CPET, measuring tHb-mass, administering i.v. iron and then repeating CPET is feasible in anaemic, iron deficient patients within their usual pre-operative pathway. Pre-operative administration of intravenous iron to iron deficient anaemic patients is associated with increases in [Hb], tHb-mass and peak exertional oxygen consumption. Appropriately powered studies are required to determine the relationship between such responses and the degree of iron deficiency, whether $\dot{V}O_{2\ AT}$ is also beneficially impacted, and whether such improvements might be associated with improved perioperative outcome.

8.3 Introduction

Anaemia, defined by the World Health Organisation (WHO) as a haemoglobin concentration ([Hb]) <130 $g{\cdot}l^{-1}$ in men and <120 $g{\cdot}l^{-1}$ in women, is identified in around 30% of elective surgical patients and is associated with adverse perioperative outcomes (18). However, it is unclear whether this association is directly causal (156); anaemia may be associated with more severe or advanced disease, for instance, or with increased requirements for red cell transfusion (which may independently impact outcome) (17,18).

One mechanism by which anaemia may directly impact surgical outcome is through its effects on aerobic exercise capacity. Pre-operative exercise capacity is associated with surgical outcome, with less fit patients at a greater risk of complications and suffering a higher mortality rate (302) (303). Maximal oxygen uptake ($\dot{V}O_{2\ max)}$ is dependent on adequate oxygen delivery to respiring tissues: a function of blood oxygen (O_2) content (haemoglobin content and its saturation with oxygen) and blood flow (cardiac output, and regional distribution). Whilst debated, cardiac output is believed to primarily limit maximal exertional oxygen consumption in otherwise healthy subjects (204). However, in the presence of severe anaemia, oxygen content may impact aerobic exercise capacity. The O_2 carrying capacity of arterial blood is largely influenced by the haemoglobin content (or total haemoglobin mass, tHb-mass). [Hb] is determined by the plasma volume (PV) in which the tHb-mass is carried. As such, it shows a greater day-to-day fluctuation than does tHb-mass, and consequently is a less precise measure of O_2 carrying capacity (34). The

hypothesis that the association of anaemia with impaired perioperative outcomes may be in part mediated through impacts on physical fitness thus merits investigation.

Cardiopulmonary exercise testing (CPET) represents the gold standard method by which $\dot{V}O_{2\ max}$ is measured. Cross-sectional cohort studies have reported lower exercise capacity (peak oxygen uptake, $\dot{V}O_{2\ peak}$) and anaerobic threshold (VO_{2AT}) in anaemic elective surgical patients (263). An increase in [Hb] should increase oxygen delivery to the tissues and might therefore affect the transition of aerobic to anaerobic glycolysis. However, this is not clear cut, with several studies conducted in athletes (35) and diabetic patients (60) suggesting little correlation of [Hb] with oxygen consumption ($\dot{V}O_2$) at peak and at anaerobic threshold (33). One of the reasons that tHb-mass has a better correlation is due to the dual role it plays in this; on the one hand it determines [Hb] in concert with the total blood volume but it also raises blood volume via erythrocyte volume and this double effect explains the superior correlation described by *Schmidt* and *Otto* (33–35).

In elite athletes all other elements of oxygen delivery are optimised, and thus increasing blood volume and haemoglobin content have both improved $\dot{V}O_{2\ peak}$, $\dot{V}O_{2\ AT}$ and physical performance (1). This may not hold true in patients, amongst whom other factors may be limiting. Nonetheless, there are some (albeit limited) data to support a similar favourable impact on exercise capacity after red cell transfusion in adult patients with stable haematological conditions (288) and in children with severe beta-thalassemia (247). Exercise capacity also improves in patients with chronic heart failure and iron deficiency in response to intravenous (i.v.) iron (197). However, the effect of i.v. iron on CPET variables in anaemic preoperative patients remains unexplored. This is of importance given that impairments in CPET-derived physical fitness are associated with an increased risk of adverse postoperative outcome (304) and thus optimising functional status preoperatively may reduce these risks. It is unclear why and/or if the anaerobic threshold is affected by changes in oxygen content. This may be physiologically plausible via increases in exercise via other mechanisms or changes in cytochromes involved in electron transport.

Therefore, we aimed to explore the relationship between exercise capacity, anaemia and haemoglobin content in an elective surgical cohort. The primary objectives were twofold:
1. To evaluate the feasibility of performing CPET before and after i.v. iron administration in iron deficient and anaemic elective surgical patients.
2. To test the hypothesis that augmenting tHb-mass and [Hb] through intravenous iron therapy may improve peak oxygen consumption ($\dot{V}O_{2\ peak}$) and oxygen consumption at anaerobic threshold ($\dot{V}O_{2\ AT}$) as quantified by CPET.

8.4 Methods

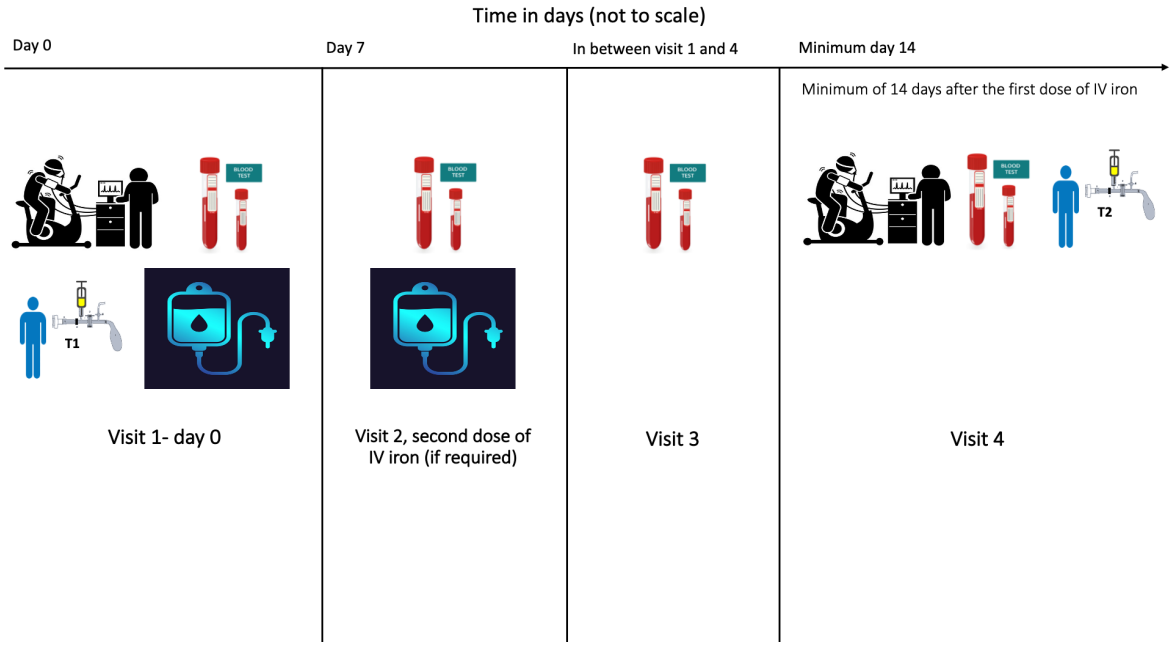
The study took place at University Hospital Southampton (UHS) NHS Foundation Trust between February and October 2018. Ethical approval was granted by the London - Surrey Research Ethics Committee and NHS Health Research Authority (REC reference 17/LO/2061). Local permissions were received from the University of Southampton (ERGO ID: 31688), University Hospital Southampton NHS Foundation Trust (R&D CRI 0357) and Southampton Centre for Biomedical Research Clinical Research Facility. The study was performed in accordance with the ethical standard set by the Declaration of Helsinki. Written informed consent was obtained from all participants. It was registered on ClinicalTrials.gov PRS with the unique Protocol ID- NCT 033 46213.

We aimed to recruit 25 patients in order to ensure that the feasibility of exploring the effect of i.v. iron on CPET variables, could be reasonably explored. For clarity, this study was not specifically powered to detect such differences in $\dot{V}O_{2\ AT}$ or $\dot{V}O_{2\ peak}$, but data from it were intended to be used in order to design a future adequately powered study. This number was based previous related work by wright et al who recruited 20 subjects (288). We aimed to recruit slightly above this to allow for any dropouts or lost datasets.

Adults >18 years (and over 50 kg) who were having pre-operative CPET as part of routine clinical care were recruited. Inclusion criteria were anaemia with a [Hb] <130 $g{\cdot}l^{-1}$ *and* iron deficiency (being iron restricted/deplete) or the functionally iron deficient (see Appendix C- UHS protocol for i.v. iron (POAS)). Patients were recruited via clinical surgical teams, from the perioperative optimisation of anaemia before surgery (POAS) service or the clinical CPET service. Excluded were pregnant women; prisoners; those with known allergy/hypersensitivity to Monofer™ (Iron Isomaltoside 1000) or any of its excipients, or to any parenteral iron products; those suffering from haemochromatosis or other iron overload states, acute liver or renal failure, active infection or haemoglobinopathies (e.g., Sickle Cell Anaemia or Thalassemia); those with other causes of anaemia (e.g. haematological malignancy, haemolysis, hypothyroidism); those receiving a red cell transfusion before a second CPET could be performed; and those unable to perform CPET or in whom such testing was contraindicated (Appendix C- UHS protocol for i.v. iron (POAS)).

8.4.1 Study pathway (see Figure 17)

After consent patients underwent a baseline CPET and tHb-mass testing (often but not always on the same day) in addition to a standard panel of bloods (Appendix D). They then received dose 1 of intravenous iron. Dosing was based on the UHS POAS protocol. Iron Isomaltoside 1000 (Monofer®) was used with the maximum allowable dose administered as per the European Medicines Agency Summary of product characteristics (SmPC). In instances where a second dose was required this occurred exactly one week after the initial infusion. All infusions were undertaken in the Welcome Trust clinical research facility at UHS and were overseen by the lead investigator. Participants came back a further 2 times (spread out once surgery date was known) for repeat blood tests before coming for their final visit (4^{th} visit) to repeat CPET and tHb-mass measurements (always in that order due to the small risk of CO gas worsening CPET performance). The study period ended at the end of the final visit (see Figure 17).



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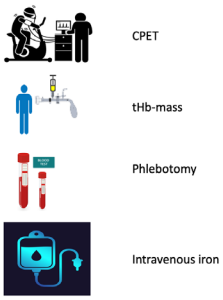


Figure 17: Study procedures

8.4.2 Optimised Carbon Monoxide Rebreathing Method

Subjects completed a baseline carbon monoxide rebreathing test (oCOR), (details of this method can be found in Chapter 4). Briefly, an intravenous cannula was inserted into the subject's upper limb. Subjects were then seated and inactive for 15 minutes before inhaling 0.8-1ml·kg^{-1} of carbon monoxide (CO) mixed with 3 litres of 100% oxygen via a glass spirometer (BloodTec, Bayreuth, Germany), which was rebreathed (via a CO_2 scrubber) for 2 minutes whilst wearing a nose clip. A portable CO gas detector (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) was used to detect possible CO leakage at the nose, mouthpiece and spirometer during rebreathing.

Carboxyhaemoglobin percentage was determined in venous blood samples drawn into Na-heparinised syringes (RAPIDLyte, Siemens Healthcare Diagnostics Inc, USA) before and at 6 and 8 minutes after administration of CO gas (analysis using a laboratory blood gas analyser; Radiometer, ABL800 FLEX). Each sample was analysed three times within 1 hour of collection. The analyser was subjected to regular maintenance and quality control checks; the accuracy of which has been evaluated elsewhere (122). [Hb] and haematocrit

values were measured using HemoCue (HemoCue AB, Radiometer, Sweden) and the blood gas analyser (Radiometer, ABL800 FLEX, Copenhagen) respectively.

8.4.3 Cardiopulmonary exercise test (CPET)

Patients cycled on an electromagnetically braked ergometer (Ergoline 2000, Ergoline GmbH, Bitz, Baden-Württemberg, Germany). With respiratory gas analysis made by calibrated metabolic carts (Geratherm Respiratory GmbH; Love Medical Ltd, Manchester, UK). Breath-by-breath $\dot{V}O_2$ and carbon dioxide output were recorded, concurrently with minute ventilation, tidal volume, respiratory rate and end-tidal gas tensions for O_2 and CO_2. Patients were connected to appropriate monitoring equipment and rested for an initial 3 min period, thereafter, completing 3 mins of unloaded cycling. Subsequently, patients performed a symptom-limited incremental ramp test set to 10–20 $W \cdot min^{-1}$ (this was based on patient weight and age allowing adjustment for clinical status and current activity levels) to deliver an intended test duration of 8–12 min before volitional exhaustion. Test cessation occurred at patient exhaustion or when the cadence reduced below 40 r.p.m. for more than 30 s despite verbal encouragement. After stopping CPET, patients completed a period of unloaded cycling to 'cool down'.

The anaerobic threshold ($\dot{V}O_{2\,AT}$ expressed in millilitres $\dot{V}O_2$ per kilogram per minute, $ml \cdot kg^{-1} min^{-1}$) was determined by a clinical exercise physiologist and consultant physician (independently of each other) both skilled in CPET interpretation, using the modified V-slope method with corroboration by ventilatory equivalents and end-tidal gas tensions for O_2 and CO_2 (263). The highest average $\dot{V}O_2$ throughout the final 20s of exercise was recorded as the $\dot{V}O_2$ peak ($ml \cdot kg^{-1} min^{-1}$) (305).

8.4.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 8.4.2c for Apple Macintosh OSX 10.14.4) and SPSS Statistics (version 25 for Apple Macintosh Chicago, IL, USA). The D'Agostino & Pearson normality test (omnibus K2 test) for normal distribution was used. Paired t-tests were used to compare variables measured before and after intravenous iron if normally distributed and included tHb-mass, $\dot{V}O_{2\,peak}$, transferrin levels, exercise time and peak wattage. Wilcoxon signed rank test was used to compare non-parametric data which included [Hb], transferrin saturation (TSAT) and iron levels. All tests were two-sided with a significance level of 0.05. Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). As this was a pilot study no formal a priori power calculation was performed. However, the sample analysed (n= 20) is in keeping with previous studies in this area (288).

Differences in between variables were assessed using the t-test or Mann-Whitney test, where appropriate. Test-retest data (repeated measures in one and the same patient) were presented using Bland-Altman plots with limits of agreement (277). Additionally, a specific approach to compute reliability statistics to compare test-retest performance was used (see Chapter 4 for further detail) (83).

G*Power was used for a post hoc power calculations using an alpha error of 0.05 and a beta error of 0.8. The primary end point was a change in $\dot{V}O_{2\ peak}$ of 0.25 $ml \cdot kg^{-1} min^{-1}$.

8.5 Results

8.5.1 Baseline characteristics and data loss

Twenty-six patients were recruited, of whom 6 withdrew prior to the study completion (see Table 10). Of the remaining 20 patients who underwent all aspects of the study protocol, 1 had an uninterpretable tHb-mass test and 3 had aspects of their CPET that were uninterpretable. All 20 patients are included in the final analysis but only 17 have complete datasets. The 20 participants had a mean ± SD age of 68 ± 10 years, height 165 ± 9 cm, weight 76.5 ± 20 kg and BMI (median and [range]) 26.4 [23.6-30.7] $kg \cdot m^2$. There was a mean ± SD of 25 ± 7 days between baseline and the final testing visit (Table 11).

Reason for non-completion	Number of patients
Surgery dates expedited preventing second CPET.	3
Did not attend follow up (no reason given).	1
Admitted as an emergency due to rectal bleeding during the study and received a red cell transfusion meaning they were ineligible to continue.	1
Started but did not want to perform a repeat CPET.	1

Table 10: Study protocol deviations

Completed SN	Patient study ID	Gender	Age (years)	Height (cm)	Weight (kg)	BMI ($kg \cdot m^2$)	Days between V1 and V4	Type of surgery
1	1	M	55	186.6	95.7	27.5	13	Colorectal
2	4	F	80	168.5	72.1	25.4	23	Colorectal
3	6	M	79	168.7	83.7	29.4	20	Colorectal (non-cancer)
4	7	M	65	181.2	125.1	38.1	33	Orthopaedics
5	8	M	72	169	87.6	30.7	27	Colorectal
6	9	F	69	161.2	71	27.3	29	Colorectal
7	10	M	72	172.8	76.6	25.7	28	Colorectal
8	12	F	49	146.4	53.2	24.8	21	Colorectal (non-cancer)
9	13	M	82	171.4	78.3	26.7	22	Colorectal
10	14	M	75	158.5	59	23.5	42	UGI
11	15	M	83	170	64.2	22.2	24	Colorectal (exenteration)
12	16	M	73	169	87.1	30.5	22	Colorectal
13	17	F	80	157.3	47.1	19	33	Colorectal
14	18	F	65	158.3	114	45.5	34	Colorectal
15	19	F	66	156.5	63.7	26	14	Colorectal
16	20	F	66	168.5	68	24	14	HPB
17	21	F	55	159.1	55.3	21.8	21	Colorectal
18	22	F	65	155	78.5	32.7	25	Spinal
19	25	F	51	167	92	33	24	Colorectal
20	26	F	74	162.1	57.2	21.8	24	Colorectal (non-cancer)
Mean (±SD) or median [IQR]			**68 ± 10**	**165 ± 9**	**76.5 ± 20**	**26.4 [23.6-30.7]**	**25 ± 7**	
SN= Study number								

Table 11: Individual patient demographics

8.5.2 Safety

No serious adverse events (SAEs) occurred during the study related to either CPET or i.v. iron infusion.

8.5.3 Haematological variables

Following administration of i.v. iron, both [Hb] and tHb-mass rose in all but 4 patients (subjects 5 and 18 for [Hb] and subjects 13 & 19 for tHb-mass: (see Table 12, Table 13 and Figure 18). [Hb] increased from (mean ± SD) 109 ± 14 $g{\cdot}l^{-1}$ to 116 ± 12 $g{\cdot}l^{-1}$ (6.2% rise: mean difference of 7.3 ± 6 $g{\cdot}l^{-1}$, p= <0.0001). tHb-mass rose from 497 ± 134g to 546 ± 139 g (9.3% rise: mean difference 49 g (95% CI: 29 to 69.2, p= <0.0001). Similarly, serum iron concentration rose from 7.2 ± 4.3 to 12.3 ± 4.2 $\mu mol.l^{-1}$ (p< 0.0001) and transferrin saturation from 13.1 ± 8.5 to 25.3 ± 7.6% (p< 0.0001, with transferrin concentration decreasing from 3.1 ± 0.63 to 2.2 ± 0.31 $g{\cdot}l^{-1}$ (p< 0.0001) (see Table 13).

8.5.4 Cardiopulmonary exercise testing variables

Mean ± SD $\dot{V}O_{2\ AT}$ was 9.1 ± 1.7 $ml{\cdot}kg^{-1}min^{-1}$ at baseline and 9.8 ± 2.5 $ml{\cdot}kg^{-1}min^{-1}$ after i.v. iron (mean difference of 0.6 ± 1.5 $ml{\cdot}kg^{-1}min^{-1}$, p= 0.1). $\dot{V}O_{2\ peak}$ rose significantly from 15.2 ± 4.0 $ml{\cdot}kg^{-1}min^{-1}$ to 16.3 ± 4.4 $ml{\cdot}kg^{-1}min^{-1}$ (mean difference 1.0 ± 1.6 $ml{\cdot}kg^{-1}min^{-1}$, p= 0.02) (Figure 19).

The mean ± SD ramped exercise time was 475 ± 130 seconds at baseline and 493 ± 140 seconds at final testing visit which was not statistically different. Mean ± SD peak work rate (watts) increased from 92 ± 34 watts at baseline to 98 ± 35 watts post iron (p= 0.0161).

8.5.5 Relationships between haematological variables and oxygen uptake

The baseline [Hb] was negatively associated with $\dot{V}O_2$ at the AT r= -0.6165 p= 0.006. The Visit 4 (V4) [Hb] was not r= -0.4581 p= 0.0559. The baseline and V4 [Hb] were unrelated to $\dot{V}O_{2\ peak}$ r= -0.09983, p= 0.7030 and r= -0.08508 p= 0.7454 respectively.

The baseline tHb-mass was negatively associated with $\dot{V}O_2$ at the AT (r= -0.7022 P 0.0017) however, the visit 4 values showed no correlation (r= -0.4549 p= 0.0665). The baseline and V4 tHb-mass were unrelated to $\dot{V}O_{2\ peak}$ r= -0.1482, p= 0.5838 and r= -0.1970 p= 0.4646 (see Figure 20 & Figure 21).

SN	Patient ID	Baseline exercise time (seconds)	V4 exercise time (seconds)	Baseline peak wattage	V4 peak wattage	Pre $\dot{V}O_{2\,AT}$	Post $\dot{V}O_{2\,AT}$	$\Delta\dot{V}O_{2AT}$	Pre $\dot{V}O_{2}$ peak	Post $\dot{V}O_{2}$ peak	$\Delta\dot{V}O_{2}$peak	Pre [Hb]	Post [Hb]	Δ[Hb]	Pre tHb-mass	Post tHb-mass	ΔtHb-mass
1	1	577	604	188	196	9.8	12.6	2.8	19.2	22.8	3.6	91	100	9	*	*	*
2	4	474	519	81	85	9.5	10.5	1	15.4	15.5	0.1	112	124	12	622	756	134
3	6	433	485	61	81	7	7	0	*	*	*	123	129	6	592	640	48
4	7	697	734	120	122	6.2	7.3	1.1	10.8	12.3	1.5	113	119	6	729	783	54
5	8	694	736	115	124	7.2	9.7	2.5	14.4	15.6	1.2	126	123	-3	695	700	4.8
6	9	192	254	36	45	*	*	*	5.4	6.6	1.2	94	106	12	345	395	50
7	10	390	487	96	124	7.9	8.5	0.6	15.5	17.8	2.3	114	128	14	597	644	47.1
8	12	292	250	76	66	10.9	12.1	1.2	18.5	16.9	-1.6	95	108	13	271	352	81.1
9	13	392	388	67	68	9.9	7.7	-2.2	12.6	10.6	-2	102	109	7	580	679	99.3
10	14	337	356	59	63	10.3	10.2	-0.1	13.5	16.9	3.4	83	89	6	264	288	24.3
11	15	599	568	100	96	11.2	13.1	1.9	20.1	20.5	0.4	108	119	11	460	491	31
12	16	*	*	*	*	*	*	*	*	*	*	87	93	6	527	554	26.5
13	17	463	362	51	64	9.7	10.1	0.4	15.6	16.7	1.1	109	110	1	431	424	-6.8
14	18	420	468	106	118	8	7.6	-0.4	11.6	12.6	1	125	128	3	608	678	70
15	19	604	645	93	98	9.3	8.6	-0.7	17.8	18.6	0.8	114	122	8	450	515	65
16	20	560	475	128	120	10.9	11.3	0.4	21.1	20.6	-0.5	116	117	1	527	554	26.5
17	21	453	570	112	128	11.6	15.8	4.2	20.3	23.7	3.4	90	114	24	368	478	110.2
18	22	542	610	91	104	10.4	9.8	-0.6	14.1	15.1	1	125	125	0	447	493	45.7
19	25	452	454	100	94	7.5	7.4	-0.1	*	*	*	123	128	5	605	577	-28
20	26	462	405	73	71	7.2	6.5	-0.7	13.1	13.6	0.5	122	127	5	443	521	78
Mean ±SD Median [IQR]		**475 ± 130**	**493 ± 140**	**92 ± 34**	**98 ± 35**	**9.1 ± 1.7**	**9.8 ± 2.5**	**0.6 ± 1.5**	**15.2 ± 4.1**	**16.3 ± 4.4**	**1.0 ± 1.6**	**109 ± 14**	**116 ± 12**	**7.3 ± 6.0**	**497 ± 134**	**546 ± 139**	**49 ± 41**
*** Indicates unusable data due to being uninterpretable or due in one case to equipment failure**																	

Table 12: Individual patient data from cardiopulmonary exercise testing, total haemoglobin mass and haemoglobin concentration.

Variable	Baseline	Final visit	Δ	Statistical test	p-value
[Hb]	109 ± 14.1	116 ± 12.0	7.3 ± 6.0	Paired t-test	<0.0001
tHb-mass (grams)	497 ± 134	546 ± 139	49.3 ± 41.3	Paired t-test	<0.0001
Iron (μmol.l^{-1})	7.2 ± 4.3	12.3 ± 4.2	6.3 ± 3.5	Paired t-test	0.0001
Transferrin (grams.l^{-1})	3.1 ± 0.63	2.2 ± 0.31	0.88 ± 0.5	Paired t-test	<0.0001
Transferrin saturation (%)	13.1 ± 8.5	25.3 ± 7.6	16.2 ± 6.2	Paired t-test	0.0011
$\dot{V}O_{2\,AT}$ (ml·kg^{-1}min^{-1})	9.14 ± 1.7	9.77 ± 2.5	0.63 ± 1.51	Paired t-test	0.0964
$\dot{V}O_2$ peak (ml·kg^{-1}min^{-1})	15.2 ± 4.1	16.3 ± 4.4	1.0 ± 1.6	Paired t-test	0.0165
Peak WR (watts)	92 ± 34	98 ± 35	6 ± 9.9	Paired t-test	0.0161
Exercise time (seconds)	475 ± 130	493 ± 140	17.7 ± 58.7	Paired t-test	0.2041
[Hb], haemoglobin concentration (g·l^{-1}); t-Hb-mass, total haemoglobin mass; Δ, change pre and post iron supplementation. Data reported as mean ± SD or median [range]					

Table 13: Cardiopulmonary exercise testing variables pre and post intravenous iron.

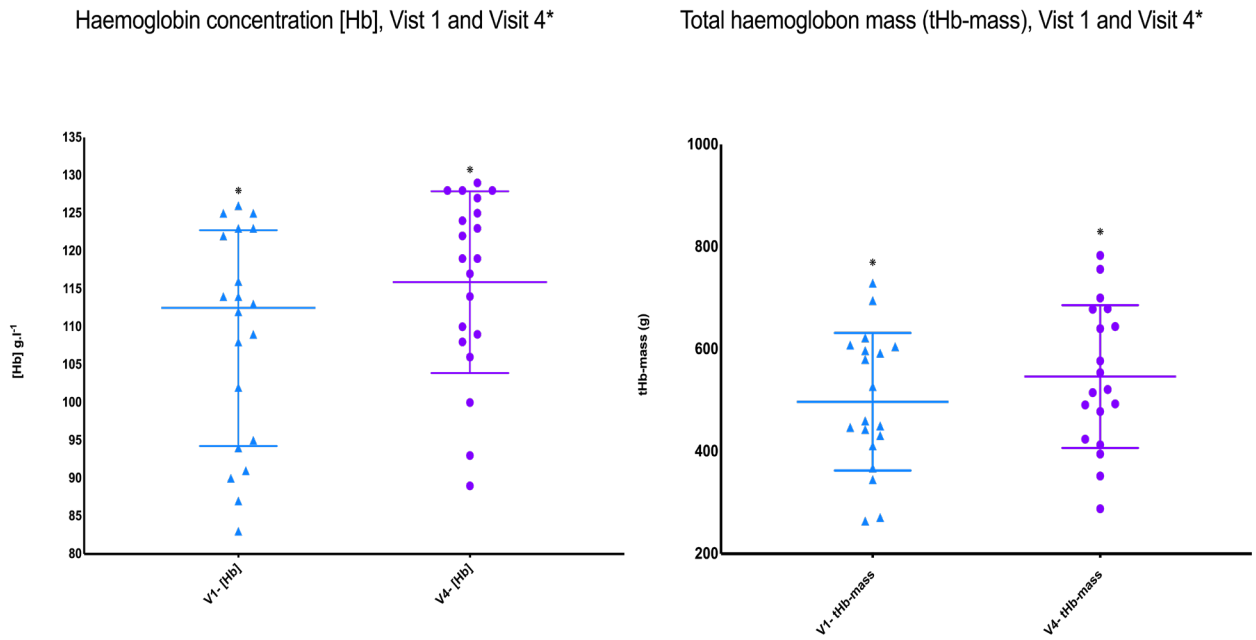


Figure 18: Haemoglobin concentration [Hb] and total haemoglobin mass (tHb-mass) pre- and post- intravenous iron infusion. Data expressed as mean with bars representing standard deviation. *= statistically significant difference.

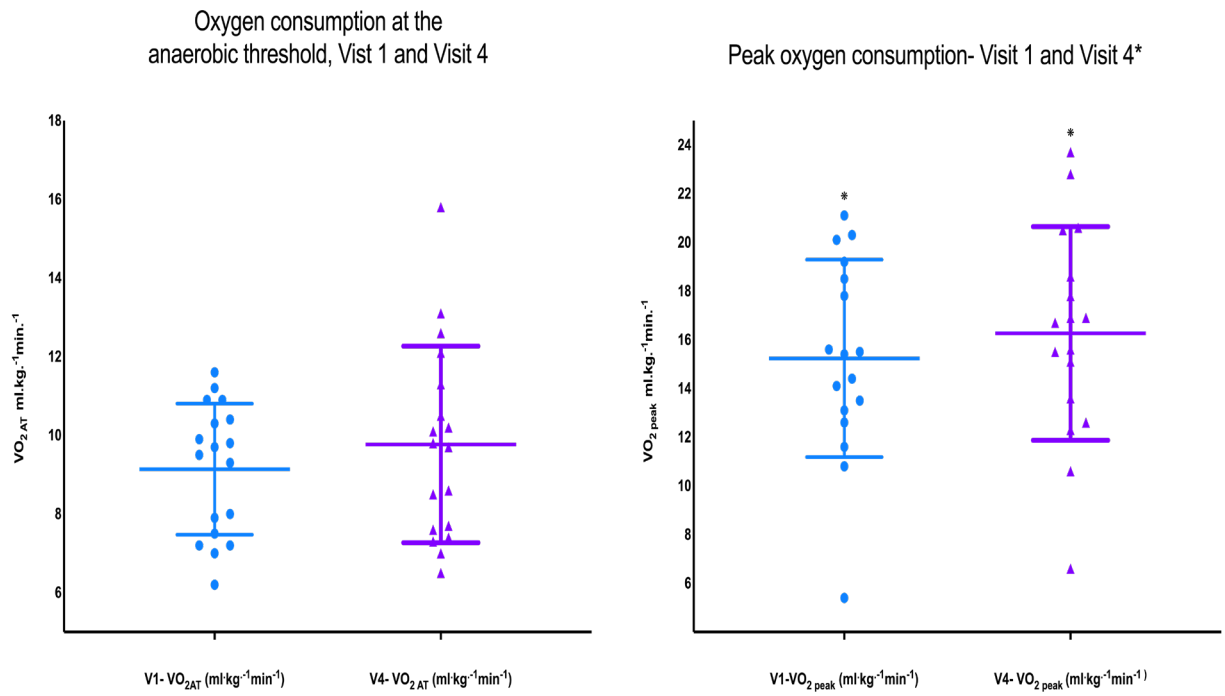


Figure 19: CPET variables; anaerobic threshold and peak oxygen consumption compared between baseline visit 1 and visit 4. *= statistically significant difference.

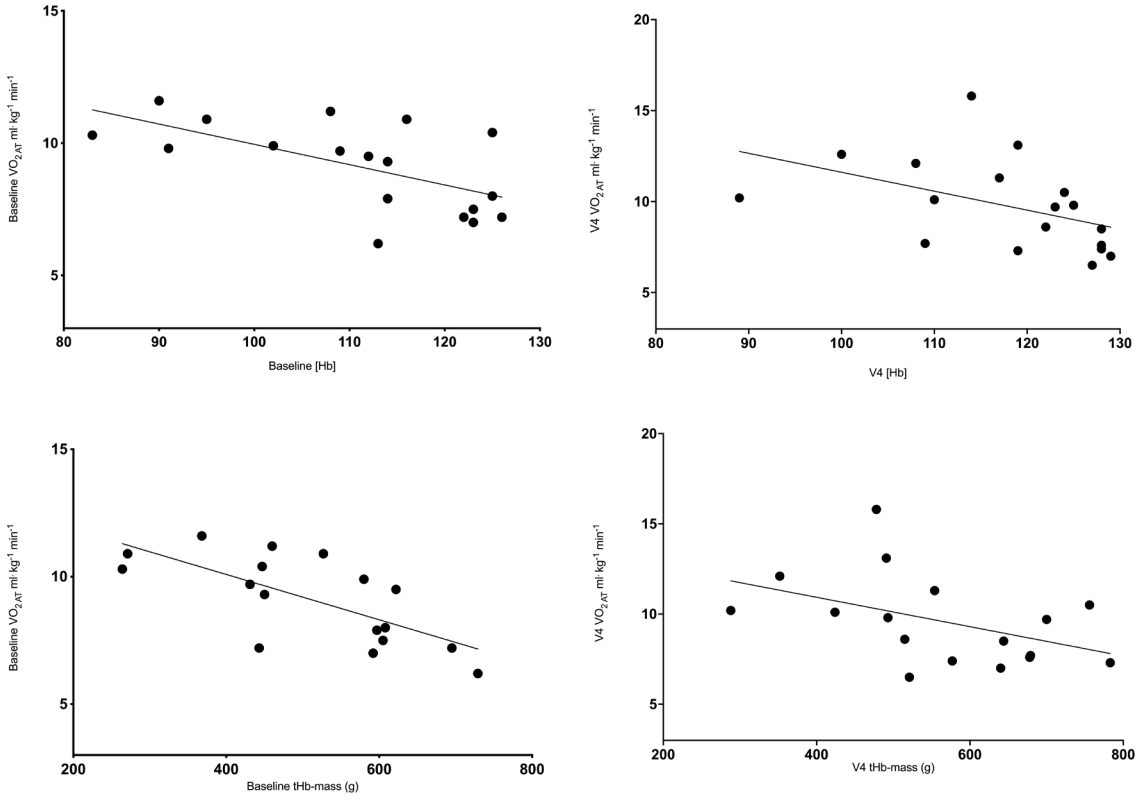


Figure 20: Unadjusted relationship between haematological variables and the anaerobic threshold ($\dot{V}O_{2\,AT}$).

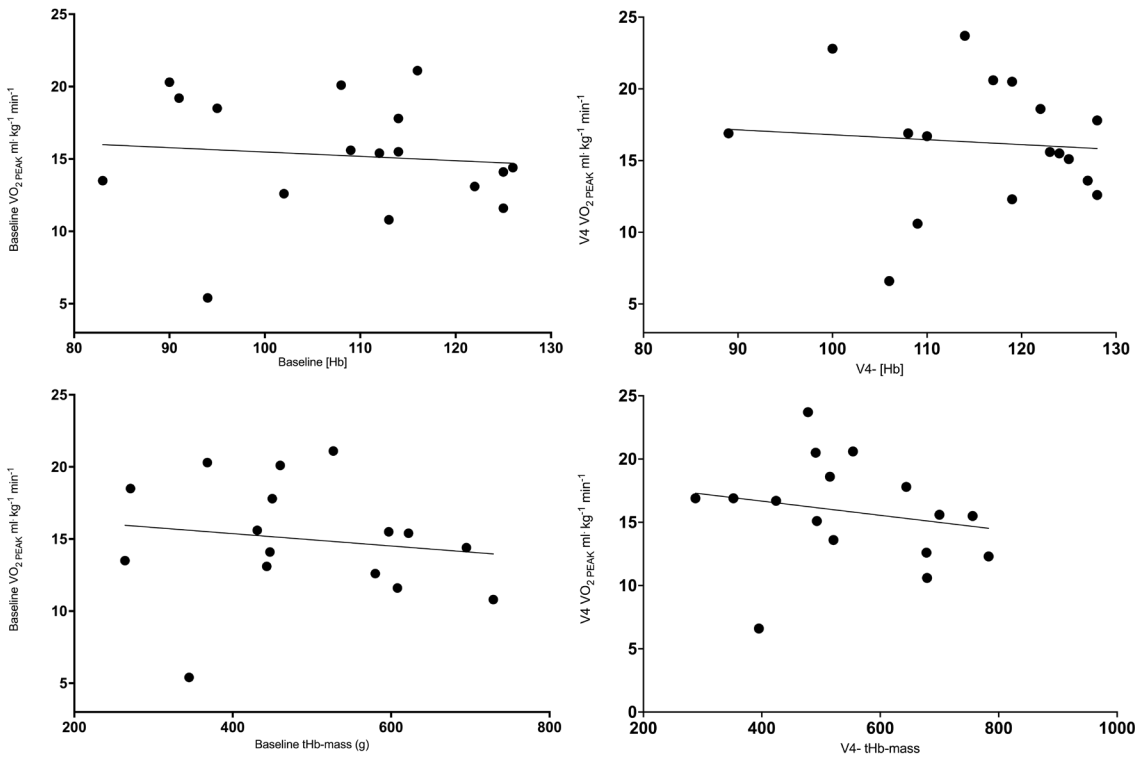


Figure 21: Unadjusted relationship between haematological variables and the peak oxygen consumption ($\dot{V}O_{2\,Peak}$).

8.6 Discussion

It is feasible to assess changes in CPET variables, tHb-mass and iron indices in response to intravenous iron in iron-deficient anaemic preoperative patients. To our knowledge, this is the first study to demonstrate such feasibility. Importantly, this was achieved without disruption to a patient's normal perioperative care pathway. Administration of i.v. iron was associated with significant increases in $\dot{V}O_{2\ peak}$ and peak work rate, [Hb] and tHb-mass (Figure 18) which have again not been previously sought or reported in such patients.

Quantifying the response of tHb-mass to i.v. iron in perioperative patients has not been performed before. This approach may allow a more precise assessment than relying on [Hb] alone (which is known to be impacted by changes in PV independent of tHb-mass (28)).

The rise in [Hb] after i.v. iron is in keeping with other perioperative studies using intravenous iron to treat anaemia (20,21,306–308). UK national guidance (154) and international consensus (137) on the management of perioperative anaemia and iron deficiency support the use of intravenous (i.v.) iron (if oral iron is 'not appropriate' or planned surgery is <6 weeks away) as an alternative to red cell transfusion, albeit that data relating to the efficacy of this approach are equivocal (20,21,308,309). The PREVENTT study showed a modest increase in [Hb] 4.7 $g{\cdot}l^{-1}$ (vs. 7.3 $g{\cdot}l^{-1}$ in the current study) following a 1000 mg dose of ferric carboxymaltose (Ferrinject®) vs. placebo (22). However, not all patients had iron-deficiency anaemia only 76% had TSATs <20% and 57% a ferritin <100 $ng.ml^{-1}$ compared to 90% and 100% respectively in our study. In addition, the median time from treatment to surgery in PREVENTT was 14 days (range 5–212 days) after i.v. iron administration but 25 days (range 13-42 days) in our study- which may have allowed a greater time for tHb-mass (and [Hb]) to rise. This time window is less than the time required for i.v. iron preparations to have their full treatment effect (around 6 weeks) (182,183), although a haematopoietic effect can be seen at just 5 days (184). Indeed, an RCT in inflammatory bowel disease (IBD) patients demonstrated a significant [Hb] response following intravenous iron at 14 days with a continued improvement in [Hb] up to 8 weeks (181). Similarly, another recent RCT comparing Iron Isomaltoside 1000 (Monofer®) with iron sucrose reported a mean change in [Hb] 14 days post treatment of 15 $g{\cdot}l^{-1}$ increasing to ~25 $g{\cdot}l^{-1}$ at 8 weeks (the duration of the study) (310).

So as not to disrupt the routine care pathway, we stipulated a priori a minimum of 10 days between iron administration and repeat CPET (see Methods). The minimum was in fact 13 days (Table 11): a timepoint by which significant increases in [Hb] have previously been demonstrated (182,183). The lower increase in [Hb] of 7.3 $g{\cdot}l^{-1}$ and 49 g for tHb-mass reflects the possibility that we may have not performed measurements at the optimum treatment response time with further increments in both [Hb] and tHb-mass likely given a longer timeframe between repeated CPETs. However, the [Hb] rise we observed is in keeping with other prospective perioperative studies where i.v. iron has been administered preoperatively (8 $g{\cdot}l$, (20); 15.5 $g{\cdot}l$, (21); 8 $g{\cdot}l^{-1}$, (311) & 9 $g{\cdot}l^{-1}$, (312)) (20), although lower than others (22 $g{\cdot}l^{-1}$ (313)). This lesser impact might perhaps have been due to ongoing tumour-related blood loss.

To our knowledge this is the first time that tHb-mass has been measured in perioperative patients after receiving intravenous iron. We observed a mean increase in tHb-mass of 49g. Unfortunately, what constitutes a 'normal' or clinically meaningful rise in an anaemic subject remains to be defined. Nonetheless, we have previously described and demonstrated that the relationship of tHb-mass with perioperative performance is greater

than that seen with [Hb] (1–3,28,34). Where [Hb] or tHb-mass did not increase post i.v. iron, the differences were within the laboratory test variation for the respective variables. Table 12 documents the individual patients in whom this was applicable (patients 5, 13, 18 & 19). Patient 5 had only borderline iron deficiency anaemia and therefore perhaps unsurprisingly went from [Hb] 126 to 123 $g \cdot l^{-1}$, tHb-mass 695-700 g. The other 3 patients had small variations within laboratory normal ranges so could represent technical error, non-response to i.v. iron or perhaps greater Hb loss may have been occurring in such subjects.

8.6.1.1 Cardiopulmonary exercise testing variables

$\dot{V}O_{2\ peak}$ and peak work rate also rose following i.v. iron ($p = 0.02$ in both cases), findings which *may* have clinical significance, and which are hypothesis generating with regard to i.v. iron improving haemoglobin mass and resulting in greater physiological fitness and possibly resilience to surgery. Improvements in $\dot{V}O_{2\ peak}$ have been demonstrated in chronic heart failure alongside improvements in functional status (NYHA classification) following treatment with i.v. iron (257), while others have shown improvements in 6 minute walk test distance (6MWD) following i.v. iron therapy (179,256). Similar findings in 6MWD performance have been observed in patients with pulmonary hypertension following i.v. iron (314), although a post-operative study comparing i.v. and oral iron in patients after total knee arthroplasty found no difference in 6MWD (315). Observational data are suggestive of impaired exercise capacity being related to the extent of iron deficiency (316) and thus likely amenable to improvement. For example, the EFFECT-HF study (197) (a multi-centre randomised trial in patients with CHF and iron deficiency) showed a beneficial effect on change in $\dot{V}O_{2\ peak}$ following i.v. ferric carboxymaltose compared to standard care, irrespective of anaemia status. However, this effect was highly sensitive to the imputation strategy used for $\dot{V}O_{2\ peak}$ among patients who died and so remains to be further assessed. In the perioperative setting, a recent pre-specified sub study of the Measurement of Exercise Tolerance before Surgery (METS) study demonstrated that anaemia (defined using [Hb]) explained only 3.8% of the variation in $\dot{V}O_{2\ peak}$ in a multi-variate regression model (263). We have shown similarly low explained variance of [Hb] in $\dot{V}O_{2\ peak}$ (9%) and $\dot{V}O_{2\ AT}$ (6%), highlighting that other factors may play a key role as fitness determinants, with tHb-mass being one such important candidate (29).

Elevating tHb-mass in elite athletes is consistently associated with a proportional increase in $\dot{V}O_{2\ max}$ (217) with such increases being underpinned by changes in arterial oxygen content and systemic oxygen transport. Early animal data is suggestive of iron improving exercise performance (190). However, there are mixed results in athletes with regard to iron improving performance with some studies supporting improved performance (231) (oral iron) (230) (i.v. iron) and others demonstrating no changes in CPET performance (192,232) (i.v. iron). A meta-analysis of iron supplementation in non-anaemic iron deficient athletes (IDNA) concluded that exercise performance is increased with iron therapy (194).

Previous work in anaemic patients receiving red cell transfusions has demonstrated increases in CPET variables but these data are complicated by the known significant effect of blood volume increases on those same variables aside from any effects of the increased O_2 carrying capacity that a transfusion affords (288). Numerous historical studies in elite athletes have examined the relationship between allogenic red cell transfusions (317) and exercise variables, which we have previously reviewed elsewhere (1). Interestingly, many of these although showing some improvement in exercise performance were underpowered and debate remains around the relative contributing factors with regard to improved O_2

delivery in these circumstances, namely the blood volume vs. the haemoglobin mass changes.

The heterogenous nature of the cohort (different surgical specialities) limits our ability to perform sub-group analysis for anaemia severity and impact on exercise performance. Another potential weakness was the time between i.v. iron and repeat CPET not being long enough for the full treatment effect to be observed (see above).

8.7 What does this study tell us?

Administration of i.v. Iron to iron-deficient preoperative patients is associated with increases in

- [Hb], tHb-mass and $\dot{V}O_{2\ peak}$

8.8 Unanswered questions and future research

In theory, anaerobic threshold might rise after i.v. iron due to increased physical activity related to improved aerobic capacity (a training effect). Alternatively, impacts of iron deficiency on the enzymes of the electron transport chain might be mitigated (318,319). Whilst anaerobic threshold did not change significantly in our study, this might have related to underpowering; post hoc analysis suggests that 101 subjects would be required with an alpha error of 0.05 and a beta error of 0.8 for a significant change in the anaerobic threshold. Further appropriately powered studies are required to examine the effect of intravenous iron upon exercise variables in a preoperative setting. In addition, the utility of alternative physiological end points may be more appropriate to guide and assess interventional changes following anaemia and/or iron optimisation. For example, an incremental exercise test to symptom limitation (ramp) may not be sensitive enough to evaluate the efficacy of some interventions and does not reflect activities of daily life which are in general sub-maximal and continuous in nature. Therefore, use of constant work rate tests at a certain percentage of the maximal work rate achieved on an incremental exercise test may be more appropriate to assess exercise tolerance and more sensitive to changes in fitness than $\dot{V}O_{2\ peak}$ or $\dot{V}O_{2\ AT}$. These sorts of tests should be included in any future studies in this area (320,321). In addition, wellbeing factors are also important and potential candidate mechanisms for i.v. iron and future work should include a robust quality of life analysis. Ultimately the question that remains elusive is whether improved exercise performance via improvement in any haematological domain, (be it red cell mass or otherwise) improves perioperative outcomes. This should be the focus of future research in this area.

8.9 Conclusion

Performing a baseline CPET, measuring tHb-mass, administering i.v. iron and then repeating a CPET is feasible in anaemic, iron deficient perioperative patients within their usual clinical pathway. Our data suggest that improvements in cardiorespiratory performance may result. Further appropriately powered prospective studies are required to ascertain whether changes in anaerobic threshold also result, and whether improvements in tHb-mass and performance in turn lead to reductions in perioperative morbidity.

Chapter 9 - Conclusions and further work

The aim of this thesis was to explore measuring total haemoglobin mass (tHb-mass) in a variety of settings. The ultimate aim was to explore the role of monitoring changes in tHb-mass perioperatively, with a particular focus on blood manipulation in the perioperative setting.

The data driven chapters of this thesis have achieved the overarching aim which was to test the hypothesis that increasing tHb-mass before surgery might increase physiological reserve defined using CPET. The objectives and hypotheses from each chapter are set out below with discussion and recommendations for future work section by section.

9.1 Chapter 5

Objective: To evaluate the oCOR test under diverse pathological conditions that could represent disorders of plasma volume. In the case of liver disease carbon monoxide mixing times and hence sampling time periods for the oCOR may be affected.

The hypothesis was that in patients with chronic liver disease (CLD) due to changes in regional (e.g. enteric) and global blood flow dynamics (270–275), and the presence of expanded arteriovenous shunts (276). Patients with CLD may have delayed admixture of CO gas leading to an inadequate rise in carboxyhaemoglobin within the standard sampling time frame of 6-8 minutes described in the classical technique. Another hypothesis was that venous sampling (chosen throughout this thesis due to ease of getting blood from subjects, local expertise, and reliability within the time frame) as opposed to capillary sampling would not unduly change the sampling time period.

> **The study concluded with: The oCOR method can be safely used to measure total haemoglobin mass in patients with chronic liver disease and ascites, without adjustment of blood sample timings. Further work might refine and validate appropriate dosing regimens.**

This study demonstrated that blood sampling did not have to be adjusted in this particular cohort of patients with chronic liver disease and ascites. Based on the findings from a similar study in patients with cardiac failure (left ventricular ejection fraction <30%) (121) it would seem prudent to consider the presence of both of these conditions if and when further studies look to use the oCOR in the clinical arena. Analysis of this work and the study in patients with cardiac failure however does lead me to conclude that the original methodological description of the oCOR is satisfactory. The very small (<1%) underestimation found by *Ahlgrim* et al is clinically negligible and the data presented in Chapter 5 supports adequate mixing within the traditional time periods described (121). This does not mean that this would hold true in all perioperative and or critical care subjects and a logical next step would be to repeat this methodological study in these cohorts.

An interesting future study that could follow this work would be to measure repeated oCOR tests within the same day pre and post paracentesis. Our previous work (28) supports the hypothesis that the incidence of dilutional anaemia would be high in this cohort and that tHb-mass ought to remain the same, whereas [Hb] may indeed differ

significantly. Chapter 6 describes in detail how repeat measurements could be safely performed in the same subject within a 'normal' working day. The ability to quantify the proportions of red cell mass, red cell volume, plasma volume and total blood volume would be especially useful as a means to guide therapy in a number of conditions not limited to heart failure, chronic liver disease and critical illness without the need to use expensive and potentially hazardous radiolabeling techniques (see section 2.6.3).

Chapter 5 highlighted the difficulties in prescribing set doses for unwell, frail and/or deconditioned clinical subjects when it comes to measuring tHb-mass using the oCOR. The original studies were carried out in healthy young subjects and athletes (5,6,74). There are multiple factors which could affect CO uptake kinetics and the spread of CO throughout the body to various compartments that may differ in older deconditioned subjects. The five compartments have been described by Bruce and Bruce: namely, the lungs, arterial blood, mixed venous blood, muscle tissue and non-muscle tissue. The time for blood to pass through each compartment is governed by the ratio of the compartmental volume to the blood flow through each compartment (322,323). Although this has been mathematically modelled there remains no data that we know of to guide differences in individuals aside from our own work and the crude delineations of 'trained and 'untrained' used in the original work by *Schmidt* and *Prommer*.

When one has not previously had their tHb-mass measured it can be difficult to work out an appropriate dose. There are no guidelines for dosing regimens in clinical subjects, although there are descriptions in the sports literature and common-sense recommendations. This work to our knowledge is the first to describe in detail the problems associated with dosing and the significance this has on the precision of measurement in the clinical setting. As referenced in Chapter 5 very few studies have been performed and of those, to our knowledge only one other reported the doses used in the paper (63). Further work should look to establish the best way to classify dosing and to ensure safety and the ability to repeat measurements within a short time period (see Chapter 6.

Using the oCOR to guide therapy is an exciting prospect in patients with disordered plasma volume and there are numerous studies that might explore this, especially with regard to diuretic therapy and or the need for red cell transfusion. If patients with significant plasma volume excess are receiving unnecessary transfusion this might have detrimental longer-term effects and this ought to be pursued, the oCOR is an effective tool to pursue this.

I recognise that throughout this thesis alternative methods such as those described by *Lundby* et al (69) or indeed different techniques such as the use of Cr^{51} red cells have not been employed to explore accuracy (see 2.7). However, as explored in 2.6.3 none of the radiolabeling techniques measure tHb-mass directly and they have aforementioned disadvantages and logistical constraints. For completeness further work could explore these experiments. Another consideration would have been to have performed all these experiments on a set of healthy controls looking to match age and sex where possible. Again, this was considered but felt unnecessary to answer the question posed by this study; this is not to say that the study could not have been improved by using a matched cohort of control subjects.

9.2 Chapter 6

Objective: To refine the performance characteristics of the oCOR in healthy volunteers, particularly in relation to interval testing and its safety within short time frames.

The second observation was that the oCOR test as described by *Schmidt* and *Prommer* and used widely within the athletic community had a safety stipulation that a period of 12 hours ought to be left between tests to allow carboxyhaemoglobin levels to return to baseline. I found one study that had attempted to challenge this but their protocol involved activity in all cases to increase pulmonary ventilation and blood flow (126). If the oCOR were to be used more widely within medicine and particularly within any acute setting, then repeatability within a short time frame would be essential.

My hypothesis was therefore that carboxyhaemoglobin levels would adequately return to a safe enough level to allow repeat testing using supplemental oxygen alone within a 3-hour period. This would comfortably allow repeat testing within a single day.

> **The study concluded with: The oCOR method is a safe technique that can be repeated within three hours when carbon monoxide is suitably cleared between tests in healthy non-athletic individuals. Using oxygen therapy alone adequately achieves this. This method has minimal bias and good precision making it attractive for regular monitoring of blood volume derivatives. Clearance using oxygen alone can safely be used in healthy volunteers with a high degree of precision and safety and it is possible that this could translate to clinical subjects.**

For blood volume and tHb-mass measurement in the clinical setting to be practicable, particularly in acute settings it has to be easy to perform, reliable and repeatable. Prior to testing safe repeatability in clinical subjects (outside of the recommended time of twelve hours between testing) I wanted to repeat and adapt the experiments of *Naef* et al. I have demonstrated that this is entirely possible (and safe) with just a three-hour window breathing oxygen in between oCOR tests. As mentioned above the safety margins are only broad guidelines and there exist no clinical guidelines that one must adhere to when using the oCOR in clinical subjects. Whilst I am not necessarily advocating starting with baseline COHb levels in excess of 5%, further studies might wish to experiment with safety margins for repeat testing. The importance of demonstrating effective washout without necessarily changing pulmonary ventilation with exercise is of great relevance to the clinical setting where this will often be impractical or even impossible.

This work allows other researchers to confidently use the oCOR in the clinical setting and opens avenues to pursue further studies that combine the dosing issues raised in Chapter 5 (see above) with the repeatability intervals mentioned in Chapter 6. Future studies should seek to investigate the feasibility of duplicate tHb-mass testing in a single day in specific patient groups, and in the perioperative period in particular. A range of ages and comorbidities should be sought. Modification of the oCOR for use in ventilated subjects in the theatre or intensive care environment would also be of great value. The ability to measure tHb-mass in the context of short-term changing parameters (bleeding for example) are important and studies using the oCOR to facilitate this ought to be pursued.

9.3 Chapter 7

Objective: To test the hypothesis that valid measurements of tHb-mass could be made using a "single breath" modification of the oCOR technique.

The third observation was that the oCOR is reliant upon an awake, compliant, spontaneously breathing subject who is able to obey simple commands. This limits its use somewhat in the critical care and perioperative space. We wished to explore if the method could be adapted to a 'single breath' technique that *may* allow the measurement of tHb-mass in subjects on a mechanical ventilator in the future.

My hypothesis was that delivery of a single carbon monoxide bolus into the breathing circuit of a participant without the need for re-breathing could be used to reliably measure tHb-mass, so long as all exhaled gas could be analysed. We thus sought to develop such a technique.

> **The study concluded with: Although it is feasible to measure tHb-mass using a 'single breath technique' it cannot yet be reliably assessed using a single CO breath. Further refinement of the technique will be required if it is to be used to measure tHb-mass in mechanically ventilated patients.**

The possible reasons for only 50% of subjects having a tHb-mass that was within 50g of the original oCOR were discussed in Chapter 7.6. Briefly, these were the possibility that a 10 second breath hold was simply too short to get adequate mixing of CO within the circulation. That the 6 subjects did not take an adequate breath in at the time the CO was injected into the breathing circuit, possibly due to a lack of practise prior to injecting the CO gas into the system.

The goal and hope were that this study would lead to work aiming to further refine the technique to improve accuracy and to assess precision. As described in Chapter 5 adequate dosing is vital to ensure accuracy. Additionally, the method as described *may* indeed be precise and simply require a calculation to match the standard oCOR technique akin to that described by *Ahlgrim* et al in patients with an EF<30% (121). However, this would of course require repeated measurements in the same subjects in the same way as described in Chapter 6. Even if this work is done it would need to be performed in a larger number of subjects to confidently assess precision.

We actively pursued follow up work with Professor *Schmidt* and a study has now been completed in Germany using the data presented here. This study has now been published and is included in Appendix E. It concluded that:

> **A 'single breath' of CO with a subsequent 30 s breath hold provides almost as exact a measure of haemoglobin mass as the established optimized CO-rebreathing method when applied to healthy subjects. The modified method has now to be checked in ventilated patients before it can be used to quantify the contributions of blood loss and of dilution to the severity of anaemia.**

Additional data was added by Professor *Schmidt* and as this was not directly generated by myself these data were not included within Chapter 7. The study, collaboration and write up were however my work and therefore a brief discussion follows:

The premise was exactly the same however they used the data as described in Chapter 7 to make modifications to the process. At least four CO-rebreathing tests were carried out by 11 subjects in a randomized order. Two of the tests consisted of the established CO-rebreathing method over 2 min (oCOR). In the third and fourth tests, the subjects inhaled a CO bolus followed by a breath hold for 15 s ($Proc_{new}15s$) or 30 s ($Proc_{new}30s$). To evaluate possible influences of the test arrangement, that is, collection and analysis of the expired air after inhaling the CO bolus, CO exhalation was determined from six subjects in a fifth test approach using the same methodology as described above after a conventional 2 min CO-rebreathing procedure ($oCOR_{+20min}$).

The significant modifications that were made for the follow-on part of the study undertaken at the University of Bayreuth were as follows:

- A mask was used instead of a mouthpiece, with a CO injection port placed directly into the mask.
- The subjects breathed with the mask on for 10 minutes at rest prior to the CO gas being injected.
- They held their breath for 15 or 30 seconds (2 different experiments)

This resulted in a similar amount of CO gas ligated to Hb by minute 7: corresponding to 94.0 ± 2.1%, 90.4 ± 4.7% and 85.2 ± 6.0% of the inhaled CO volume, respectively.

The resultant tHb-mass calculated from the originally described 7-minute point was similar between oCOR 843g ± 293, $Proc_{new}15s$ 819g ± 285 and $Proc_{new}30s$ 838g ± 301. When the procedures in this study were compared for $PROC_{new}15s$ for 15 s, the initial CO exhalation after 1 min was twice as high as that in $Proc_{new}30s$, indicating that ∼10% ($Proc_{new}30s$ ∼4%) of the inhaled CO did not diffuse into the blood. After 7 min, that is, when the CO mixing in the blood was completed and therefore used in the established oCOR for blood sampling after the test, the loss of CO was ∼13% in Procnew15s and only ∼8% in $Proc_{new}30s$. Interestingly, a small but systematic underestimation by approximately 25 g compared with the reference method was obvious in $Proc_{new}15s$. This was not seen when $PROC_{new}30s$ was compared to oCOR suggesting that that the smaller CO volume taken up from the blood during $Proc_{new}15s$ may have been the cause and equally could have been the cause in the study described in Chapter 7.

To adapt this methodology and translate it to a subject on mechanical ventilation is the logical next step. CO is applied to the inhalation path, and breathing is interrupted in the inhalation position for 15 or 30 s. In healthy subjects, there is little risk of interrupting breathing for 30 s, and the risk can be classified as very low also in ventilated patients. Since the oxygen consumption during the 30-s breath interruption is only approximately 150 ml, the arterial O_2 saturation does not change during this period (324); but in any case, it must be checked during and after the test. In severely anaemic patients, the test might be used with great caution after extensive validation.

The work of this thesis; testing mixing times in chronically unwell patients, demonstrating safety in repeated oCOR testing within shorter time periods and the above study based on the work from Chapter 7 ought to give future researchers confidence to explore the single breath technique in critically unwell patients being mechanically ventilated.

9.4 Chapter 8

Objective: To test the hypothesis that intravenous iron will augment tHb-mass (measured by oCOR) and thereby improve physical fitness (measured using CPET) in patients preparing for surgery.

The final observation was that anaemia is associated with adverse perioperative outcomes and impaired physical fitness. Correction of anaemia before surgery through intravenous iron therapy is becoming common practice.

The relationship was explored between exercise capacity, anaemia and haemoglobin content in an elective surgical cohort. Specifically, the aim was to test the hypothesis that augmenting haemoglobin through intravenous iron therapy would improve preoperative physical fitness (cardiopulmonary exercise testing (CPET) variables).

> **The study concluded with: Performing a baseline CPET, measuring tHb-mass, administering i.v. iron and then repeating a CPET is feasible in anaemic, iron deficient perioperative patients within their usual clinical pathway. Our data suggest that improvements in cardiorespiratory performance may result. Further appropriately powered prospective studies are required to ascertain whether changes in anaerobic threshold also result, and whether improvements in tHb-mass and performance in turn lead to reductions in perioperative morbidity.**

The findings of this chapter are noteworthy. Firstly, the fact that recruitment was achievable within a dynamic perioperative system with predominantly cancer patients was of note and the time frame between initial i.v. iron dose and repeat CPET and tHb-mass testing was in excess of the minimum 10 days that were set when designing the study and was also considerably longer (at mean 25 days) than the largest perioperative trial of i.v. iron which achieved a median of 14 and 15 days in the interventional and placebo arms respectively (22). We tried to leave the i.v. iron as long as was practically possible within the perioperative window without testing the patient on the day of surgery which was felt to have been inappropriate. This testing protocol was deemed unachievable by previous investigators and therefore as part of this feasibility study could go a long way to changing the structure of future perioperative interventional trials.

The rise in both [Hb] and tHb-mass was significant and greater than that seen in the PREVENTT trial (22). Perhaps the most important finding was that CPET derived exercise parameters improved with i.v. iron therapy and in the case of $\dot{V}O_{2\ peak}$ this was statistically significant. As described above, future research might use this study to power a randomised controlled trial with change in $\dot{V}O_{2\ peak}$ as the primary outcome measure. Wider and more important patient related outcome measures however might be: length of hospital stay, complications, re-admission rates, psychological well-being parameters and other yet undescribed outcomes which would need to be thoroughly explored in the design of such a trial.

9.5 Final comments

The measurement of blood goes beyond mere scientific intrigue, after all:

> 'The life of the flesh is in the blood' Leviticus 17:11 (9)

The limits of human performance and the extent to which blood volume manipulation may affect performance have gone relatively unstudied in clinical medicine and more so in the perioperative context. My hope is that the work of this thesis will go some way toward using a superior target measure (in total haemoglobin mass) for future studies that may go on to use novel therapies to augment oxygen delivery via increasing red cell mass. The concept of 'fitness' and its link to perioperative outcomes is of particular interest and studies that combine multiple augmentation strategies to further explore this link are warranted.

Appendix A - Studies assessing anaemia in surgical patients

Table 14: Studies Assessing Anaemia and Transfusion Requirements in Surgical Patients- taken from (141) used with permission Elsevier licence number 4418991469141

Study	*Location (Dates of Data Collection)*	*Population*	*Findings*
Benoist et al. (2001)	France (1990–1997)	212 patients who underwent elective resection for colorectal cancer	Preoperative Hb ≤ 125.0 g/L was a significant risk factor for perioperative red cell transfusion (P <0.0001). In patients with anemia, the risk of transfusion was ≥47% in patients with ≥1 other risk factor. In non-anaemic patients, the risk of transfusion was <11% in patients with ≤1 risk factor but increased to 47% in those with ≥2 risk factors.
Feagan et al. (2000)	Canada (May 1996–April 1999)	201 patients undergoing primary hip arthroplasty who had an Hb concentration between 98.0 and 137.0 g/L and did not pre-donate blood; all patients were randomized to epoetin (various doses) or placebo	Percentage of patients requiring transfusion was greater in the placebo group (44.9%) than in either the high-dose (11.4%) or low-dose (22.8%) epoetin groups. The number of units of blood transfused was highest in the placebo group.
Stowell et al. (1999)	USA (March 1996–August 1997)	490 patients scheduled for total hip or knee replacement surgery and randomized to epoetin or PAD	Percentage of patients requiring transfusion was greater in the PAD group (19.2%) than in the epoetin group (12.9%).
			Number of units of blood transfused was also higher in the PAD group (0.36 U) than in the epoetin group (0.25 U).
Tanaka et al. (1999)	Japan (1995–1998)	31 patients with RA who were unable to donate autologous blood because of Hb <110.0 g/dL; all patients were treated with epoetin	All epoetin responders were able to donate the required 773 mL of autologous blood. Non-responders were able to donate only a mean 264 mL of autologous blood.
			Epoetin responders did not require allogeneic blood. Non-responders required a mean 720 mL of allogeneic blood.
Monk et al. (1999)	USA (Not given)	79 patients undergoing radical retropubic prostatectomy	As Hct increased, the percentage of patients requiring transfusion decreased.
Kettelhack et al. (1998)	Germany (February 1992–August 1993)	109 patients with moderate anemia and right-sided colon cancer scheduled for hemicolectomy and randomized to receive epoetin or placebo	Risk of red cell transfusion was greater in patients with Hb <115.0 g/L than in those with higher values.
Mercuriali et al. (1998)	Italy (Not given)	40 anemic patients scheduled for hip replacement surgery who were randomized to epoetin (various doses) or placebo	Amount of whole blood patients could donate increased as the epoetin dose increased. Patients given high dose epoetin were able to donate the most blood, whereas those receiving placebo donated the least.
Churchill et al. (1998)	USA (January 1992–December 1993)	2,590 patients with major joint and limb construction, divided into subgroups based on	Admission Hct was a significant predictor of RBC transfusion in all 4 patient subgroups.

		procedure (total hip vs. total knee replacement) and PAD status (depositor or non-depositor)	
Lent and Neuss (1997)	Germany (1988–1993)	617 patients who underwent transurethral resection for obstructive prostatopathy	Only 14 patients (2.3%) required transfusion. Patients with the lowest (≤100.0 g/dL) and highest (>140.0 g/L) Hb levels received the fewest transfusions (2 for the lowest and 3 for the highest).
Andrews et al. (1997)	United Kingdom (Not given)	100 patients attending a preadmission clinic before undergoing total hip or knee replacement surgery; all anemic patients were given iron, and nonanemic patients were randomized to iron or placebo	Anemic patients required more units of blood (2.8 U) than did the nonanemic patients given iron (1.7 U) or placebo (1.8 U).
Sowade et al. (1997)	Germany (Not given)	72 patients undergoing elective open-heart surgery who had contraindications to PAD were randomized to epoetin or placebo	Percentage of patients receiving allogeneic blood was lowest in the nonanemic patients given epoetin and highest in the anaemic patients given placebo.
Bombardini et al. (1996)	Italy (1979–1983; no normovolaemic haemodilution); (1984–1993; normovolaemic haemodilution)	6,141 patients undergoing total hip replacement, including 1,488 who did not receive normovolaemic haemodilution and 4,653 who did	21% of patients given haemodilution required red cell transfusion compared with 82% of patients not given haemodilution.
			The mean number of blood units required was higher in patients not given haemodilution.
Tryba (1996)	Germany (Not given)	125 patients with Hct ≤0.39 scheduled for elective orthopaedic surgery who were anticipated to require ≥3 U of blood; all patients were randomized to receive epoetin and IV iron, IV iron only, or no treatment	Percentage of patients receiving allogeneic blood was lower in the epoetin group (33%) than in controls (58%).
Price et al. (1996)	USA (Not given)	204 patients scheduled for elective orthopaedic surgery with baseline Hct ≤0.39 randomized to epoetin or placebo	Percentage of patients requiring allogeneic blood was substantially higher in the placebo group. When stratified by both treatment and baseline Hct (<0.33% or 0.33), the percentage requiring allogeneic blood was lowest in the epoetin recipients with Hct ≥0.33 and highest in the placebo recipients with Hct <0.33
de Andrade et al. (1996)	USA (April 1993–August 1994)	316 patients scheduled for elective orthopaedic (hip/knee) surgery with Hb ≤150.0 g/L who were expected to require ≥2 U of blood; patients were randomized to placebo or epoetin (various doses)	Among patients with Hb levels between 100.0 g/L and 130.0 g/L, transfusion was required in 16% of those given high-dose epoetin, 23% of those given low-dose epoetin, and 45% of those given placebo.
			Mean number of allogeneic blood units transfused was highest in the placebo recipients with Hb levels between 100.0 g/L and 130.0 g/L and lowest in the patients given low dose epoetin who had Hb levels >130.0 g/L.
			Regardless of Hb levels, the placebo group required a higher mean

			number of allogeneic blood units than did either of the epoetin groups.
Mercuriali et al. (1994)	Italy (Control patients: February 1989–March 1991; epoetin recipients: March 1991–December 1992)	23 patients with RA who were unable to deposit autologous blood because of anaemia (Hct <0.34) and were scheduled for primary total hip or total knee replacement	Mean number of allogeneic blood units transfused was lower in the epoetin group.
Canadian Orthopaedic Perioperative Erythropoietin Study Group (1993)	Canada (March 1990–December 1991)	208 patients undergoing elective primary or revision hip arthroplasty who were randomized to placebo or epoetin (various durations of treatment)	Percentage of patients requiring transfusion was highest (74%) in the placebo group, intermediate (55%) in the 9-day epoetin group, and lowest (33%) in the 14-day epoetin group.
Goodnough et al. (1993)	USA (Not given)	385 patients scheduled for elective orthopaedic surgery for whom blood type/crossmatching was requested; of these patients, 249 pre-donated autologous blood and 136 did not	Percentage of patients requiring red cell transfusion was lowest in the nonanemic autologous blood donor group and highest in the anemic non-autologous blood donor group.
Goodnough et al. (1992)	USA (Not given)	281 patients scheduled for orthopaedic surgery for whom blood type/crossmatching was requested	Anemic patients required more allogeneic blood than did nonanemic patients.
Keating et al. (1998)	USA (January 1993–August 1997	279 patients undergoing unilateral and 280 patients undergoing bilateral total knee replacement	Percentage of patients requiring red cell transfusion was higher in the bilateral group than in the unilateral group. Furthermore, the percentage of patients requiring transfusion decreased as the Hb level increased.
			Logistic regression showed that the Hb level before surgery and before donation was a significant predictor of transfusion risk in both groups of patients.

Hb = haemoglobin; Hct = haematocrit; IV = intravenous; PAD = preoperative autologous blood donation; RA = rheumatoid arthritis; RBC = red blood cell.

*Data originally reported as conventional values are provided in SI units.

Table 15: The relationship between anaemia and outcome in perioperative care since 2002. Studies all used the WHO definition of anaemia unless otherwise stated.

Study	***Cohort***	***Study Population size***	***Prevalence of anaemia***	***Important outcome measures reported***
#Baron – BJA 2014 (18)	Non-cardiac, non – neurological surgery Prospective cohort	46, 539	28.7%	*In hospital mortality-Severe anaemia ([Hb] <80 g.l^{-1}) [odds ratio (OR) 2.82 (2.06–3.85) or moderate anaemia (80-100 g.l^{-1} women & 80-110 g.l^{-1} men) 1.99 (1.67–2.37) for increased mortality. Anaemia is associated with greater admission to ICUs and increased intensive care resource use.* ***Anaemia independently associated with mortality.***
#Saager- Anesth Analg 2013 (143)	Non-cardiac surgery Retrospective database study (propensity matched cohort)	574,860	25.3%	*30- day mortality. With propensity matching and adjustment for mediator variables OR 1.24 (1.1.0-1.40)*
#Jung- Gastric Cancer 2013 (325)	Non-cardiac Gastric cancer Retrospective cohort	588	21.3%	*Comorbidity, POD2-Hb change, LOW-Hb, and postoperative transfusion were found to be independent risk factors for postoperative complications, suggesting that both anaemia and transfusion are important factors in the development of complications after gastric cancer surgery.* ***Note: this study did not specifically look at preoperative anaemia and outcome.***
#Gupta- Ann Surgery 2013 (144)	Patients over 65, elective vascular surgery Retrospective-database study	31,857	47%	*30-day mortality and cardiac events- 2.4% and 2.3% respectively compared to 1.2% and 1.2% in non-anaemic* patients.*
#Oshin- Vasc Endovasc Surg 2013 (326)	Vascular surgery Retrospective-database study	360	53.6% <140g.l^{-1} used for men and <120g.l^{-1} used for women	*In peripheral arterial surgery, preoperative low Hb is associated with major adverse cardiac events (MACE) and death. Further investigation is necessary to elucidate whether this relationship is causal. Low Hb (OR for each 1 g/dL drop below the mean = 1.4 [1.13-1.7]; P = .002) was an independent risk factors for MACE and for death (OR for each 1 g/dL drop below the mean = 1.5; 95% CI [1.14-1.86]; P = .002).*
#Musallam- Lancet 2011 (17)	Non-cardiac surgery Retrospective database study	227, 425	30.44%	*30-day mortality- OR 1.42 (1.31-1.54) Composite postoperative morbidity also higher OR 1.35 (1.30-1.40).* ***Anaemia independently associated with mortality.***

#Beattie-Anaesthesiology 2009 (147)	Non-cardiac surgery Retrospective cohort	7,759	39.5%	*Nearly five-fold increase in the odds of post- operative mortality- after adjustment for confounders- odds ratio for mortality 2.36 (1.57-3.41)*
#Melis- J Surg Res 2009 (327)	Oesophageal cancer resections Retrospective cohort	413	47.6%	*Preoperative **anaemia** did not predict worsened short-term outcomes, but increased the chances of red blood cell transfusion, which were significantly associated with higher overall **complications** and increased risk of surgical site infections.*
#Wu- JAMA 2007 (150)	Non-cardiac surgery* aged >65 Retrospective database study	310,311	42.8%	*Adjusted risk of 30-day post- operative mortality and cardiac morbidity begins to rise when haematocrit levels decrease to less than 39% or exceed 51%.*
#Seicean-Spine (Phila Pa 1976) 2013 (328)	Orthopaedics spinal surgery Prospective cohort (NSQIP)	24,473	24%	*Patients with all levels of anaemia had significantly higher risk of nearly all adverse outcomes than non-anaemic patients in unadjusted and propensity-matched models.* *All levels of anaemia were significantly associated with prolonged length of hospitalization and poorer operative or 30-day outcomes in patients undergoing elective spine surgery.*
#Greenky-2012	Orthopaedics-joint arthroplasty Retrospective cohort	15222	19.6%	*Joint infection occurred more frequently in anaemic patients at an incidence of 4.3% in anaemic patients compared with 2% in non-anaemic patients.* *Thirty-day (0.4%), ninety-day (0.6%), and 1-year (1.8%) mortality rates were not higher in patients with preoperative anaemia.* *Anaemic patients had increased hospital stays averaging 4.3 days compared with 3.9 days in non-anaemic patients.*
#*Dubljanin-Raspopović-* Cent Eur J Med *2011* (329)	Hip fracture patients >65 yrs. Retrospective cohort	343	53.9%	*In multivariate analysis anaemia was associated with age, gender (female), type of fracture (intertrochanteric) and American Society of Anesthesiologists (ASA) classification (3 or 4), while severity of anaemia was associated with recovery of ambulatory ability at discharge.* *There was no difference in the incidence of postoperative complication, in-hospital mortality and length of hospital stay between the groups at discharge.*

#Vochteloo- BMC Musculoskelet Disord 2011 (330)	Hip fracture patients >65 yrs. Prospective from 2008 (2005-2008 retrospective)	1262	42.5%	*In univariate **analysis**, the 3- and **12**-month mortality rate, delirium incidence and discharge to a nursing home rate were significantly worse in preoperatively anaemic **patients**.* *In multivariable regression **analysis**, **anaemia** at **admissio n** was a significant risk factor for discharge to a nursing home and readmission < 90 days, but not for mortality.*
#Gruson- BJS 2002 (331)	Hip fracture, patients >65 yrs. Prospective cohort	395	45.6%	*Hospital length of stay and mortality rate at six and twelve months were significantly higher for patients who were anaemic on admission* *There were no differences in the recovery of ambulatory ability and of basic and instrumental activities of daily living status at three, six, and twelve months between the two patient groups.*
Taurianen- World J Surg 2017 (332)	Cardiac surgery Prospective cohort	2761	*23.9%*	*Patients with **preoperative anaemia** had an increased prevalence of significant comorbidities and were **associated** with higher unadjusted risk of early and late adverse events.* ***However, after propensity matching and adjustment for severity of bleeding preoperative anaemia** was not **associated** with an increased **mortality** risk (HR 1.10, 95% CI 0.86-1.39).*
#Ranucci- Ann Thorac Surg 2012 (145)	Cardiac surgery Retrospective- only classified severe anaemia those with Hct <30	13,843	2.98%#	*Retrospective propensity-matched study that compared the 401 severely anaemic patients (Hct <30) with a propensity matched group of 401 patients, the 'control' group- Severely anaemic patients, as expected, were more likely to receive allogeneic blood products during the hospital stay. They had a significantly higher rate of stroke. Major morbidity (p <0.001) and operative mortality (p <0.014) were significantly higher in severely anaemic patients, who experienced significantly longer mechanical ventilation time and ICU stay.*
#*Zhang- Ann Thorac Surg 2013* (333)	Cardiac surgery- patients who had had a recent MI Retrospective cohort	655	66%	*Before risk adjustment, in-hospital mortality, and the incidence of major adverse events (MAE) were significantly higher in patients with anaemia versus no anaemia (mortality, 5.1% versus 1.4%, p = 0.02; MAE, 8.6% versus 3.1%, p = 0.009). After risk adjustment, the association between preoperative anaemia and mortality or MAE became insignificant (mortality odds ratio 2.34, 95% confidence interval: 0.56 to 9.87, p = 0.25; MAE odds ratio 1.13, 95%*

				confidence interval: 0.46 to 2.79, p = 0.78)
#Hung- Anaesthesia 2011	Cardiac surgery Retrospective cohort	2688	54.4%	*Compared with non-anaemic **patients**, **anaemia** was significantly associated with **transfusion** (791 (54.1%) vs. 275 (22.4%), p < 0.001, OR (95% CI) 3.4 (2.8-4.1)), death (45 (3.1%) vs. 13 (1.1%), p = 0.0005, OR 2.4 (1.2-4.5)), and prolonged ICU **stay** (287 (19.6%) vs. 168 (13.7%) p < 0.001, OR 1.3 (1.0-1.6)).*
#Boening- Ann Thorac Surg 2011 (334)	Cardiac surgery Retrospective cohort	3311 Anaemia defined as: haematocrit less than 33% or Hb ≤ 11 g/dL	5.6%	*The 30-day mortality of anaemic patients (12.9%) was significantly higher (p < 0.001) than the mortality of nonanemic patients (2.2%).* *Logistic regression (cofounding factors) (odds ratio 3.727, confidence interval: 2.196 to 6.324).* *Anaemia was a risk factor for perioperative morbidity (major adverse cardiovascular events) after CABG surgery (odds ratio 2.199, confidence interval: 1.423 to 3.397).*
#Shirzad- Cent Eur J Med 2010 (335)	Cardiac surgery Retrospective cohort	4432	14.6% <120g.l^{-1} used for men and women	*Mortality was increased in anaemia patients. For Hb<100g.l-1 OR=* 3.785 *For Hb>100 g.l^{-1}<119.9 g.l^{-1} OR=* 2.046
#Munoz- Transfus Altern Transfus Med 2010	Cardiac surgery Retrospective cohort	576	36.5%	*Postoperatively, anaemic patients received transfusions and inotropic support and stayed longer than four days in the recovery unit more frequently than non-anaemic patients, but there were no differences in the composite outcome variable (stroke, myocardial infarction, renal failure, or death).*
#Van Straten- Circulation 2009 (146)	Cardiac surgery Retrospective cohort	10,025	16%	*Anaemia was a predictor of early mortality (hazard ratio 1.48, 95% CI 1.08 to 2.02, whereas the preoperative haemoglobin level as a continuous variable was not.* *The preoperative haemoglobin level, entered as a continuous variable and as a dichotomous variable (anaemia), was an independent risk factor for late mortality.*
#*De Santo- J Thorac Cardiovasc Surg 2009*	Cardiac Surgery Prospective cohort	1047	28%	*Unadjusted odds ratios (Ors) for in-hospital death, cardiac morbidity, and **acute kidney injury** were 3.8 (95% confidence interval [CI] 2.0-7.3), 1.7 (95% CI 1.1-2.8), and 4.0 (95% CI 2.1-7.6), respectively. Adjusting for **anaemia** in confounders proved an independent predictor of **acute kidney injury** (OR 2.06; 95% CI 1.14-3.70), whereas the cardiac morbidity and in-hospital mortality were independently predicted by **kidney** function. No dose-response relationship emerged*

				*between **anaemia** severity and **acute kidney injury**.*
#Karkouti- Circulation 2008 (148)	Cardiac surgery Retrospective cohort	3500	26%	*Composite outcome of in-hospital death, stroke, or acute kidney injury Unadjusted OR 3.6 (2.7 to 4.7) Adjusted OR 2.0 (1.4-2.8)*
#Bell- Ann Thorac Surg 2008 (336)	Cardiac surgery Retrospective cohort	36,339	16.9%	*Unadjusted odds of thirty-day operative mortality for patients with **preoperative haemoglobin** level less than 10 g/dL were 2.37 times higher than for patients with **haemoglobin** levels of 10 g/dL or greater (95% confidence interval: 1.84 to 3.05; p < 0.0001). Multivariable effect upon 30-day operative mortality decreased considerably (odds ratio = 1.29, 95% confidence interval: 0.99 to 1.68; p = 0.064).*
#Kulier- Circulation 2007 (149)	Cardiac surgery Retrospective but from the Prospective dataset from the EPI II Study (337)	4804	28.1% Male 35.9% Female	*Low preoperative haemoglobin was a significant independent predictor only for noncardiac (P= 0.001) but not for cardiac outcome (P= 0.398). This was greatest for renal complications. Increased length of hospital stay if HB <110 g.l^{-1}*
#Cladellas- Eur Heart J 2006 (338)	Cardiac surgery-	201	20.9% <120g.l^{-1} used for men and women	***Pre-operative** HB <12 g/dL was identified as an independent predictor for **in-hospital mortality** (OR, 3.23; 95% CI, 1.09-9.55; P = 0.03). Also adjusting for EuroScore, **pre-operative** HB remained significant (OR, 3.64; 95% CI, 1.32-10.06; P = 0.01).*
Zindrou- Lancet 2002 (339)	Cardiac surgery Retrospective	2059	*Not reported for WHO definition. 2.6% had Hb <100g.l^{-1}*	*The crude mortality rate was five times higher among those with a haemoglobin value of 100 g/L or less (nine individuals, 17%) than among those with a higher haemoglobin concentration (69, 3·4%; p=0·001). Adjusted OR of 3.17 (high [Hb] vs. low [Hb]*
Carson- Lancet 1996 (19)	Non-cardiac surgery Retrospective patients who refused red cell transfusion for religious reasons	1958	*Not reported*	*Thirty-day mortality- seminal work- clear biological gradient of worsening mortality with reduced preoperative [Hb] Mortality was 1·3% (0·8–2·0) in patients with preoperative [Hb] ≥12 g.l^{-1} and 33·3% (18·6–51·0) if <60 g.l^{-1} The increased risk of death was more pronounced in patients with coronary artery disease 'Even mild anaemia may be associated with some increase in death risk'*

Study was included in the 2015 systematic review and meta-analysis by Fowler et al (156)
*Anaemia was defined by haematocrit (<39%)

Appendix B - Reasons for experimental failure

A total of 150 experiments were planned. Only 142 took place, in 7 experiments the test was not performed due to the CO gas supply running out and in 1 experiment the patient's blood clotted prior to analysis. Of the 142 experiments performed on 25 subjects 122 were suitable to be included on the final analysis. Six experiments had a ΔCOHb% of <4.5% so they were discarded as per standard laboratory practise in our laboratory. Five experiments had a significant leak identified or gas was lost on injection (1 experiment) and 3 experiments had technical problems with the blood gas analyser calibration and were unsuitable due to inaccurate readings and 6 experiments were performed in a subject who subsequently was found out to be a smoker.

Appendix C- UHS protocol for i.v. iron (POAS)

Adult Monofer infusion guideline for patients undergoing surgery	Version: 2
Date Issued:	March 2020
Review Date:	March 2022
Document Type:	Guideline

Executive Summary

Preoperative anaemia (defined by Hb<130g/L) increases perioperative morbidity and mortality in addition to increasing the transfusion risk. Intravenous iron is one of the therapies used in its management. All patients with preoperative anaemia should be referred back to primary care for investigation of the cause where appropriate.

Iron deficiency is normally treated satisfactorily by the use of oral iron supplements. However there are four circumstances when parenteral iron is recommended to replace iron stores, when; (i) oral iron supplements are tolerated poorly by some patients leading to non-adherence; (ii) there is insufficient absorption of iron from the gut due to an underlying illness such as renal disease or Crohn's disease; (iii) the rate of iron loss exceeds the total absorption rate of iron from the gut; or (iv) surgery is planned <8 weeks and cannot be delayed due to urgency of the treatment. Those patients requiring intravenous iron are primarily seen as day case patients at Victoria House, however for the preoperative patients,

they are treated on Surgical Day Unit, F level theatres recovery or on the surgical wards at Southampton General Hospital, or at Lymington Hospital.

1 Scope and Purpose

This guideline is relevant to patients undergoing surgery. It is aimed at doctors, pharmacists and nurses involved in the administration of Monofer to patients over 18 years of age.

2 Details of Procedure to be followed

The prescriber will be able to use this guideline, along with basic patient details, to calculate the appropriate Monofer dose for an individual patient. This should be prescribed on the electronic prescribing system (for SGH/PAH site) and on paper non-EPMA prescription chart for Lymington Hospital. The infusion should be prepared and administered according to the prescription and the administration recorded.

3 Roles and Responsibilities

Medical staff

- It is the admitting doctor's responsibility to ensure that treatment with intravenous iron is appropriate and other causes of anaemia have been excluded (including monitoring liver function tests).
- Review the contraindications and cautions to treatment. If any of these apply to the patient, then there must be documentation of the risk-benefit balance.
- It is the initiating doctors' responsibility to ensure that the appropriate monitoring (including haemoglobin and serum ferritin) is performed, reviewed and patients followed up appropriately. The frequency of monitoring will need to be decided on a case-by-case basis.
- It is the responsibility of the medical team to ensure that the iron infusion is prescribed.
- It is the consultant's responsibility to ensure that the guideline is followed, and specialist advice requested if required.

Nursing staff

- It is the admitting nurse's responsibility to weigh the patient (if not done within the last 6 weeks) to enable accurate calculation of the iron dose.
- Check for contraindications and cautions and ensure there has been proper consideration if any apply.
- Ensure that the infusion is prepared and administered safely and appropriately.
- The nursing staff will inform the patient about the risk of skin extravasation and discolouration. Patients will be asked to inform them immediately if they experience any pain at the cannula site.
- It is the responsibility of the discharging nurse to ensure that patients are supplied with verbal and written information about the treatment.

Pharmacy Staff

- It is the responsibility of the ward pharmacist to highlight any deviation from the guideline to the patient's medical team.
- It is the responsibility of the surgical pharmacist or their supervisor to ensure these guidelines remain updated.

4 Related Trust Policies

Medicines – prescribing, acquisition, storage, and administration.

5 Communication Plan

The guideline will be available on the Trust intranet.

6 Consent

Explain the procedure to the patient and gain verbal informed consent.

7 Therapeutic indications in perioperative setting

Monofer is indicated for the treatment of iron deficiency in the following circumstances:

- When oral iron preparations are ineffective or cannot be used.
- Where there is a clinical need to delivery iron rapidly.

The diagnosis of iron deficiency must be based on appropriate laboratory tests (serum ferritin, transferrin saturation and haemoglobin).

8 Process for Monitoring Compliance/Effectiveness

There is prospective data collection and periodic analysis of safety and efficacy of Monofer in perioperative care setting by the Perioperative Anaemia Service. Any areas of concern identified will be fed back to the appropriate clinicians.

9 Arrangements for Review of the Policy

Every three years.

10 References

1. Summary of Product Characteristics Monofer. Available at www.medicines.org.uk Last updated 15/10/2015 accessed February 2016
2. Personal Communication. Tim Greer. Pharmacosmos UK Ltd. 18/02/2016
3. 'Intravenous iron for the treatment of pre-operative anaemia in adults' Royal National Orthopaedic Hospital NHS Trust July 2018
4. 'A protocol for use and administration of intravenous iron isomaltoside (Monofer)' Barking, Havering, and Redbridge University Hospitals NHS Trust July 2019
5. 'Protocol for intravenous iron isomaltoside in the perioperative care setting for the treatment of iron deficiency anaemia where oral iron is not appropriate' The Newcastle upon Tyne Hospitals NHS Foundation Trust January 2018
6. 'Intravenous iron isomaltoside for correction of pre-operative iron deficiency anaemia in adults undergoing cardiac surgery' Royal Papworth Hospital August 2018
7. 'Guidelines for the use of parenteral iron in adults' Royal United Hospitals Bath June 2017

Appendix A – Clinical indications

Monofer therapy in preoperative patients is indicated in anaemic patients with absolute or functional iron deficiency.

Oral iron should be used as a first line treatment option where possible and is indicated in iron deficiency anaemia (IDA) where surgery is not urgent.

The diagnosis of iron deficiency must be based on laboratory tests.

The use of Monofer in the preoperative setting is for patients with IDA who have one of the following and fit the criteria for the anaemia pathway:

- Undergoing major surgery where the interval between diagnosis of IDA and date of procedure is predicted to be short (time to surgery <8 weeks)
- IDA with history or poor inadequate response to oral iron therapy after eight weeks of treatment
- IDA with a history or poor compliance or intolerance to oral iron due to side effects
- Functional IDA
- Mixed functional and true iron deficiency anaemia

Patients with non-anaemic iron deficiency are considered as a case-by-case scenario. (For e.g., blood refusers, high risk patient, high risk surgery)

Anaemia: Hb<130g/L for both men and women

Iron deficiency anaemia: Ferritin <30mg/l

Functional iron deficiency anaemia (FIDA): Ferritin>100mg/l + TSAT <20%

Mixed IDA/FIDA (Ferritin 30-100 + TSAT<20%)

Appendix B – Monofer Prescribing and Administration Guideline

The purpose of this guideline is to advise medical and nursing staff on the prescribing and administration of Monofer. Please refer to the Summary of Product Characteristics (available at www.medicines.org.uk) for full prescribing information.

Monofer (iron (III) isomaltoside 1000) is indicated for the treatment of iron deficiency when oral iron preparations are ineffective or cannot be used.
Intravenous iron preparations must always be prescribed by brand.

1. **Dosage and frequency of use**

The dose of Monofer is expressed in milligrams (mg) of elemental iron. The iron need and the administration schedule for Monofer must be individually established for each patient. After an iron deficient state has been corrected, patients may require repeated therapy to maintain acceptable haemoglobin and/or iron parameters.

For patients ≥50kg, dose is 20mg/kg rounded down to the nearest 100mg in the anaemic patient, with a maximum of 2000mg. For obese patients use ideal body weight.

For dosing for patients with weight <50kg: follow the Ganzoni formula:

Iron need (mg of iron) = body weight (kg) x (150-Hb)/10 x 2.4 + 500

2. **Patient monitoring before the infusion**

- The Hb level should be checked and recorded before the first dose is given.
- A set of observations (including blood pressure, pulse rate and temperature) and the MEWS score should be measured and recorded before administration. If the patient has any signs or symptoms of infection alert the appropriate doctor. They must decide whether or not it is appropriate to delay giving the dose until any infection has resolved.

3. **Method of administration**

Ensure adrenaline (epinephrine) is available prior to administration in case of an anaphylactoid reaction. Hydrocortisone 100mg IV and chlorpheniramine 10mg IV should also be prescribed as prn.

Monofer is available as iron (lll) isomaltoside solution for injection containing 100mg iron in 1ml. This is available as 1ml, 5ml and 10ml ampoules.

Doses should be diluted in 100mL **sodium chloride 0.9%** (although volumes up to 500mL are acceptable where appropriate) and administered by intravenous infusion. Only sodium chloride 0.9% should be used for dilution and flushing.

Doses up to 1000mg must be administered over more than 15 minutes. Doses exceeding 1000mg must be administered over 30 minutes or more.

No other therapeutic agents should be added.

Storage and Handling:
Inspect vials visually for sediment and damage before use and only use if sediment-free.
Store vials at room temperature.
Each vial is intended for single use only. Any unused product or waste material should be discarded.

4. **Cautions, contraindications, and side effects**

Parenterally administered iron preparations can cause hypersensitivity reactions including anaphylactoid reactions, which may be potentially fatal. If allergic reactions or signs of intolerance occur during administration, the treatment must be stopped immediately. Hypersensitivity reactions have also been reported after previously uneventful doses of parenteral iron complexes. The patient should be observed for adverse effects for at least 30 minutes following each Monofer injection.

FISHBANE reaction – typically when this occurs, a patient will get a small amount of iron and will complain of chest and back pain. It is nothing more than a mild, self-limited innocent arthralgia myalgia reaction that goes away. It is never associated with hypotension, tachycardia, periorbital oedema, or stridor. The management of the Fishbane reaction is to stop the infusion and the symptoms will resolve. After a period of several minutes, re-challenge the patient. It will not recur, and it should not be considered a serious adverse event.

Any side effects, however minor, should be reported to the doctor. The patient should be advised to telephone the UHS medicines helpline (02381 206907) or the unit/ ward if they experience any side effects at home after the infusion.

Contraindications:
- Known hypersensitivity to the active substance, to Monofer or to any of its excipients
- Known serious hypersensitivity to other parenteral iron products
- Non-iron deficiency anaemia
- Iron overload or disturbances in utilisation of iron
- Acute infection, ongoing bacteraemia, or flare of a rheumatological inflammatory condition
- Decompensated liver cirrhosis and hepatitis
- Concomitant administration with oral iron preparations (allow a minimum five-day gap as absorption of oral iron might be decreased)

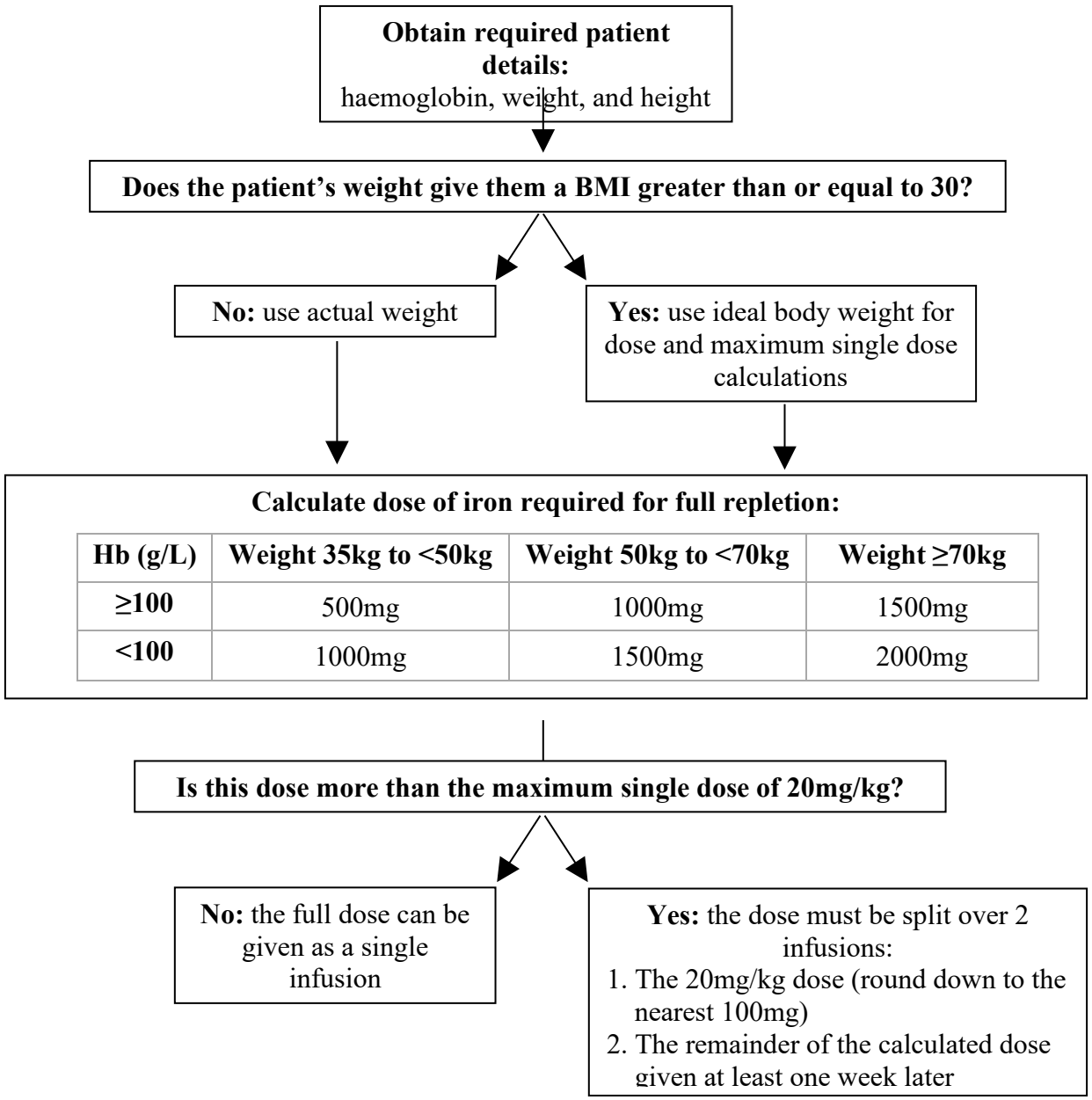
Cautions:
- Chronic infection
- Asthma, eczema, or atopic allergies
- Hypotensive episodes may occur if the intravenous injection is administered too rapidly.
- Pregnancy/lactation (seek specialist advice)

Please refer to the summary of product characteristics for a full list of contraindications, cautions and side effects.

In the event of a drug extravasation then standard procedures should be instituted (stop the infusion, aspirate residual medicines through the cannula and elevate the limb) and the Medicines Advice Service (extension 6908 and 6909) should be contacted for further advice. While the risk of tissue damage is low there is a risk of permanent skin staining.

5. Documentation on the managed care unit

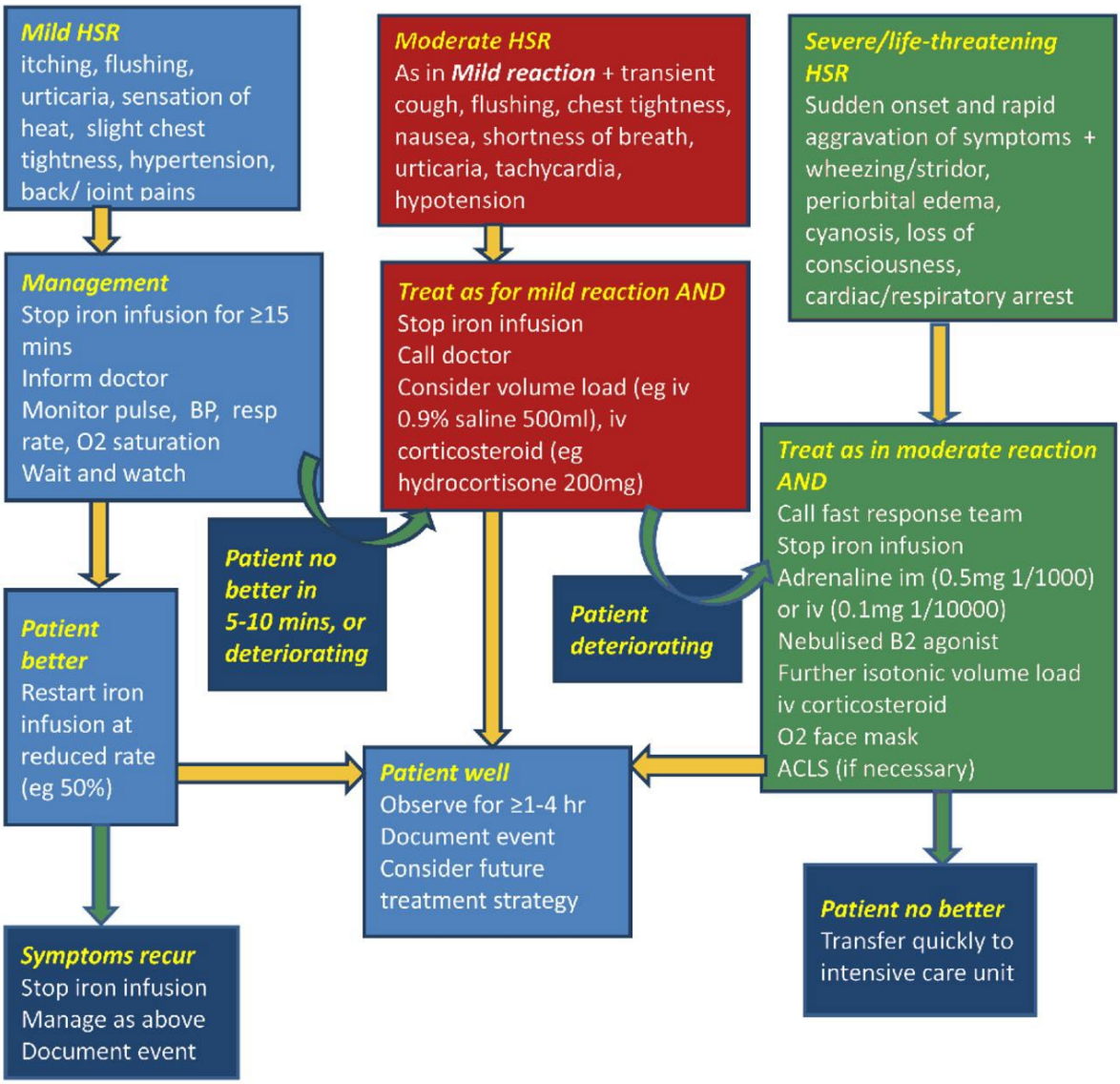
- Record in medical notes: Attendance, clinical observations, patient status and supply of a patient information leaflet.
- Ensure HMR is written.
- Every patient who receives Monofer should be provided with a patient information leaflet and details of who to contact if in the event of a possible adverse reaction.
- Once iron repletion has occurred it is the responsibility of the referring doctor to ensure regular assessments are completed to ensure that iron levels are corrected and maintained.



Height (imperial)	Height (metric)	Weight for BMI of 30 (kg)	IBW (M) (kg)	IBW (F) (kg)
5ft	152	69.7	50	45.5
5ft 1in	155	72.0	52.3	47.8
5ft 2in	157	74.4	54.6	50.1
5ft 3in	160	76.8	56.9	52.4
5ft 4in	163	79.3	59.2	54.7
5ft 5in	165	81.8	61.5	57
5ft 6in	168	84.3	63.8	59.3
5ft 7in	170	86.9	66.1	61.6
5ft 8in	173	89.5	68.4	63.9
5ft 9in	175	92.1	70.7	66.2
5ft 10in	178	94.8	73	68.5
5ft 11in	180	97.6	75.3	70.8

6ft	183	100.3	77.6	73.1
6ft 1in	185	103.1	79.9	75.4
6ft 2in	188	106.0	82.2	77.7

Appendix C – Grading and management of acute hypersensitivity reactions to intravenous iron infusions



Taken from Rampton, D. et al. (2014) Hypersensitivity reactions to intravenous iron: guidance for risk minimization and management. *Haematologica* 99(11) pp. 1671-6.

https://hosted.bmj.com/media/images/UK-MONF-0818-00017-InternationalConsensusSummaryLeavepiece-Digital-UK-Aug2018.pdf

https://www.ncbi.nlm.nih.gov/pubmed/27022297

Adult Monofer infusion guideline for patients undergoing surgery

Version: 1

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Target audience:	Prescribers creating prescriptions for Monofer for perioperative anaemia service Nurses administering Monofer.
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Does this document replace or revise an existing document	Yes
Should this document be made available on the public website?	No
Is this document to be published in any other format?	No

The Trust strives to ensure equality of opportunity for all, both as a major employer and as a provider of health care. This document has therefore been equality impact assessed to ensure fairness and consistency for all those covered by it, regardless of their individual differences, and the results are available on request.

Appendix D - POAS bloods sent

Hb, WBC, Plts, Neutrophils, MCV, RDW % , Platelet crit (PCT), Monocytes, MCHC, Triglyceride, TSAT %, Transferrin, Iron, Creatinine, Total protein, Albumin, Thyroxine, LDL, Cholesterol, Calcium/adjusted, Ferritin, Folate, B12

Appendix E- Publication PDFs

Plumb *et al. Extrem Physiol Med (2016) 5:5*
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Extreme Physiology & Medicine

REVIEW Open Access

'Blood doping' *from Armstrong to prehabilitation:* manipulation of blood to improve performance in athletes and physiological reserve in patients

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Abstract

Haemoglobin is the blood's oxygen carrying pigment and is encapsulated in red blood corpuscles. The concentration of haemoglobin in blood is dependent on both its total mass in the circulation (tHb-mass) and the total plasma volume in which it is suspended. Aerobic capacity is defined as the maximum amount of oxygen that can be consumed by the body per unit time and is one measure of physical fitness. Observations in athletes who have undergone blood doping or manipulation have revealed a closer relationship between physical fitness (aerobic capacity) and total haemoglobin mass (tHb-mass) than with haemoglobin concentration ([Hb]). Anaemia is defined by the World Health Organisation (WHO) as a haemoglobin concentration of <130 g/L for men and <120 g/L for women. Perioperative anaemia is a common problem and is associated with increased mortality and morbidity following surgery. Aerobic capacity is also associated with outcome following major surgery, with less fit patients having a higher incidence of mortality and morbidity after surgery. Taken together, these observations suggest that targeted preoperative elevation of tHb-mass may raise aerobic capacity both directly and indirectly (by augmenting preoperative exercise initiatives-'prehabilitation') and thus improve postoperative outcome. This notion in turn raises a number of questions. Which measure ([Hb] or tHb-mass) has the most value for the description of oxygen carrying capacity? Which measure has the most utility for targeting therapies to manipulate haemoglobin levels? Do the newer agents being used for blood manipulation (to increase tHb-mass) in elite sport have utility in the clinical environment? This review explores the literature relating to blood manipulation in elite sport as well as the relationship between perioperative anaemia, physical fitness and outcome following surgery, and suggests some avenues for exploring this area further.

Keywords: Blood doping, Blood manipulation, Anaemia, Perioperative, Surgery, Total haemoglobin mass, Autologous blood transfusion, Recombinant human erythropoietin rHuEPO, Prehabilitation, Altitude, $\dot{V}O_{2max}$, Cycling, Hypoxia-inducible factors, Surgical outcomes

Background

Haemoglobin is the blood's oxygen carrying pigment. Erythropoietin is the hormone that stimulates human haemoglobin (and red blood corpuscle) synthesis. Its synthetic recombinant form (rHuEPO) is commonly used in clinical practice to augment haemoglobin levels, as is the use of agents that support haemoglobin synthesis (such as intravenous or oral iron, vitamin B12 or folic acid) when these are deficient. Hypoxia-inducible factor (HIF) is a transcriptional regulator that (amongst other effects) drives erythropoietin synthesis, and whereby enhances haemoglobin levels. The first recorded human blood transfusion took place in 1795 [1] and homologous blood transfusion is widely used in clinical practice for anaemic patients, including during the perioperative period. Some elite athletes have illegally tried to enhance

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their performance by increasing their haemoglobin levels and thereby increasing their oxygen carrying capacity via the so-called 'blood doping'. Such activities have often taken place prior to rigorous safety trials (being properly performed for medical benefit) that are a measure of the risks that such athletes are prepared to take to achieve success [2]. For example, there is evidence that the new HIF activators are being abused within elite sport [3–6].

Aerobic capacity is defined as the maximum amount of oxygen that can be consumed by the body per unit time and is one measure of the physical fitness. $\dot{V}O_{2max}$ is classically defined as *'a plateau in oxygen uptake attained during maximal exercise despite further increases in exercise workload, thereby defining the limits of the cardiorespiratory system'* [7]. However, many individuals do not reach a plateau in oxygen uptake despite maximum exertion, and the term $\dot{V}O_{2peak}$ is used instead, being the highest measured oxygen consumption during exercise, typically averaged over a 30 s period. $\dot{V}O_2$ at anaerobic threshold is defined as *'the highest sustained intensity of exercise for which the measurement of oxygen uptake can account for the entire energy requirement.'* An alternative definition is *'the exercise intensity at which lactate starts to accumulate in the blood stream'* [8]. These oxygen uptake variables are in part dependent on the oxygen carrying capacity of the blood, which is in turn dependent on blood haemoglobin levels.

Anaemia is defined by the World Health Organisation (WHO) as a haemoglobin concentration of <130 g/L for men and <120 g/L for women [9]. Perioperative anaemia is common, with a quoted prevalence varying between 16 and 47 % reported in different patient cohorts (see Table 1), and is associated with increased morbidity and mortality following surgery. Using data from the European Surgical Outcomes Study (EuSOS) [10], Baron et al found that the presence of moderate anaemia was associated with a higher likelihood of in-hospital mortality than when it was absent, after adjustment for co-morbidities and the severity of the surgery [odds ratio (OR) 1.99–95 %; confidence intervals (CI) 1.67–2.37] [11]. Both $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$ are positively correlated with outcome following major surgery: less physically fit patients having a higher incidence of mortality and morbidity after surgery. Much of the literature in this area is derived from studies reporting cardiopulmonary exercise testing (CPET) variables. The underlying hypothesis of these studies has been that patients with greater physiological reserve defined by CPET variables (most commonly $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$) are better able to withstand the physiological challenge of surgery. Given that the oxygen uptake variables $\dot{V}O_{2peak}$ and $\dot{V}O_{2AT}$ are correlated with [Hb], it may be that some of the physical fitness–outcome relationship is mediated through haemoglobin related effects rather than cardiorespiratory function.

Prehabilitation is the process of enhancing functional capacity of the individual to enable him or her to withstand a subsequent stressor. This may be achieved through a single well-defined intervention (e.g. structured aerobic exercise programme) [20] or may encompass a package of smaller integrated steps leading to overall functional improvement, the so-called 'aggregation of marginal gains' [21–23]. Such interventions have a role in prehabilitation within clinical medicine in general, and before major surgery in particular. However, whilst the efficacy of such approaches in improving physical fitness is becoming clearer [20, 24], it is currently uncertain whether they will be effective in improving clinical outcomes in the perioperative setting. As we learn more about the relationship between physical fitness, defined by CPET-derived variables, and responses to prehabilitation in different patient groups, it may be that lessons learned from elite athletes could be applied to improving outcomes in patients around the time of surgery.

Table 1 Prevalence of preoperative anaemia

Study	Cohort	Study population	Prevalence of anaemia (%)
Baron—BMJ 2014 [11]	Non-cardiac, non-neurological surgery	46,539	28.7
Sagger—Anesth Analg 2013 [12]	Non-cardiac surgery	574,860	25.3
Gupta Ann Surgery 2013 [13]	Patients over 65 elective vascular surgery	31,857	47
Musallam Lancet 2011 [14]	Non-cardiac surgery	227,425	30.44
Van Straten—Circulation 2009 [15]	Cardiac surgery	10,025	16
Beattie—Anaesthesiology 2009 [16]	Non-cardiac surgery	7759	39.5
Karkouti—Circulation 2008 [17]	Cardiac surgery	3500	26
Kulier—Circulation 2007 [18]	Cardiac surgery	4804	28.1 male 35.9 female
Wu JAMA 2007 [19]	Non-cardiac surgery	310,311	42.8

Broadly based on WHO definition of anaemia, <130 g/L for men and <120 g/L for non-pregnant women

Measuring haemoglobin concentration vs. total haemoglobin mass

Traditionally, the concentration of circulating haemoglobin [Hb] has been used as a clinical measure of the blood's oxygen carrying capacity. However, a low [Hb] may be due to a reduced amount of haemoglobin (absolute mass of circulating haemoglobin; tHb-mass) or an increased volume of dilution (plasma volume). Thus, [Hb] may be stable and tHb-mass low in the context of acute bleeding, [Hb] normal or elevated but tHb-mass low in the context of dehydration, or [Hb] low but tHb-mass normal or high in the context of excess plasma volume (fluid). Therefore, the use of [Hb] to define blood oxygen carrying capacity may be misleading under some circumstances.

tHb-mass represents the absolute mass of circulating haemoglobin in the body, the measured [Hb] being dependent upon tHb-mass and blood volume (BV) [sum of plasma volume (PV) and total red cell volume]. The proportion of oxygen carried in solution in plasma is trivial (0.3 ml per 100 ml of plasma) under normal physiological conditions, whereas each gram of Hb binds up to 1.39 ml of oxygen. Thus, tHb-mass is the principal determinant of total blood O_2-carrying capacity and may provide additional information regarding the clinical status of patients than that provided by [Hb] alone.

It appears that tHb-mass is of greater utility in blood manipulation in elite athletes trying to improve sporting performance as it is more stable and predictable over time and also has a more direct correlation with performance. The question addressed by this review is whether tHb-mass, in comparison with haemoglobin concentration, is a more precise and accurate variable to guide targeting of haemoglobin manipulation, if the aim is to improve physiological reserve in patients in order to improve clinical outcomes. We also explore the techniques of blood manipulation in elite athletes and whether any of these techniques may be useful from a prehabilitation perspective within clinical medicine.

Haemoglobin manipulation in sport

Athletes and coaches are constantly pursuing *legal* means, such as training at altitude to augment oxygen carriage through an increase in [Hb] and thereby improving sea-level performance. However, recent revelations relating to high profile individuals within professional cycling, including Floyd Landis, Tyler Hamilton [25] and Lance Armstrong [26], have highlighted the *illegal* methods used by some athletes to improve performance, often in advance of the efforts of regulatory authorities to constrain them and of their adoption into clinical medicine [27]. It is legitimate to question whether such methods are safe (or at least fall within the broad margins of safety), if they are effective and if they could have wider applicability within clinical medicine.

Whilst a variety of agents have been used to manipulate haemoglobin levels (e.g. blood, recombinant human erythropoietin (rHuEPO), Continuous Erythropoietin Receptor Activator (CERA), hypoxia-inducible factor (HIFs) agents and possibly even 'gene doping' (although we do not yet have definitive evidence of this [28, 29]), the basic aim remains the same that increasing oxygen delivery (DO_2) through elevating haemoglobin levels will augment maximum oxygen uptake ($\dot{V}O_{2max}$) and perhaps more importantly (for endurance events) increase the workload at which anaerobic threshold (AT) is reached. There is still debate around the factors that limit $\dot{V}O_{2max}$, with candidate mechanisms including central control, cardiac limitation, mitochondrial utilisation and total oxygen delivery (the product of cardiac output and blood oxygen content). However, whilst there remains uncertainty about the dominant controlling factor, many authorities agree that in highly trained athletes, DO_2 is a factor that contributes to $\dot{V}O_{2max}$ limitation [30–32] and that $\dot{V}O_{2max}$ is also, at least in part, dependent on a number of underlying genetic factors that are not amendable to modification through training [33]. Therefore, blood manipulation to augment DO_2 has been seen as a logical, albeit illegal, approach to augmenting $\dot{V}O_{2max}$ and thereby improving athletic performance. It is notable in this regard that tHb-mass displays a much stronger relationship with $\dot{V}O_{2max}$ than does [Hb] [34, 35] and may therefore be a more useful marker of intervention efficacy. Here, the relationship between different physiological measures of physical fitness and performance merits consideration. Whilst the majority of sports research has focused on $\dot{V}O_{2peak}$ or $\dot{V}O_{2max}$ as the accepted gold standard indices of cardiorespiratory fitness, other variables may have an important role in determining performance, particularly in endurance events. As exercise increases above a threshold submaximal work rate, anaerobic respiration begins to contribute to Adenosine Triphosphate (ATP) production and this is both inefficient (relative to aerobic respiration) and unsustainable (due to progressive lactic acidosis). Therefore, when discussing performance, although a high total aerobic capacity ($\dot{V}O_{2peak}$/ $\dot{V}O_{2max}$) is important for success in endurance sports, submaximal indices of fitness, such as the lactate or anaerobic threshold (LT/AT) and exercise efficiency/economy, may also be critical determinants of performance. For example, two athletes with the same $\dot{V}O_{2max}$ do not necessarily perform to the same level in an endurance performance test or race: the athlete with the higher $\dot{V}O_{2AT}$ is likely to perform better. Furthermore, the efficiency or economy with which work is done relative to energy expenditure may be important. For example, Lucia et al

showed that a range of $\dot{V}O_{2max}$ levels amongst elite cyclists could be compensated for by differences in efficiency [36]. Whilst improvements to $\dot{V}O_{2max}$ are important, very few athletic competitions are performed at $\dot{V}O_{2max}$ and it cannot therefore be assumed that performance will be enhanced to the same degree as $\dot{V}O_{2max}$ increases. Intriguingly, the premise that improvement in physiological variables (i.e. aerobic capacity) enhances athletic performance (i.e. races or gold medals won) has not been well investigated. Having said that, the effects of blood manipulation on a range of physiological variables, including to $\dot{V}O_{2max/peak}$ and $\dot{V}O_{2AT}$, are both of relevance for athletes and may have significance in clinical contexts [37].

What is 'blood manipulation'/'blood doping'?

The World Anti-Doping Agency (WADA) defines blood manipulation as the reintroduction of blood or blood products allogenic (homologous) or heterologous, the artificial enhancement of oxygen transportation or delivery and any form of intravascular manipulation of the blood or its components by physical or chemical means [3]. Blood doping is complex and rapidly evolving, as highlighted by the recent WADA amendments to the 2014 prohibited list consequent on the emergent use of Xenon and Argon as HIF activators. It was reported that Russian athletes used HIF activators at the 2014 winter Olympics in Sochi [3]. The earliest reports of 'blood doping' in the scientific literature date back to 1945–1947 [38, 39]. The first alleged use in elite sport was in the 1960s, when a French four times Tour de France winner (1961–1964) was named as one of the first cyclists to use the technique [40]. The first reported use in athletics comes from around the time of the 1968 Mexico City Olympic Games.

It has been said, "Increasing the oxygen transport capacity of the exercising skeletal muscles, either by means of training or doping, is the most powerful tool for improving athletic performance in aerobic sports [41]". Below we will briefly outline some of the methods of blood manipulation used in sport.

Autologous blood transfusion

The link between the O_2-carrying capacity of the blood and indices of exercise capacity such as $\dot{V}O_{2max}$ has recently been reviewed elsewhere [34]. Haematocrit (Hct) is also known as packed cell volume (PCV) or erythrocyte volume (ECV) and is the volume percentage of red blood cells within the blood. There does not appear to be a simple linear correlation between haematocrit and increased $\dot{V}O_{2max}$. Brun et al showed that a "low" haematocrit (Hct) (<40 %) was associated with a higher aerobic capacity [42]. However, this must be interpreted with caution, as the lowest Hct was only 36.8 % (i.e. not actually *that low*). It is probable that lower Hct levels, such as those seen in patients rather than athletes or healthy volunteers, would result in a reduced oxygen carrying capacity and therefore reduced $\dot{V}O_{2max}$. By the 1970s, it was becoming well known that increasing tHb-mass could increase $\dot{V}O_{2max}$. It later became clear that other factors were also important, for example, changes in diastolic function and changes in blood volume (BV) [43].

A 1982 review documented all published studies comparing exercise testing variables pre-phlebotomy, and post transfusion, at that time. It is apparent from Table 2 that a *significant* increase in [Hb] was associated with an increase in $\dot{V}O_{2max}$. The author concluded that at least 2 units of blood were needed with frozen blood being superior to refrigerated blood [44]. Of the 14 studies in Table 2, *only 5* of them showed statistically significant improvements in [Hb] and $\dot{V}O_{2max}$ post autologous transfusion [39, 45–48]. The results of studies failing to find such a relationship between [Hb] and exercise capacity may in part be explained by the small quantity of blood re-infused, insufficient time for the body to achieve equilibrium [Hb] after venesection, and inadequate storage of the RBCs [44].

In general, autologous blood transfusion seems to improve performance, but there are very few studies addressing this question directly. Improved 5-mile treadmill run times (mean improvement of 44 s) with reduced self-reported perceived exertion after autologous blood transfusion were demonstrated by Williams et al [47]. Berglund et al demonstrated a significant fall in the race times of cross-country skiers when compared to matched controls pre- and post autologous blood transfusion [49]. Brien et al took 6 well-trained runners and improved their 10 km time by an average of 1 min. Using a double-blind cross-over design, each runner received a 400 ml autologous transfusion of blood or saline repeated again 5 days apart with a 10 km race 5 days after each treatment. Five out of the 6 runners had faster race times after transfusion [50].

Recombinant human erythropoietin: rHuEPO

There are more data available for rHuEPO and a number of studies have shown correlation between improved performance and rHuEPO use. In 1991, Ekblom et al showed an improved $\dot{V}O_{2max}$ post rHuEPO injection in 15 volunteers [51]. Similar results were shown by Audran et al. Table 2 from this paper shows the increase in Hct and [Hb] from day 0 to day 24 and subsequent rise in $\dot{V}O_{2max}$ with reduction in maximum heart rate [52]. Parisotto et al attempted to develop a blood profile to detect athletes who were abusing rHuEPO and were able to

Table 2 Summary of studies of blood doping and exercise

Authors	Date	Storage technique	Volume infused[a] (ml)	Time of reinfusion post phlebotomy	Hb or Hct vs control[b] (%)	$\dot{V}O_{2max}$ vs control (%)	End capacity[c] vs control[b] (%)
Pace et al	1947	Fresh[g]	2000	–	+26[d]	N.R	+34.7[d]
Gullbring et al	1960	Refridg	610	7 days	+0.7	N.R	+3
Robinson et al	1966	Refridg	1000	2 weeks	+4.8	+1.4	N.R
Ekblom et al	1972	Refridg	800	4 weeks	+2.1	+5.5[e]	+ 15.6[e]
		Refridg	1200	4 weeks	+1.3	+1.6[e]	+ 25.1[e]
Von Rost et al	1975	Refridg	900	3 weeks	+2.7	+9.0[e]	+ 37[5]
Bell et al	1976	Refridg	500	3 weeks	+1.0	+5.6[f]	+ 7.5
Ekblom et al	1976	Refridg	800	~5 weeks	+4.5[e]	+8.0[d]	N.R
Videman and RytÖmaa	1977	Refridg	4–600	2–3 weeks	+2.6	N.R	+ 3.8
Robertson et al	1978 Abst	N.R	1800	NR	N.R.	+12.8[d]	+ 15.6[d]
Williams et al	1978	Frozen	460	3 weeks	+3.3	N.R	+ 4.1
Cottrell	1979 Abst	Frozen	405	9 weeks	N.R.	~+2.0[f]	N.R
Roberston et al	1979 Abst	N.R	800	N.R.	+15.8[d]	+30.5[d]	+ 13.1[d]
Buick et al	1980	Frozen	900	7 weeks	+11[d]	+5[d]	+ 35[d]
Spriet et al	1980 Abst	Frozen	800	11 weeks	+7.9[d]	+3.9[d]	N.R
			1200	12 weeks	+10.7[d]	+6.6[d]	N.R
Williams et al	1981	Frozen	920	7 weeks	+7[d]	N.R	+2.5[d]

Reproduced with permission from Wolters Kluwer Health [44]

NR data not reported, *Refridg* refrigerated

[a] Whole blood or equivalent whole blood

[b] *Control* pre-phlebotomy measurement

[c] Endurance exercise capacity, physical work capacity or performance time

[d] Statistically significant ($P \leq 0.05$)

[e] No statistical analysis reported

[f] Predicted from submaximal exercise heart rate

[g] Fresh homologous blood; all other studies used autologous blood

demonstrate a predictable blood profile post rHuEPO usage. They measured tHb-mass (using Burge and Skinner's method) and found a consistent increase in Hct, [Hb] and tHb-mass 3 weeks after rHuEPO administration, which persisted for 21 days. They also found a 6.3 and 6.9 % increase in $\dot{V}O_{2max}$ compared to placebo. After a 4-week washout period, tHb-mass and $\dot{V}O_{2max}$ had returned to baseline [53]. Birkeland et al showed in a double-blind placebo-controlled trial that injection of 5000 IU of rHuEPO thrice weekly for 4 weeks improved $\dot{V}O_{2max}$ by 7 %. They found that Hct rose from a mean of 42.7–50.8 and peaked 1 day after rHuEPO was stopped. Haemoglobin concentration also increased in the rHuEPO group [54].

However, data supporting an improvement in performance following rHuEPO usage in athletes were still limited. Russell et al were the first to characterise the submaximal and maximal exercise adaptations to prolonged use of low dose rHuEPO. They compared 3 groups, (1) intravenous (i.v.) iron + rHuEPO, (2) oral iron + rHuEPO and (3) placebo. They performed exercise tests on a cycle ergometer at weeks 0, 4, 8 and 12. The relative increases in $\dot{V}O_{2max}$ at weeks 4, 8 and 12 were 7.7, 9.7 and 4.5 %, respectively, for the rHuEPO + i.v. iron group; 6.0, 4.7 and 3.1 % for the oral iron + rHuEPO group; and −0.5, −0.1 and −1.0 % for the placebo group [55].

In 2007, Thomsen et al stated that "Although the positive effect of rHuEPO treatment on $\dot{V}O_{2max}$ is clearly established, it remains unknown as to what its impact is on endurance performance". They investigated the effect of rHuEPO on $\dot{V}O_{2max}$ and time to exhaustion during cycle ergometry in healthy volunteers. rHuEPO significantly increased $\dot{V}O_{2max}$ by 9.1 and 8.1 % in week 4 and 11, respectively, with no changes in the placebo group [37].

Emerging strategies

The range of interventions aimed at increasing tHb-mass, both in development and currently available, is large and has been extensively reviewed elsewhere [2, 4, 6, 56].

Towards the end of the 1990s, interest had grown within clinical medicine and the sporting world in using

artificial oxygen carriers and perfluorocarbon emulsions [57]. However, neither has been adopted in either setting, probably due to well-recognised adverse effects and ease of detection [58].

HIF stabilisers/activators are compounds that act by mimicking hypoxia and thereby stimulating EPO synthesis via the HIF pathway. When the partial pressure of oxygen is low, HIF1α undergoes a stabilisation process, which leads to gene transcription, including that of the erythropoietin gene [59, 60]. HIF stabilisers/activators are oral compounds, which are potentially advantageous as they are simply administered and are less immunogenic than erythrocyte-stimulating agents such as rHuEPO. The number of agents available is beyond the scope of this review and has recently been summarised elsewhere [2]. There is already clear evidence of their abuse within elite sport [3–5, 56]. Cobalt is one such agent, which has adverse effects including heart, liver, kidney and thyroid toxicity as well as cancer promotion [61]. There is evidence of cobalt being abused in horse racing [62]. Xenon and argon are also both HIF activators and have both been reportedly used as performance-enhancing agents in the recent years [3, 63].

Gene therapy is also theoretically possible, but some early reports highlighting significant safety concerns including life-threatening red cell aplasia and extreme erythrocytosis have probably limited its use [64]. There are also EPO-mimetic peptides such as *Peginesatide* that are not currently in production but are nevertheless candidates for abuse [2].

Harms: what are the downsides?

Not only is blood manipulation/doping illegal, but also many of the agents used may pose health risks to the athlete. Despite this, some athletes are apparently prepared to accept such risks to increase their chances of success. As has already been noted, manipulation of haemoglobin may be associated with a variety of adverse effects including, for example, hyper-viscosity from rHuEPO and the toxic effect of cobalt. The risks associated with blood transfusion are summarised in Table 3.

Table 3 Risks associated with blood transfusion and manipulation

Theoretical	Demonstrated
Age of stored blood may affect its efficacy; the so-called 'storage lesion' [65]	Transfer of infectious diseases [40]
	Transfusion reactions/anaphylaxis
	Increase in colorectal cancer recurrence [66]
	Phlebitis [40]
	Septicaemia [40]
	Graft versus host disease (GvHD)
	Transfusion-related immunomodulation (TRIM) [67, 68]
	Hyper-viscosity PE and DVT [40]
	Air embolism [40]
	Transfusion-related acute lung injury (TRALI)
	Risk of wrong blood (storage problems) [25]
	Detraining effect [40]
	Illegal practice to blood dope [25, 26]

Haemoglobin manipulation in the clinical setting

Blood manipulation occurs commonly in clinical practice. In the UK, approximately 8000 units of blood are transfused each day [69] including homologous transfusion and transfusion of blood salvaged during major surgery. The level of anaemia that mandates blood transfusion is not well defined in all perioperative settings [67, 70–74] and there has been a shift over the last two decades towards more conservative transfusion strategies, particularly within intensive care [75, 76]. Whilst the association of anaemia with adverse outcome is well recognised, uncertainty remains as to whether this relationship is causal and about when and how to intervene in the perioperative period. It is unclear whether anaemia per se causes increased morbidity/mortality or whether anaemia is associated with other (perhaps unidentified) pathology, which is the cause of the adverse outcomes. Whilst the study by Baron et al suggests that anaemia alone (once all co-morbidities are corrected for) is associated with an increased mortality in perioperative patients [11], residual confounding cannot be excluded due to the observational design of this study. Of note, measures to correct anaemia (including transfusion) seem ineffective at reducing the incidence of adverse outcome. This may be because anaemia is not the cause of the underlying pathology, in which case correcting anaemia would not be expected to improve outcome, or alternatively that adverse consequences of the interventions used (such as blood transfusion) outweigh the benefits of correcting anaemia. It is commonly hypothesised that much of the morbidity associated with a more liberal transfusion strategy is due to the adverse effects of homologous stored blood rather than the increased oxygen carrying capacity actually being ineffective. The on-going evolution of preparation techniques for transfused blood is likely continuing to alter the risk–benefit ratio for different transfusion strategies. Amongst the multiple reasons for potential harm from transfused blood (see Table 3), age of the blood is an area that has recently been investigated [77]; however, no significant differences were found with regard to 90-day mortality between a fresh blood

group (6.1 ± 4.9 days) and when compared with standard blood (22.0 ± 8.4 days) [77]. A recent analysis of the FOCUS study comparing a liberal and restrictive strategy in hip fracture patients found no difference in 3-year mortality [70]. The balance between the theoretical benefits of augmenting DO_2 and the harms of transfusion remains unclear: "Blood transfusion is like a marriage: It should not be entered into lightly, unadvisedly, or wantonly, or more often than is absolutely necessary" [78].

Two large observational datasets in non-cardiac surgery have shown that anaemic patients spend more time in intensive care, suffer more complications, stay in hospital longer, consume greater hospital resource and are more likely to die [11, 14]. The same pattern is also reflected in data from cardiac surgical practice [17, 79]. NHS England has issued guidance on the management of patients who present for elective surgery. The poor outcome associated with anaemia has led to the recommendation that these patients have their surgery delayed until treatment of their anaemia has occurred [80].

Whilst anaemia is clearly important, some authors have questioned its reliability as an independent marker of ill health as it may often be linked to an underlying acute or chronic disease that may yet be undiagnosed. Interestingly, Baron et al accounted for this: after adjusting for co-morbidities and severity of the surgery, patients with moderate anaemia had a higher in-hospital mortality [odds ratio 1.99 (95 % confidence interval 1.67–2.37)] [11]. Despite this, there is currently no convincing evidence that treating the absolute value of [Hb] improves outcome.

Importantly, the majority of studies in perioperative transfusion have examined the 'very anaemic' and attempted to move them to the 'slightly less anaemic'. Little work has been done to manipulate 'normal physiology' to target supra-optimal DO_2 values (through Hb augmentation) in this population. Manipulation of tHb-mass, in contrast to [Hb], in patients who are about to undergo a physiological challenge, may improve their resilience to such an insult. Equating 'performance' gains by elite athletes to the ability of patients to survive surgery involves a substantial conceptual leap, but recent preliminary work has shown that transfusion can improve exercise variables in anaemic patients [81]. It remains uncertain whether clinical outcomes will alter alongside changes in tHb-mass and whether the closer relationship between tHb-mass (compared with [Hb]) and performance in the athletic context, will be mirrored for patient outcomes in the clinical context. Indeed it may be that the metabolic cost of modern surgery has been overestimated, thus allowing for an adequate DO2 at 'low' O_2 carrying states. The recent POM-O study was a randomised controlled trial of patients undergoing major elective surgery. Patients were randomised to a postoperative protocol (fluid, with and without dobutamine) targeted to achieve their individual preoperative oxygen delivery value (goal-directed therapy) or standardised care (control). Maintaining DO_2 appeared to be the important factor regardless of whether patients were in the goal-directed therapy group (fluids and dobutamine) or the usual care group [82].

Prehabilitation

Interest in prehabilitation has grown off the back of the success of enhanced recovery programmes (ERPs). ERPs were set up to try improving surgical outcomes by implementing care pathways. These pathways did not focus on discovering new knowledge but placed importance on integrating the best evidence into practice. It has been shown that exercise testing in patients before surgery is feasible and that physiological gains can be made in only short periods of time [12, 83, 84], although the clinical benefits remain uncertain [24]. CPET may play a role in guiding prehabilitation [85].

In a recent study, AT improved by a mean difference of 1.2 ml kg^{-1} min^{-1} in anaemic patients who had CPET pre- and post the transfusion of autologous blood [81]. The clinical effects of an increase in $\dot{V}O_2$ at the AT of around 1 ml kg^{-1} min^{-1} are uncertain and this was a small single-centre study (with consequent elevated risk of bias). However, the results were consistent with a study in thalassemic patients, showing improved exercise duration and $\dot{V}O_{2peak}$ [86]. Pilot et al showed that autologous transfusion following hip arthroplasty improved early postoperative exercise testing variables, but this effect was equivocal by day 23. However, this study was not randomised and is therefore subject to risk of both confounding and bias [87]. This area needs further research. There is an on-going study looking at intravenous iron infusion in major abdominal surgery [88]. The transfusion trigger in major elective surgery remains unknown, particularly with regard to outcomes such as ability to mobilise postoperatively [89]. Within the hip fracture population, postoperative anaemia is associated with increased length of stay, reduced ambulation and reduced functional independence [90]. However, evidence is lacking for a liberal transfusion strategy in non-cardiac surgery ([Hb] 80–100 g/L) and this is consistent with the literature in critically unwell patients with septic shock [75].

The effects of prehabilitation training on the mitochondrial architecture, redox state and muscle capillary network remain unstudied in surgical patients and the additional effect of augmenting tHb-mass is also unknown. Training effects are of course different from the effects of blood manipulation and optimising the

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most critical steps in mitochondrial oxygen transport by training may be superior to using rHuEPO to enhance gene expression and induce angiogenesis; however, this remains to be elucidated. Exercise modifies mitochondrial biogenesis, not only by upregulating antioxidant enzymes but also by increasing mitochondrial number, thereby allowing for a lower level of respiratory activity for the same degree of ATP generation [91, 92]. Interestingly, Jacobs et al found that improvement in exercise performance after six sessions of high intensity training (HIT) over the span of 2 weeks was primarily attributed to enhanced oxidative potential in the skeletal muscle with no measurable effect on tHb-mass [91].

It may be that for some patients DO_2 may be critical to VO_2 and survival, whereas in another group of patients, anaemia, fitness levels and DO_2 are part of a broader pattern of resilience. The biological pathways whereby regular physical activity might confer resilience include: (1) serving as a buffer against stress and stress-related disorders/chronic diseases, (2) optimising neuroendocrine and physiological responses to physical and psychosocial stressors, (3) promoting an anti-inflammatory state and (4) enhancing neuroplasticity and growth factor expression [93].

Are we then aiming for the total package of broader gains from 'fitness' or can we just look at specific targets such as tHb-mass? Whilst the complex biological mechanisms that relate 'fitness' to resilience remain opaque, it could be postulated that differences or gains (from targeted therapy or intervention) seen by patients on the CPET bike are simply a result of the other unmeasured markers of physical fitness. In the 1990s–2000s, when professional cycling had a widespread doping problem, highly trained elite athletes were experiencing significant gains over and above the increased gains in fitness from simply having a greater DO_2 (primarily from doping, giving them a far greater tHb-mass). It is likely that patients would benefit from increased fitness levels and pilot data suggest that physical training does indeed return fitness levels back to baseline after a physiological insult such as chemotherapy [20]. Whether increasing tHb-mass offers benefit to all patients, or only those in whom it is initially subnormal remains to be elucidated.

Future directions

We know that the manipulation of tHb-mass is possible via a number of strategies. There is good evidence that CPET variables can be improved in line with gains in tHb-mass in athletes. We also know that fitness relates to outcome following surgery. What is not known is if the same relationship between physical fitness (CPET variables) and tHb-mass exists in patients who are awaiting major surgery or critically ill in hospital. The measurement of tHb-mass and correlation to CPET variables has not been studied within clinical medicine. It is also not clear which method would be best to manipulate tHb-mass in patients and what would represent the 'optimal' tHb-mass in each individual, balancing the risks of an increased tHb-mass with the theoretical benefits of improved oxygen delivery. We hypothesise that tHb-mass is a more accurate variable to guide and quantify potential intervention than [Hb] as it is relatively more stable [94–97] and is not affected by changes in plasma volume which may vary greatly in the perioperative period.

Informative studies might focus on measuring tHb-mass in different patient groups and quantifying the relationship with CPET variables. Initial work to establish safety margins for different levels of haemoglobin mass in the perioperative period and the safest method for achieving them would be valuable. Preliminary studies to establish the strength of relationship between tHb-mass and outcome in comparison with that of [Hb] would also be of value. It may then be justifiable to explore whether manipulation of tHb-mass, rather than [Hb], has a positive effect on surgical outcomes in adequately powered randomised controlled trials.

It is not known what an increased tHb-mass does to mitochondrial function or if the benefits of training that come from a high intensity programme, in terms of improved mitochondrial content and function [91], can be augmented by boosting tHb-mass via rHuEPO, autologous blood transfusion or iron therapy. The ergogenic effects of EPO independent of its effect on boosting tHb-mass warrant further study and it is likely that there is a complex interplay between erythropoietin concentrations, reticulocyte migration and gene expression that may affect CPET variables and possibly outcome.

Conclusions

In performance sport, blood doping continues to be a problem. Novel agents are continually being developed and the regulatory bodies struggle to catch up with the dopers. The success of such strategies raises the question as to whether some of these approaches may have utility in clinical practice.

In particular, the closer relationship between tHb-mass (rather than [Hb]) and $\dot{V}O_{2max}$ raises the question as to whether we should be targeting this variable with blood manipulation techniques in the clinical setting.

The recognised association between low levels of physical fitness and adverse clinical outcomes in the perioperative context offer a specific clinical setting in which it may be valuable to address these questions. Furthermore, preoperative exercise training interventions (prehabilitation) may be optimised by such an approach.

Observational studies clarifying the relationship between tHb-mass and physical fitness and clinical outcomes in patients are required before interventional studies using this variable to target blood manipulation strategies are justified. The newer agents being used for blood manipulation in elite sport may have utility in this respect in the clinical environment.

Health warning

Blood doping/boosting or manipulation of the blood in anyway is inherently dangerous and can result in death. The authors strongly discourage anyone from undertaking any form of blood manipulation except under the close supervision of a trained medical specialist as part of a research trial or as a planned medical intervention for ill health.

The authors would also advocate that anyone manipulating their blood within the rules of the World Anti-Doping Authorities (WADA) such as by altitude training or artificial hypobaric environment usage do so under the close supervision of a medical professional experienced in the manipulation of haemoglobin. https://www.wada-ama.org/.

Abbreviations

AT: anaerobic threshold; BV: blood volume; CERA: Continuous Erythropoietin Receptor Activator; CPET: cardiopulmonary exercise testing; EPO: erythropoietin; ECV: erythrocyte volume; [Hb]: haemoglobin concentration; Hct: haematocrit; HIFs: hypoxia-inducible factors; LT: lactate threshold; PCV: packed cell volume; RCV: red cell volume; rHuEPO: recombinant human erythropoietin; tHb-mass: total haemoglobin mass; TRIM: transfusion-related immunomodulation; $\dot{V}O_{2max}$: maximum oxygen uptake; $\dot{V}O_{2peak}$: peak oxygen uptake; WADA: World Anti-Doping Agency.

Authors' contributions

JP conceived the review, coordinated it and helped to draft the manuscript. JO helped drafting the manuscript. MG helped with drafting and reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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ORIGINAL RESEARCH

Physiological Reports

Application of the optimized carbon monoxide rebreathing method for the measurement of total haemoglobin mass in chronic liver disease

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Abstract

Background: Anemia is common in liver cirrhosis. This generally infers a fall in total hemoglobin mass (tHb-mass). However, hemoglobin concentration ([Hb]) may fall due to an expansion in plasma volume (PV). The "optimized carbon monoxide rebreathing method" (oCOR) measures tHb-mass directly and PV (indirectly using hematocrit). It relies upon carboxyhemoglobin (COHb) distribution throughout the entire circulation. In healthy subjects, such distribution is complete within 6–8 min. Given the altered circulatory dynamics in cirrhosis, we sought in this pilot study, to assess whether this was true in cirrhosis. The primary aim was to ascertain if the standard timings for the oCOR were applicable to patients with chronic liver disease and cirrhosis. The secondary aim was to explore the applicability of standard CO dosing methodologies to this patient population.

Methods: Sixteen patients with chronic liver parenchymal disease were studied. However, tHb-mass was determined using the standard oCOR technique before elective paracentesis. Three subjects had an inadequate COHb% rise. In the remaining 13 (11 male), mean ± standard deviation (*SD*) age was 52 ± 13.8 years, body mass 79.1 ± 11.4 kg, height 175 ± 6.8 cm. To these, mean ± *SD* dose of carbon monoxide (CO) gas administered was 0.73 ± 0.13 ml/kg COHb values at baseline, 6 and 8 min (and "7-min value") were compared to those at 10, 12, 15 and 20 min after CO rebreathing.

Results: The "7-min value" for median COHb% (IQR) of 6.30% (6.21%–7.47%) did not differ significantly from those at subsequent time points (8 min: 6.30% (6.21%–7.47%), 10 min: 6.33% (6.00%–7.50%), 12 min: 6.33% (5.90%–7.40%), 15 min: 6.37% (5.80%–7.33%), 20 min: 6.27% (5.70%–7.20%)). Mean difference in calculated tHb-mass between minute 7 and minute 20 was only 4.1 g, or 0.6%, $p = .68$. No subjects reported any adverse effects.

Conclusions: The oCOR method can be safely used to measure tHb-mass in patients with chronic liver disease and ascites, without adjustment of blood sample timings. Further work might refine and validate appropriate dosing regimens.

Appendix E

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JP received a grant from the Association of Anaesthetists of Great Britain and Ireland to undertake studies on tHb-mass in the clinical setting. SK received a bursary form the Royal College of Surgeons of England to undertake this study as part of her Masters of Medical Sciences degree at the University of Southampton. MPWG was funded in part from the British Oxygen Company Chair of the Royal College of Anaesthetists awarded by the National Institute of Academic Anaesthesia. JP and MG work within the University of Southampton NIHR Respiratory Biomedical Research Unit, which received a portion of funding from the UK Department of Health Research Biomedical. Research Units funding scheme. The work was also supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre. All funding was unrestricted.

KEYWORDS
anemia, chronic liver disease, optimized carbon monoxide rebreathing method (oCOR), total hemoglobin mass (tHb-mass)

1 | INTRODUCTION

The concentration of hemoglobin in the circulation ([Hb]) is determined by its total circulating mass (tHb-mass) and the plasma volume (PV) in which it is suspended. Anemia, defined as a reduction in [Hb] to <120 g/l in nonpregnant females and <130 g/l in males (WHO, 1968), is common in patients with chronic liver disease (CLD), with a reported prevalence of 75% (Gonzalez-Casas, Jones, & Moreno-Otero, 2009). To this, a fall in tHb-mass (due to gastrointestinal blood loss, hematinic deficiency, hemolysis, and bone marrow suppression) may contribute (Gkamprela, Deutsch, & Pectasides, 2017). So, too, may a rise in PV (Otto, Plumb, Clissold, et al., 2017), although this element is often ignored in clinical practice, largely due to difficulties in measuring PV.

The tHb-mass can be measured directly by the methods of Burge and Skinner (Burge & Skinner, 1995), modified by Schmidt and Prommer (the optimized carbon monoxide (CO) rebreathing method (oCOR)) (Schmidt & Prommer, 2005) (see methods and Appendix 1 & 2). Here, binding of a known volume of CO to Hb, and measurement of carboxyhemoglobin concentration (COHb) allows tHb-mass to be measured and, using this and knowing hematocrit, PV to be derived. Predominantly applied to monitor athletes' responses to altitude training (Gore et al., 2013), it has more recently been used in clinical medicine (Ahlgrim et al., 2018; Diaz-Canestro, Haider, Lundby, & Montero, 2019; Koponen et al., 2013; Otto, Plumb, Clissold, et al., 2017; Otto, Plumb, Wakeham, et al., 2017; Wrobel, Pottgiesser, Birkner, Deibert, & Ahlgrim, 2016). Data regarding oCOR application to CLD patients were limited (Wrobel et al., 2016), but we demonstrated a poor relationship between [Hb] and tHb-mass in CLD (r = .410, p = .11) with PV explaining much of the variance in [Hb] (Otto, Plumb, Clissold, et al., 2017).

The accuracy of the oCOR technique depends on the completeness of COHb mixing in the circulation after CO inhalation (Garvican et al., 2010; Gore, Hopkins, & Burge, 2005) and this appears complete by 8 min (Gore et al., 2013; Schmidt & Prommer, 2005). As such, measurement of COHb% at 6 and 8 min (averaged to yield the "7 min" value) is commended (Schmidt & Prommer, 2005), and was used in our study. However, mixing is delayed in disease states such as cardiac failure (Ahlgrim et al., 2018) and polycythemia (Wachsmuth, Soria, Jimenez, & Schmidt, 2019). Admixture might be slowed down in CLD due to changes in regional (e.g. enteric) and global blood flow dynamics (Drazen et al., 2010; Iwakiri & Groszmann, 2006; Mayr et al., 2018; Murray, Dawson, & Sherlock, 1958; Scheinfeld, Bilali, & Koenigsberg, 2009; Takahashi et al., 2014), and due to the presence of expanded arteriovenous shunts (Schrier, 1988).

Meanwhile, the applicability of the standard oCOR dosing regimen has not been reported in CLD patients. The desired absolute rise in COHb from baseline- 4.0%–6.5% (ΔCOHb%) with peak levels <10% (Naef, Steiner, & Wehrlin, 2014; Plumb et al., 2018; Turner, Pringle, et al., 2014) represents a tradeoff between precision and safety. Cirrhosis leads to higher baseline COHb% due to elevated levels of inducible heme oxygenase (HO-1) driving increased heme conversion to biliverdin, with associated breakdown of CO production (Iwakiri

& Groszmann, 2006). Because most hemoximeters estimate COHb% to only a single decimal place, resulting in smaller increments in COHb% could result in lower precision of measurement (Turner, Pringle, et al., 2014; Turner, Richardson, Maxwell, & Pringle, 2014b). Meanwhile, COHb > 15% can be associated with headaches and changes in visual-evoked potentials (Stewart, 1975).

Traditional oCOR methodology distinguishes between "trained" (athlete) and "untrained" (nonathlete, but healthy) subjects with 1.0 and 0.8 ml/kg doses of CO, respectively, being safely used for men and 0.7 and 0.6 ml/kg doses for women (Schmidt & Prommer, 2005). Of note, CLD patients are likely to be significantly more deconditioned than the "untrained" group in the original paper (Schmidt & Prommer, 2005) and, as such, further dose reduction might be indicated. "Appropriate dose reduction" has also been commended "for patients with significant anemia and or/morbid obesity" although these states were not clearly defined (Gore et al., 2005; Turner, Pringle, et al., 2014). Further, when body mass index is >30 kg m^2, the use of ideal body weight (IBW) is advocated when calculating the CO dose to be administered (Wachsmuth et al., 2019). Such elements pose problems for the study of CLD patients. Firstly, up to three quarters may be anemic (Gonzalez-Casas et al., 2009) which, if due to low tHb-mass, could suggest the need for a lower CO dose. The fact that [Hb] relates poorly to tHb-mass in CLD (Otto, Plumb, Clissold, et al., 2017) further confounds CO dose-estimation. Finally, ascites and/or edema may confound measured body weight and prevalent sarcopenic and/or low body fat mass confound "ideal body weight." The potential for delivering COHb% values beyond those desirable is therefore high.

In this prospective, pilot observational clinical study, we sought to address both of these issues. We sought primarily to ascertain if adequate circulatory mixing of CO occurs within the standard time frames (Schmidt & Prommer, 2005). Our secondary aim was to explore the applicability of standard CO dosing methodologies to this patient population.

2 | MATERIALS AND METHODS

The study was conducted at the University Hospital Southampton NHS Foundation Trust between May 2015 and October 2017. Ethical approval was granted by the London—Camden and Kings Cross Research Ethics Committee (REC reference: 13/LO/1902), with an amendment allowing venous sampling for up to 20 min after CO inhalation being likewise approved. Written informed consent was obtained from all participants.

2.1 | Subjects

Sixteen patients with CLD and diuretic-refractory ascites were recruited by the clinical team from those undergoing elective day-case paracentesis. We have previously published data derived from venous samples at 6 and 8 min after CO

TABLE 1 Hemoglobin carbon monoxide measures

Patient number	[Hb] (g/l)	tHb-mass (g)	CO "dose given" (ml)	CO dose (ml CO/kg body weight)	Absolute change in COHb% (pre-CO exposure and 7 min later)
1*	98	396	50	0.6	7.2
2	104	628	48	0.5	4.5
3	105	431	44	0.6	5.5
4	117	664	64	0.8	5.8
5*	113	581	40	0.7	4.0
6	93	662	64	0.7	5.9
7	132	590	52	0.8	4.5
8	101	518	46	0.6	5.0
9	144	883	60	0.7	4.2
10[$]	184	799	64	1.0	4.9
11	100	648	72	0.8	6.7
12	118	787	70	0.9	5.5
13	121	828	70	0.8	4.7
M ± *SD*. Aside from [Hb] Median [IQR]	113.0 [100.5–126.5]	647.0 ± 148.9	57.23 ± 11.00	0.73 ± 0.14	5.20% ± 0.96%

*Female patient

[$]Patient has polycythaemia rubra vera (PCV) and requires regular bloodletting for treatment CO (carbon monoxide); [Hb] (concentration of hemoglobin in venous blood); COHb% (percentage of hemoglobin in the form of carboxyhemoglobin).

Appendix E

inhalation in 16 subjects (Otto, Plumb, Clissold, et al., 2017). In 5 of these, there was no venous sampling after 8 min. We thus obtained full data (venous sampling at 6 & 8 min then additionally at 10, 12, 15, and 20 min after CO inhalation) in an additional 5 patients, and report these data for the total of 16 patients (Table 1).

2.2 | Optimized carbon monoxide rebreathing method (oCOR)

Here tHb-mass was determined by oCOR on the morning immediately prior to abdominal paracentesis (Schmidt & Prommer, 2005) (see also Appendix 1 & 2). Venous COHb% was measured before and after 2 min rebreathing a known CO volume (0.5 to 1.0 ml/kg, with dose determined according to fitness, frailty, gender and [Hb] as described below). We sought a minimum ΔCOHb% of 4.0% to avoid inaccuracies in tHb-mass measurements (see introduction).

The tHb-mass was calculated using the difference in COHb% in blood samples obtained before and (averaged) 6 and 8 min after the inhalation of the CO bolus, and using blood samples drawn between 8 and 20 min. For these calculations, CO diffusion to myoglobin and CO exhaled until the respective time point was considered as previously published (Prommer & Schmidt, 2007) (e.g. in patients studied for 20 min, CO volume diffused to myoglobin ranged from 1.4 to 2.9 ml, and exhaled CO volume between 1.0 and 5.1 ml). For detailed calculation of tHb-mass and blood volume derivatives see Appendix 1 & 2 and for further explanation see (Prommer & Schmidt, 2007).

2.3 | Dosing of carbon monoxide

Carbon monoxide dosing aims to achieve an absolute ΔCOHb% of between 4.0% and 6.5%, between 6 and 8 min (the mean being referred to as the "7 min value") after CO administration. We assumed that all the CLD patients were further debilitated and deconditioned beyond being simply "untrained" (see above), and thus used doses of 0.8 and 0.6 ml/kg CO for men and women, respectively. Two patients did not appear debilitated (World Health Organization performance status zero) (West & Jin, 2015), one of whom also who had polycythemia rubra vera (PRV) with a [Hb] of 184 g/l and dosing was increased to 0.9 and 1.0 ml/kg, respectively, in these.

It is advocated that consideration be given to further dose reduction in anemic subjects (<130 g/l in men, and <120 g/l in women (WHO, 1968)), and we followed this approach. In total, CO dose was reduced from the 0.8 or 0.6 ml/kg recommended for "untrained subjects" in 5 individuals (subjects 2, 3, 6, 8 & 9, Table 1) due to them being anemic (5 subjects, see Table 1) and then further reduced if their performance status was low (high number on the Zubrod scale) at the discretion of the investigators. This occurred in 3 subjects (2, 3 & 8 Table 1). If a subject's BMI is >30 kg m^2, it is suggested that CO dosing is based on IBW (see introduction). However, this did not apply to any of our subjects.

2.4 | Statistical analysis

Statistical analysis was performed using GraphPad prism version 7.0c for Apple Macintosh. The Shapiro–Wilk test for normal distribution was used. Values are presented as mean ± standard deviation (*SD*), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). Paired *t* tests were used to calculate differences in tHb-mass at different time points. The Mann–Whitney U test was used to calculate differences in COHb% at different time points. Repeated measures ANOVA was used to compare calculated tHb-mass data for each time point.

3 | RESULTS

3.1 | Carbon monoxide dosing

The mean ± *SD* CO dose administered was 0.73 ± 0.13 ml/kg (range 42–76 ml), with weight-indexed doses ranging from 0.5–1.0 ml/kg. Mean dose to males was 0.75 ± 0.14 ml/kg, and to females 0.65 ± 0.07 ml/kg.

In three subjects, ΔCOHb% was <4% (2.6%, 2.7% and 3.2%). Their demographics are shown in Table 2. Two were women, being reflected in their somewhat lower heights and weights. However, no variables differed significantly from the 13 subjects in whom ΔCOHb% was sufficient to allow valid calculation of tHb-mass ($p = .08$ for BMI, $p = .46$ for [Hb]).

TABLE 2 Demographics of the three excluded patients

Patient	Age (years)	Gender	Height (cm)	Weight (kg)	BMI (kg m^2)	[Hb] (g l)	CO dose in ml	ΔCOHb%
1	49	M	176	65.7	21.2	105	46	3.2
2	50	F	162	57.8	22.0	128	36	2.6
3	50	F	165.5	66.3	24.2	129	40	2.3

Note: The demographic details of the three subjects in whom the rise in COHb% was < 4%.

The doses received were in accordance with the description in the methods with the two females receiving 0.6 ml/kg, and the one male (who was anemic [Hb] 105 g/l), receiving 0.7 ml/kg). In the remaining 13 subjects, venous COHb% increased from a mean of 1.62% ± 0.77% at baseline to a median value of 6.3 (IQR 6.21–7.47) at "7 min" giving a mean ΔCOHb% of 5.26% ± 096% (range 4.0%–7.2%). Table 1 shows the doses administered and the ΔCOHb% for each patient, the latter being adequate for all 13 subjects (Table 1).

One patient had a peak COHb% of 10.1% after 6 min, exceeding that which most investigators would aim for. They suffered no ill effects, however, and their COHb% level was below 10% at 12 min.

The tHb-mass could not be reliably calculated for the 3 patients in whom ΔCOHb% was < 4%. This left 13 patients (11 male; mean 52 ± 13.8 years, body mass 79.1 kg ± 11.4 kg, height 175 ± 6.8 cm, body mass index 25.8 ± 3.0 kg m^2) for the final analysis. All had established CLD: nine alcoholic cirrhosis (one of whom also suffered hepatocellular carcinoma), one cryptogenic cirrhosis, one with chronic hepatitis C, one Budd–Chiari Syndrome and one nonalcoholic steatohepatitis.

In the 13 in whom COHb rise was > 4% (absolute value), median [Hb] was 113 g/l (IQR 101–127 g/l), mean ± *SD* tHb-mass 647.3 g ± 148.9 g, PV 4221 ml ± 104 and blood volume 6,101 ± 1,194 ml. Ten patients (77%) were anemic (mean ± *SD* [Hb] 107 ± 9.6 g/l versus. 153.3 ± 27.2 g/l for those not anemic) (Table 1 and Appendix 1).

3.2 | Carbon monoxide wash-in curve

Figure 1 shows the COHb% values from baseline to 20 min after CO inhalation. The peak occurred at either 6 or 8 min after inhalation (median 6.33% (IQR 6.25%–7.46%) and 6.30% (IQR 6.20%–7.47%) respectively. The values were 6.30% (6.21–7.47) at seven minutes, 6.33% (6.00%–7.50%) at 10 min, 6.33% (5.90%–7.40%) at 12 min, and 6.37% (5.80%–7.33%) at 15 min, The value at 7 min did not differ from that 20 min after CO inhalation (6.27% (5.70%–7.20%), $p = .20$). In keeping, calculated tHb-mass was also near-constant over this time period (mean ± *SD* being 647.3 ± 149.1 g at 6 min, 648.6 ± 149.1 g at 8 min, and 653.4 ± 162.0 g, 659.7 ± 163.2 g, 653.5 ± 164.9 g and 651.4 ± 167.9 g at 10, 12, 15, and 20 min, respectively (Figure 2)). The calculated tHb-mass at minutes 7 and 20 were similar ($p = .68$) and differed by only 4.1g, or 0.6%, while repeated measures ANOVA confirmed no difference in tHb-mass between any other timepoints ($p = .48$).

4 | DISCUSSION

We have clarified, for the first time that the optimized carbon monoxide rebreathing method can be used to calculate tHb-mass in patients with chronic liver disease without modification of the timing of venous sampling. Thus, COHb% values at 6 and 8 min giving a "7 min" (the mean of minutes 6 and 8) do not fall significantly over the next 12 min, and calculated tHb-mass thus does not differ significantly ($p = .68$). This suggests that the mixing of CO in the blood is complete by "7 min" after inhaling the CO-bolus, and that any blood sample between min 7 and min 20 can be used for the calculation of tHb-mass (Figures 1 and 2). While some COHb% values appear to still be rising between minutes 6 and 8, this difference has little impact on calculated tHb-mass. For example, calculated tHb-mass in patient 2 (see Table 1) is 396.1 g versus 389 g for "minute 7" and 8, respectively.

These data suggest that any changes in COHb circulatory mixing kinetics in CLD do not necessitate adjustment of standard sample timings (Schmidt & Prommer, 2005), perhaps in contrast to the situation in heart failure (left ventricular ejection fraction < 30%) patients (Ahlgrim et al., 2018) and polycythemia (Wachsmuth et al., 2019).

For precision in tHb-mass measurement by oCOR, the rise in carboxyhemoglobin (ΔCOHb%) is variably required to exceed an absolute value of 5% (typically 0.5%–1.5% at rest rising to 5%–7% post-CO rebreathing) (Schmidt & Prommer, 2005; Turner, Richardson, et al., 2014), or to reach values of 5.5%–6% (Gore et al., 2005). Our own unpublished data suggest that values over 4.0% are essential for adequate reliability. Low doses (0.6 ml/kg) result in a ΔCOHb% of only 3.4 ± 0.4%, but yield similar tHb-mass values to those when using 1.0 ml/kg (791 versus 788 g, respectively)- suggesting that a ΔCOHb% of <4% may be methodologically acceptable (Turner, Pringle, et al., 2014). In healthy subjects, a dose of 1 ml/kg has been shown to be adequate and safe (Turner, Pringle, et al., 2014). We have safely and effectively used 0.4–1 ml/kg in patients (Otto, Plumb, Clissold, et al., 2017; Otto, Plumb, Wakeham, et al., 2017), and the only other clinical study to provide data (in patients with coronary artery disease) used doses of 0.7 ml/kg for women and 0.8 ml/kg for men (Karlsen, Leinan, Aamot, Dalen, & Stoylen, 2016) and yielded a mean ΔCOHb% of 4.5%. Recent work in polycythemia subjects used 1.7 ml/kg and yielded an approximate ΔCOHb% of 3.7%. (Wachsmuth et al., 2019). Unfortunately other clinical studies did not report the doses used (Koponen et al., 2013; Wrobel et al., 2016). We accept that we cannot comment on accuracy based on these data as multiple measures at differing doses have not been taken. Further clinical research might address this question.

We show, however, that CO-dosing to achieve ΔCOHb% values > 4% (with peaks < 10%) can be problematic in CLD patients. Mean CO doses were 0.75 and 0.65 ml/kg for males and females respectively, but ranged from 0.5–1 ml/kg (Table 1). Primary dosing was based on gender with the basic assumption that all participants were "untrained" by nature

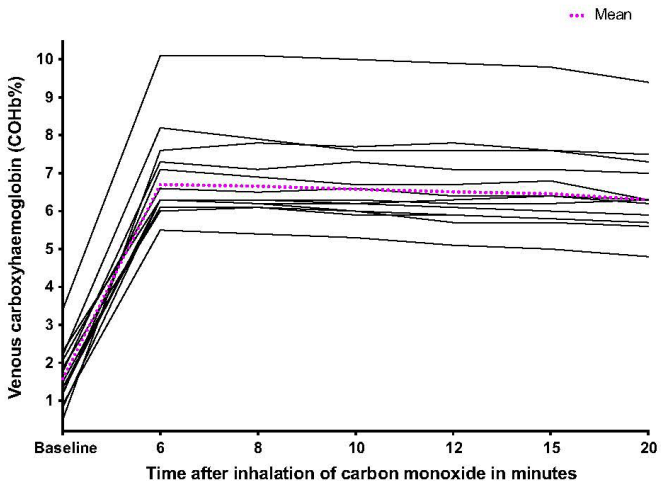

FIGURE 1 Carboxyhaemoglobin (COHb%) wash in curves from baseline to 20 minutes after CO re-breathing. Each line represents one individual. Venous carboxyhaemoglobin samples taken at baseline to 20 minutes post inhalation of carbon monoxide gas.

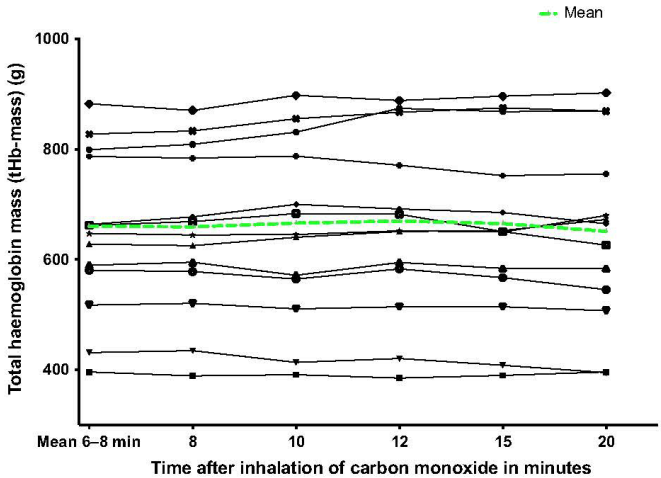

FIGURE 2 tHb-mass data calculated for different COHb% sampling time points. Each line represents one individual. tHb-mass was calculated using COHb% values in blood drawn from 6 different time points after inhalation of the carbon monoxide bolus.

of their CLD. The original recommended doses of 0.8 or 0.6 ml for men and women, respectively, were used for dosing. However, only 36% (4 subjects) of the male participants in this study received 0.8 ml/kg. In 5 subjects, the dose was reduced as described above (Table 1). In the two male patients with a [Hb] ≤100 g/l one received a CO dose of 0.7 ml/kg and the other a dose of 0.6 ml/kg (yielding a ΔCOHb% of 5.9% and 7.2% respectively). Two male patients received higher doses (one patient with JAK2 positive polycythemia rubra vera (PRV) receiving 1 ml/kg) the other was young, unusually had no other comorbidities, had a very good exercise tolerance and was deemed "relatively fit" compared to the other subjects.

Using the recommended dosing for "untrained subjects" led to the exclusion of three patients due to "under-dosing" (rise in COHb% of 2.6%, 2.7% and 3.2% respectively). Of note, two of these cases were women, one of whom had a body mass (57.8kg) lower than many other subjects- and who therefore only received 36 ml of CO gas. The remaining subject was a male who received 0.7 ml/kg CO dosing. It is possible that the calculated CO bolus will be too high or too low due to [Hb] not always correlating with tHb-mass. By way of example, in the three most anemic patients ([Hb] 100, 98, and 93 g/L), tHb-masses were 647.6 g for the person with [Hb] 100 g/l but 396 g/l 1 g in the person with [Hb] only 2 g/l lower ([Hb] 98 g/l—with the highest tHb-mass of the three

(662.3 g) being in the most anemic patient ([Hb] 93 g/l). In the 3 patients excluded from the original 16, this was the case: absolute ΔCOHb% values were 3.2%, 2.7% and 2.3%, respectively, making calculation of tHb-mass less reliable. They did not have any documented CO gas leaks or other analytical errors that could have resulted in a low ΔCOHb%. In the case of a dilutional anemia resulting in a falsely low calculated CO bolus and subsequent low ΔCOHb% (<4%), it is possible to repeat the test within only a few hours (Naef et al., 2014; Plumb et al., 2018). In our previously published work relating to CLD patients, one subject with a [Hb] of only 69 g/l was given a dose of 0.4 ml/kg producing a ΔCOHb% of 4.7% (Otto, Plumb, Clissold, et al., 2017). This highlights the unpredictability of response in severely anemic patients to bolus CO administration—perhaps because [Hb] and tHb-mass values are divorced when plasma volume is known to be deranged (Otto, Plumb, Clissold, et al., 2017).

Strengths of this study include the fact that the same two operators performed all oCOR tests in the same laboratory, using the same equipment thus reducing measurement error. Conditions were identical for all of the patients and all measurements were made prior to paracentesis.

Limitations include that fact that we did not objectively quantify "fitness." Nor did we evaluate intersubject reliability by performing multiple tests on each patient. While theoretically possible that areas with very poor perfusion may not have equilibrated by 20 min (our last sample time), there was no appreciable rise in COHb% after the initial peak at either 6 or 8 min, making this unlikely, and any possible impact small. While accuracy might have been improved, we were reluctant to use higher CO doses due to the high prevalence of anemia (77%), and because no other reports exist of the use of the oCOR method in such patients. Due to the pilot nature of this work, we cannot comment specifically on safety but it is possible that, in future, doses could be adjusted according to baseline values, such that excessively high COHb% levels are avoided. However, it is important to recognize that "safe levels" of COHb are not exclusively about peak COHb%, and that the duration of raised COHb% is also important. Carboxyhemoglobin levels have been elevated to 20% in healthy volunteer studies without reported ill effects (González-Alonso, Richardson, & Saltin, 2001).

5 | FUTURE RESEARCH

We did not measure tHb-mass and PV after paracentesis. We would advocate that future studies do so, plotting the change in PV (and weight) which occur over time. Future studies might also address the relationship between circulating albumin concentrations/oncotic pressure, ascitic volume, and PV.

There is no clinical cut off which defines "pathophysiologically low" tHb-mass in the same way in which the WHO has defined a lower limit of [Hb] to define "anaemia." Further research should define normative data for PV and tHb-mass for healthy subjects across genders and races, and over wide age ranges.

Further work is required in order to generate and validate appropriate CO dosing regimens in CLD patients. These might consider baseline COHb%; actual body mass, ideal body mass, the contribution ascitic/edema mass to overall body mass and true body composition; more precise measures of frailty and fitness; and indicators that a low [Hb] might in fact be due to PV expansion. Studies might focus on repeat measures using a variation in doses in the same subject to measure both accuracy and reliability.

Studies should also be extended across different disease groups—and most especially in those among who plasma volume may be subject to pathological variation. In those with liver disease, trials might address the use of this methodology in guiding clinical intervention (such as diuretic treatment or packed red cell transfusion).

6 | CONCLUSIONS

The oCOR method can be safely used to measure total hemoglobin mass in patients with chronic liver disease and ascites, without adjustment of blood sample timings. Further work might refine and validate appropriate dosing regimens.

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CONFLICT OF INTEREST

JP has received financial support from Siemens Healthcare Limited for consumables and hardware for research into the measurement of hemoglobin mass (2015–2018). JP was given consumables from Intersurgical UK Ltd (2015–2018); has received honoraria for speaking and/or travel expenses from Siemens, Vifor Pharma and Pharmacosmos and has received unrestricted grant funding from Pharmacosmos. JP is unaware of any direct or indirect conflict of interest with the contents of this paper or its related fields. SK has no conflicts of interest. JO has no conflicts of interest. WS is a managing partner of the company "Blood tec GmbH," but he is unaware of any direct or indirect conflict of interest with the contents of

this paper. HM consults for Google Deepmind on health technology and is on the Council of the UK Intensive Care Society but is unaware of any direct or indirect conflict of interest with the contents of this paper or its related fields. MG is the vice-president of CPX International. He also serves on the medical advisor board of Sphere Medical Ltd and the board of EBPOM Community Interest Company, Medinspire Ltd and Oxygen Control Systems Ltd. He has received honoraria for speaking for and/or travel expenses from BOC Medical (Linde Group), Edwards Lifesciences and Cortex GmBH and unrestricted research support from Sphere Medical Ltd and Pharmacosmos Ltd. He leads the Fit-4-Surgery research collaboration and the Xtreme Everest oxygen research consortium, which has received unrestricted research grant funding from BOC Medical (Linde Group), Deltex Medical and Smiths Medical. MPWG was funded in part from the British Oxygen Company Chair of the Royal College of Anaesthetists awarded by the National Institute of Academic Anaesthesia. All funding was unrestricted. The funders had no role in study design, data collection and analysis, decision to publish or the preparation of the manuscript. This work was conducted at the Southampton NIHR Biomedical Research Centre with participants studied within the Southampton NIHR Clinical Research Facility. All authors are unaware of any direct or indirect conflict of interest with the contents of this paper or its related fields. No funders had any role in study design, data collection and analysis, decision to publish or the preparation of the manuscript.

AUTHORS CONTRIBUTIONS

JP, JO, MW, MG, HM, and WS conceived the study. JP and SK conducted the study and collected the data. JP and HM drafted the manuscript. SK, JO, WS, MW, MG, and HM all contributed to the draft. JP produced the figures and tables. JP collated the article including references. The final version was read and approved by all authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ORIGINAL RESEARCH

Replicating measurements of total hemoglobin mass (tHb-mass) within a single day: precision of measurement; feasibility and safety of using oxygen to expedite carbon monoxide clearance

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Blood volume, optimized carbon monoxide re-breathing, plasma volume, red cell volume, total hemoglobin mass (tHb-mass).

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Abstract

Hemoglobin concentration ([Hb]) is a function of total hemoglobin mass (tHb-mass) and plasma volume. [Hb] may fall by dilution due to plasma volume expansion and changes in the perioperative period may therefore correlate poorly with blood loss. A simple, reliable, repeatable way to measure plasma volume and tHb-mass would have substantial clinical utility. The "optimized carbon monoxide re-breathing method" (oCOR) meets these criteria. However, it is recommended that a minimum of 12 h (when breathing room air) is left between repeat measurements. Twenty-four subjects underwent 3 days of testing. Two oCOR tests were performed (T1 and T2), 3 h apart, with a different CO clearance method employed between tests aiming to keep the carboxyhemoglobin level below 10%. The primary aim was to ascertain whether tHb-mass testing could be safely repeated within 3 h if carboxyhemoglobin levels were actively reduced by breathing supplemental oxygen ($PROC_A$). Secondary aims were to compare two other clearance methods; moderate exercise ($PROC_B$), or a combination of the two ($PROC_C$). Finally, the reliability of the oCOR method was assessed. Mean (SD) tHb-mass was 807.9 ± (189.7 g) (for T1 on day 1). $PROC_A$ lowered the carboxyhemoglobin level from the end of T1 (mean 6.64%) to the start of T2 (mean 2.95%) by a mean absolute value of 3.69%. For $PROC_B$ and $PROC_C$ the mean absolute decreases in carboxyhemoglobin were 4.00% and 4.31%, respectively. The fall in carboxyhemoglobin between T1 and T2 was greatest in $PROC_C$; this was statistically significantly lower than that of $PROC_A$ ($P = 0.0039$) and $PROC_B$ ($P = 0.0289$). The test-retest reliability for the measurement of total hemoglobin mass was good with a mean typical error (TE) of 2.0%. The oCOR method is safe and can be repeated within 3 h when carbon monoxide is suitably cleared between tests. Using oxygen therapy alone adequately achieves this.

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Introduction

Humans are obligate aerobes, the oxygen necessary for metabolism being carried by circulating hemoglobin, whose concentration [Hb] has traditionally, been used as a measure of the blood's oxygen carrying capacity.

In 1968, the World Health Organization (WHO) defined anemia as a [Hb] <130 g L^{-1} for men and <120 g L^{-1} for women (WHO scientific group, 1968). Despite evidence that these levels may actually be inappropriately low they are still widely accepted within clinical practice (Beutler and Waalen 2006; Zavorsky et al. 2012). Perioperative anemia is common and is associated with greater post-operative morbidity and mortality (Musallam et al. 2011). This may in part be due to impaired oxygen delivery. Measures of preoperative cardiopulmonary fitness, measured using cardiopulmonary exercise testing (CPET) such as peak oxygen consumption (VO_2peak) and oxygen consumption at the anaerobic threshold (VO_2AT) are inversely related to postoperative outcome. Both VO_2peak and VO_2AT fall when tHb-mass is reduced by venesection (Dellweg et al. 2008) and improve with red cell transfusion (Wright et al. 2014). However, hemoglobin concentration [Hb] is a function of the total mass of circulating hemoglobin (tHb-mass) and the volume of plasma within which it is carried (PV) and, as such, tHb-mass might be better correlated with patients' CPET performance than [Hb]. In keeping with this, we have shown that [Hb] correlates modestly (Otto et al. 2013) or not at all (Otto et al. 2017a) with peak oxygen consumption (VO_2peak) or oxygen consumption at the anaerobic threshold (VO_2AT).

In disease states in which PV expansion is common such as chronic heart or liver failure, [Hb] correlates poorly with tHb-mass (Otto et al. 2017b). tHb-mass is also more stable than [Hb] over time, being unaffected by shifts in PV (which may be exaggerated when intravenous fluids are administered perioperatively) (Eastwood et al. 2008; Garvican et al. 2010a). Thus, in the perioperative setting changes in tHb-mass may be a better guide to true blood loss than changes in [Hb]. There is an increasing recognition of the unsuitability of using [Hb] as a reliable guide to perioperative transfusion (Shander and Ferraris 2017; Plumb et al. 2016).

tHb-mass can be measured directly using the so-called optimized carbon monoxide (CO) re-breathing method (oCOR) (see Methods and Appendix I for further details). In use since 2005, it has predominantly been applied to athletes in order to monitor responses to altitude training (Gore et al. 2013), but more recently has been used in clinical medicine (Otto et al. 2017a,b; Koponen et al. 2013; Wrobel et al. 2016). There is no "normal range" for tHb-mass described for healthy subjects. Endurance athletes would hope to have upwards of 13 g kg^{-1} (males) and 11 g kg^{-1} (females).

In assessing the changes in PV and tHb-mass over the perioperative period, it would be useful to make repeated measurements over relatively short timescales. However, to date, most authors recommend that the oCOR can only be repeated after a 12-h interval (when breathing room air) (Schmidt and Prommer 2005), due to the concern that physiological clearance of administered carbon monoxide for the purposes of tHb-mass measurement would, under normal conditions, be expected to take approximately 9 h (based on a post test COHb level of 7% and a half-life of 4.8 h) (Zavorsky et al. 2012). To address this, various methods have been proposed which might enhance carbon monoxide clearance rates (Zavorsky et al. 2012; Naef et al. 2015) such that tHb-mass assessment could be more rapidly repeated while still maintaining carboxyhemoglobin levels below the proposed recommended maximum value of 10% (Otto et al. 2017a,b; Naef et al. 2015; Turner et al. 2014). With carbon monoxide toxicity exposure, concentration and time need to be considered; higher levels for a very short duration are less dangerous than lower levels for prolonged periods. In this regard, mild exercise (inducing a higher rate of pulmonary ventilation) and normobaric "hyperoxia" (supplementing oxygen at $FiO_2 > 0.21$) are two potentially effective candidate interventions to facilitate the clearance of serum carboxyhemoglobin (Prommer et al. 2008). An important study by Zavorsky et al.

(2012) demonstrated that mild exercise, hyperoxia, and increased pulmonary ventilation the so called "triple therapy" was most effective at reducing the half-life of CO administered to healthy subjects. They also found that moderate exercise (in room air) was as effective as breathing 100% oxygen at rest at clearing CO. To the best of our knowledge, only one study (in healthy athletic males), using administration of supplemental inhaled oxygen during physical activity, has demonstrated the feasibility of doing this in the context of tHb-mass assessment – here the efficacy of oxygen therapy alone, or of exercise alone was not assessed (Naef et al. 2015).

The primary aim of this study was to ascertain whether tHb-mass testing could be safely repeated within 3 h if carboxyhemoglobin levels were actively reduced by breathing supplemental oxygen alone ($PROC_A$). The secondary aims were to compare two other carboxyhemoglobin clearance procedures (gentle cycling alone, or gentle cycling in combination with oxygen administration-procedure B and C ($PROC_B$ and $PROC_C$), respectively. The final aim was to evaluate the reliability of duplicate tHb-mass measurements in healthy volunteers, who were not the elite or "sub elite" athletes largely studied by others previously (Naef et al. 2015).

Methods

The study took place at University Hospital Southampton NHS Foundation Trust between August 2016 and March 2017.

Ethical approval was granted by the West Midlands (Edgbaston) Research Ethics Committee and NHS Health Research Authority (REC reference: 16/WM/0274). Local permissions were received from the University of Southampton (ERGO ID: 19642), University Hospital Southampton NHS Foundation Trust (R&D CRI 0329) and Southampton Centre for Biomedical Research Clinical Research Facility. The study was performed in accordance with the ethical standard set by the Declaration of Helsinki. Written informed consent was obtained from all participants.

Healthy adults aged over 16 years who were physically able to perform the testing protocol were eligible for recruitment. Excluded were adults lacking mental capacity to consent, pregnant women, smokers, prisoners, subjects with a baseline carboxyhemoglobin level >5%, or patients with hemoglobinopathies. One subject was a smoker (this was not clear until after the first experiments had taken place and their data were removed). No subjects had stayed at an altitude higher than 1500 m for any time in the preceding year. This left data from 24 subjects for analysis. In total, 24 x 6 (144) measurements of tHb-mass were planned but 136 were carried out; the missing eight experiments were due to CO gas running out mid-experiment in three subjects and a blood sample clotting in one subject. Of these 136 measurements, 122 were suitable for analysis. Values being discarded if the rise in carboxyhemoglobin (ΔCOHb%) was <4.5% (standard practice in our laboratory), if significant leaks were detected (CO ppm > 5 using the Dräger Pac 7000), if the breath-holding technique was sub-optimal (determined either by a leak using the Dräger Pac 7000 or if the subject failed to breath hold for 10 seconds), or if the resting carboxyhemoglobin values were greater than 5% (Appendix 3). Fourteen subjects had three consecutive days of measurement, the remaining 10 had all 3 days within a 10-day period.

tHb-mass was measured twice (a period of 3 h separating the first (T1) and second (T2) measurements) using the optimized carbon monoxide rebreathing (oCOR) method. This protocol was repeated on each of three separate occasions, with a different protocol to enhance CO clearance being applied between tests on each day. Figure 1 outlines the experimental design and study activity. The three experimental days had to occur within a 10-day period.

Day 1

Procedure A ($PROC_A$) – Immediately after the first oCOR test, 60% oxygen was administered to seated subjects via a Venturi mask (Intersurgical Ltd, Berkshire, UK) for exactly 1 h. An identical oCOR test was repeated exactly 2 h later (3 h in total from the end of the first oCOR test).

Day 2

Procedure B ($PROC_B$) – Immediately after the first oCOR test, subjects exercised at low intensity (50 W and 60–70 revolutions per min) for 45 min on a static exercise cycle ergometer (Ergoline Ontibike 200, Germany). An identical oCOR test was repeated exactly 2 h and 15 min later (3 h in total from the end of the first oCOR test).

Day 3

Procedure C ($PROC_C$) – Immediately after the first oCOR test, subjects exercised as for $PROC_B$ were positioned on a static exercise cycle ergometer (Ergoline Ontibike 200) while breathing 60% oxygen via a Venturi mask (Intersurgical Ltd, Berkshire). An identical oCOR test was repeated exactly 2 h and 15 min later (3 h in total from the end of the first oCOR test).

The fraction of inspired oxygen chosen was 0.6, this was for pragmatic and safety reasons. First, there is a

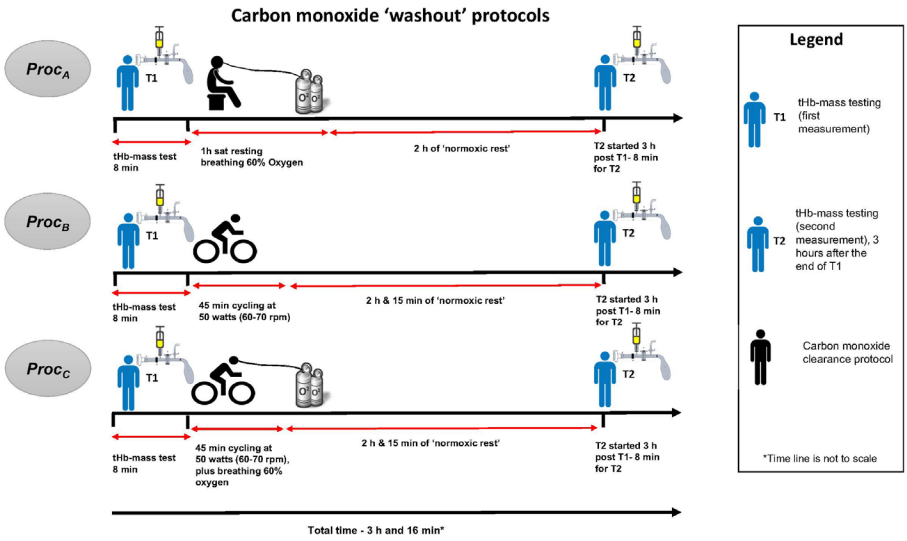

Figure 1. Schematic to describe the experimental sequence.

convenient venturi delivery device that consistently delivers 0.6 available. Higher inspired fractions of oxygen can be detrimental if breathed for long periods and in a small subset of patients can be very detrimental if they are known to retain carbon dioxide (Ridler et al. 2014; Martin and Grocott 2013). We wanted to balance safety with expedient expulsion of CO gas.

In all cases, subjects were allowed to leave the lab between tests, remaining in the hospital and avoiding formal or strenuous physical activity. Finally, triplicate measurements of tHb-mass taken on separate days (T1 of each procedure) were compared. We deliberately did not include a control procedure for clearance (i.e., no exercise and no inspired additional oxygen). It has previously been demonstrated that clearance of CO under these normal conditions is prolonged and the study by Naef et al. (2015) demonstrated this precisely.

Optimized carbon monoxide rebreathing method

Subjects completed a baseline carbon monoxide rebreathing test (oCOR) (details of this method can be found in Appendix 2). Briefly, subjects were seated and inactive for 15 min prior to sampling. Subjects inhaled 0.8–1 mL kg^{-1} of CO mixed with 3 L of 100% oxygen via a glass spirometer (BloodTec, Bayreuth, Germany) and then rebreathed (via a CO_2 scrubber) for 2 min while wearing a nose clip. A portable CO gas detector (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) was used during the rebreathing period to check for possible CO leakage at the nose, mouthpiece, and spirometer.

Prior to commencing the rebreathing technique, the investigators inserted an intravenous cannula into the subject's upper limb. Venous blood samples were taken via Na-heparinized syringes (RAPIDLyte, Siemens Healthcare Diagnostics Inc, USA) before (at baseline) and at 6 and 8 min after administration of CO gas. All three blood samples (0, 6, and 8 min), were analyzed using a laboratory blood gas analyzer (Radiometer, ABL800 FLEX) for carboxyhemoglobin percentage values. Each sample was analyzed three times within 1 h of collection. The analyzer used in this study was subjected to regular maintenance and quality control checks; the accuracy of which has been evaluated elsewhere (Turner et al. 2013). [Hb] and hematocrit values were measured using HemoCue (HemoCue AB, Radiometer, Sweden) and blood gas analyzer (Radiometer, ABL800 FLEX, Copenhagen), respectively.

The desired rise in carboxyhemoglobin is 5– 6.5% after administration of carbon monoxide gas aiming to achieve

levels below 10% at the end of the test. This represents a tradeoff between precision and safety. However, "safe levels" of COHb are not exclusively about peak COHb% but duration of high blood levels is equally, if not more important. Most hemoximeters estimate carboxyhemoglobin only to a single decimal place meaning that smaller changes in carboxyhemoglobin could result in lower precision of measurement. We use a minimum Δ% COHb of 4.5% to avoid overestimating tHb-mass. The safety limit of 10% is based on previous work by our group and professor Schmidt who developed the oCOR test. This level allows a significant margin of safety before subjects are likely to experience any significant level of CO toxicity. Previous researchers have also used this level (Otto et al. 2017a,b; Turner et al. 2014; Naef et al. 2014). Levels greater than 5% can produce symptoms such as mild headaches and when levels start to exceed 15% most individuals start to experience headaches and visual evoked potential start to change (Stewart 1975).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c for Apple Macintosh OSX) and SPSS Statistics (version 25 for Apple Macintosh Chicago, IL). The Shapiro-Wilk test for normal distribution was used. Values are presented as mean ± standard deviation (SD), unless otherwise stated. Median and interquartile range (IQR) are reported when variables were not normally distributed. Categorical variables are presented as frequency (%). Differences in carboxyhemoglobin were assessed using the Student's paired *t*-test.

This was a feasibility study so a formal power calculation was not required. The aim was to recruit 25 subjects on the advice of professor Schmidt (personal communication) based on the work of their group having performed thousands of tHb-mass measurements. They use a number of 10 test/retests to ensure personal operator quality assurance. The statistical principles of test/retest reliability measures as described by Hopkins (2000) would also suggest that 10 patients are sufficient to get a meaningful estimate of typical error (TE) for an individual tester.

Test-retest data (repeated measures in one and the same patient) are presented using Bland–Altman plots with limits of agreement (Bland and Altman 1986). Additionally, a specific approach to compute reliability statistics to compare test-retest performance was used see (Appendix 4 for further detail) (Hopkins 2000). TE of measurement for tHb-mass was calculated and expressed as coefficient of variation with 95% confidence limits (CL), derived from χ^2 distributions. All tests were two-sided and statistical significance was set at $P < 0.05$. TE includes random error (analytic error arising from using the method-specific apparatus and intra-individual biological variation) but not systematic error (bias) (Gore et al. 2013; Naef et al. 2015; Gore et al. 2005). Reported studies using the oCOR have commonly reported this method (Naef et al. 2015; Fagoni et al. 2018; Keiser et al. 2017; Garvican et al. 2010b; Eastwood et al. 2012).

Results

Baseline characteristics

Twenty-four subjects were included in the study (20 male), with age (median [range]) 23 [20–32] years, height 176 ± 11 cm, and weight 78.4 ± 12.8 kg. On day one, test 1, the average results were; [Hb] 152.4 ± 13.6 g L^{-1}, hematocrit (Hct) 46.47 ± 4.3%, and tHb-mass 807.9 ± 189.7 g.

Comparing the three carboxyhemoglobin clearance procedures ($PROC_C$, $PROC_B$, and $PROC_C$)

Table 1 shows the changes in carboxyhemoglobin for each procedure and the reliability statistics. All three carbon monoxide clearance methods achieved a reduction in carboxyhemoglobin from the end of T1 to the beginning of T2 on each of the days studied.

Baseline carboxyhemoglobin levels were higher at the start of T2 than T1 for all three procedures (median 1.15% (start of T1) vs. 2.87 % (start of T2), respectively, $P < 0.0001$). T1 baseline carboxyhemoglobin was lower in $PROC_A$ than in $PROC_B$ and $PROC_C$ ($P = 0.0010$ and 0.0013, respectively). T2 baseline carboxyhemoglobin was lowest in $PROC_C$ and it differed significantly from $PROC_A$ and $PROC_B$ ($P = 0.0039$ and 0.0289, respectively). There were no significant differences between $PROC_A$ and $PROC_B$.

The fall in carboxyhemoglobin between T1 and T2 (4.31%- absolute mean value for $PROC_C$) was greater in $PROC_C$ than $PROC_A$ (absolute mean value 3.69%) ($P = 0.0039$, (the difference between $PROC_C$ and $PROC_A$ being 0.62%) and this result was robust to analysis using one-way ANOVA with Bonferroni multiple comparison test ($P = 0.037$) (See Table 1). Although $PROC_B$ and $PROC_C$ were initially significantly different this was not the case after applying the Bonferroni test $P = 0.617$ (Fig. 2). The absolute mean fall between tests 1 and 2 for $PROC_B$ was 4.00%. Cycling with supplemental oxygen ($PROC_C$) was therefore statistically more efficient than oxygen alone ($PROC_A$) but not statistically more efficient than cycling alone ($PROC_B$) at lowering carbon monoxide levels in the blood. There was no statistical difference between oxygen alone ($PROC_A$) and

Table 1. The three clearance protocols for washing out carbon monoxide after total hemoglobin mass measurement.

	First measurement (T1)				Second measurement (T2)				Fall in COHb% from the end of T1 to the start of T2	Reliability statistic-Typical Error (TE) (95% CL)
Procedure	Baseline COHb (%)	COHb 7-min value (%)	Delta change in COHb (%)	tHb-mass (g)	Baseline COHb (%)	COHb 7-min value (%)	Delta change in COHb (%)	tHb-mass (g)		Overall 2.0 (1.67–2.59)
$Proc_A$	1.09 ± 0.63	6.64 ± 0.90	5.55 ± 0.60	807.9 ± 189.7	2.95 ± 0.55	8.45 ± 0.83	5.50 ± 0.55	789.4 ± 171.2	3.69 ± 0.72	1.61 1.21–2.41
$Proc_B$	1.39 ± 0.59	7.10 ± 0.80	5.67 ± 0.60	788.1 ± 180.1	3.10 ± 0.75	8.77 ± 0.96	5.67 ± 0.52	786.2 ± 172.5	4.00 ± 0.83	2.02 (1.53 –2.99
$Proc_C$	1.33 ± 0.46	7.10 ± 0.43	5.75 ± 0.34	775.1 ± 188.5	2.72 ± 0.51	8.27 ± 0.72	5.63 ± 0.43	775.7 ± 181.2	$4.31^{1,2,3}$ ± 0.61	2.37 (1.76 –3.6)

Mean carboxyhemoglobin (%) values at baseline and 7 min (the mean of min 6 and 8) during test 1 and 2 of each procedure. The Delta change from baseline to minute 7 and the T1–T2 fall in COHb% are shown. The resulting mean total hemoglobin mass is also shown. Typical error of measurement (TE) of tHb-mass for the three carbon monoxide clearance procedures.
COHb, carboxyhemoglobin; $PROC_{A/B/C}$, procedures A/B/C; TE, typical error of measurement; CL- confidence limits.
[1]The reason for slight discrepancy in the value generated by (COHb% 7-min value from T1 − COHb% baseline value from T2) is that not all participants had values for both tests in each experiment. There were two participants who did not have a valid test in T1 but did have a value in baseline value in T2.
[2]Significantly different compared to the fall in COHb in $PROC_{A\ \&\ B}$ ($P = 0.0039$ and $P = 0.0289$, respectively).
[3]Statistically significant using one-way ANOVA with Bonferroni multiple comparison test.

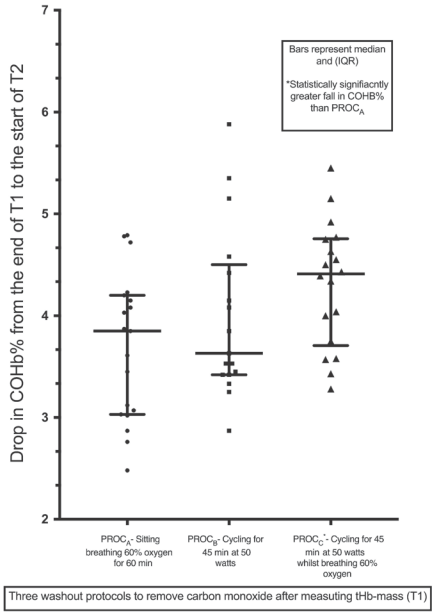

Figure 2. Comparing the fall in carboxyhemoglobin (COHb%) between test 1 and test 2 of each procedure (T1 and T2). Median with interquartile range shown for each procedure. *Denotes the significant difference between $PROC_C$ and $PROC_A$ (after multiple comparison correction using the Bonferroni method). COHb, carboxyhemoglobin; $PROC_{A/B/C}$ procedures A/B/C.

cycling alone ($PROC_B$) at lowering carbon monoxide levels in the blood.

Differences between males and females revealed a mean ± SD tHb-mass of 863.3 ± 147.0 and 541.3 ± 27.0, respectively. Further breakdown of male and female differences are tabulated in Table 2. All three procedures were relatively more efficient at clearing CO in females (see Table 2). Comparing $PROC_C$ with $PROC_A$ *and* $PROC_B$ they differed statistically as above in males ($P = 0.0458$ and $P = 0.0072$) but this did not hold true for females ($P = 0.8690$). There were no other statistically significant differences between the procedures when males and females were separated.

Test re-test reliability

The overall TE for the study was 2.0%, 95% CI (1.67–2.59). Neither the procedure nor the baseline carboxyhemoglobin level influenced the calculated tHb-mass. The

Table 2. Male and female differences between the three clearance procedures and resulting tHb-mass.

	Male	Female
Age (years)	Median 22, range 20 32	Median 22, range 22 23
Height (cm)	179.4 ± 8.9	161.0 ± 8.3
Weight (kg)	81.6 ± 11.4	63.0 ± 3.40
BMI (kg m^2)	25 ± 3.4	24 ± 2.0
Hemoglobin concentration [Hb](g L^{-1})	154.5 ± 13.8	141 ± 2.60
tHb-mass (g)	863.3 ± 147.0	541.3 ± 27.0
Blood volume (BV) (mL)	6188 ± 962.0	4220 ± 265.0
Plasma volume (PV) (mL)	3565 ± 664.3	2569 ± 182.2
Erythrocyte volume (EV) (mL)	2577 ± 524.0	1651 ± 83.20
Procedure A ($PROC_A$) fall in COHb% from the end of T1 to the beginning of T2	3.54 ± 0.72	4.25 ± 0.38
Procedure B ($PROC_B$) fall in COHb% from the end of T1 to the beginning of T2	3.90 ± 0.77	4.32 ± 1.10
Procedure C ($PROC_C$) fall in COHb% from the end of T1 to the beginning of T2	4.20 ± 0.63	4.72 ± 0.34
$PROC_A$ versus $PROC_B$- statistically significant?	No $P = 0.6543$	No $P = 0.8690$
$PROC_A$ versus $PROC_C$- statistically significant?	Yes $P = 0.0458$	No $P = 0.1119$
$PROC_B$ versus $PROC_C$- statistically significant?	Yes $P = 0.0072$	No $P = 0.5388$

All values are expressed and mean ± standard deviation unless otherwise stated.

TE of $PROC_{A\text{-}C}$ are shown in Table 1 and Figure 3 shows the Bland–Altman plots for $PROC_{A\ C}$ (Bland and Altman 1986) with limits of agreement. The bias is similar for $PROC_{A\text{-}C}$ (0.7, 4.5, and −1.1, respectively), but increased when T1 was compared across the 3 days (8.4). There were no significant differences between subjects that had the measurements over three consecutive days (14 subjects) versus within 10 days (11 subjects) with regard to the precision of repeat measures of tHb-mass.

Serum carboxyhemoglobin during $PROC_A$ and $PROC_C$ remained below 10% and ranged from 0.5 to 9.9% and 0.7 to 9.3%, respectively. Carboxyhemoglobin during $PROC_B$ ranged from 0.7 to 10.7% (Fig. 4). One subject had a serum carboxyhemoglobin above 10% (10.7% at 6 min and 10.4% at 8 min) after carbon monoxide administration during T2.

Discussion

Repeated measurements of tHb-mass can be safely made within 3 h if an adequate carbon monoxide clearance procedure is performed between tests. Administering oxygen alone (without exercise) is sufficient to achieve this and to avoid excess (>10%) carboxyhemoglobin levels being reached in healthy subjects.

Co-administering oxygen with mild exercise lowered the carboxyhemoglobin the most (Fig. 2, Table 1). This was statistically different from oxygen alone but not from cycling alone (after application of Bonferroni's test). These data are in keeping with those from a study which evaluated the effectiveness of measures to treat carbon monoxide poisoning (Goldstein 2008) and of a study which showed "hyperoxic exercise" to be superior to 6 h of normal daily activity in reducing carboxyhemoglobin (Naef et al. 2015). They are also in keeping with the study by Zavorsky et al. (2012) which demonstrated that the "triple low" of exercise, supplemental oxygen, and increased pulmonary ventilation was the most effective way to clear CO from the blood. We found no statistical difference between $PROC_A$ and $PROC_B$ but did find that $PROC_C$ was significantly better than $PROC_A$ at lowering carboxyhemoglobin levels. The fact that there was no significant difference between the other procedures may have been a consequence of the study being underpowered to detect any real difference or may represent a lack of difference between these techniques.

When males and females were compared all three procedures lowered COHb% to a greater extent in female subjects. This is in keeping with previous research and the fact that females have a lower tHb-mass. In a study examining this gender differences in CO $t_{1/2}$ disappeared when tHb-mass was normalized suggesting that CO storage explained much of the difference (Zavorsky et al. 2014). Due to the low number of female subjects within the study it is impossible to conclude further than this.

Strengths of this study include the fact that the same two operators performed all of the experiments in the same laboratory with the same equipment. Weaknesses include the fact that one subject performed the carbon monoxide clearance procedures in a different order. A

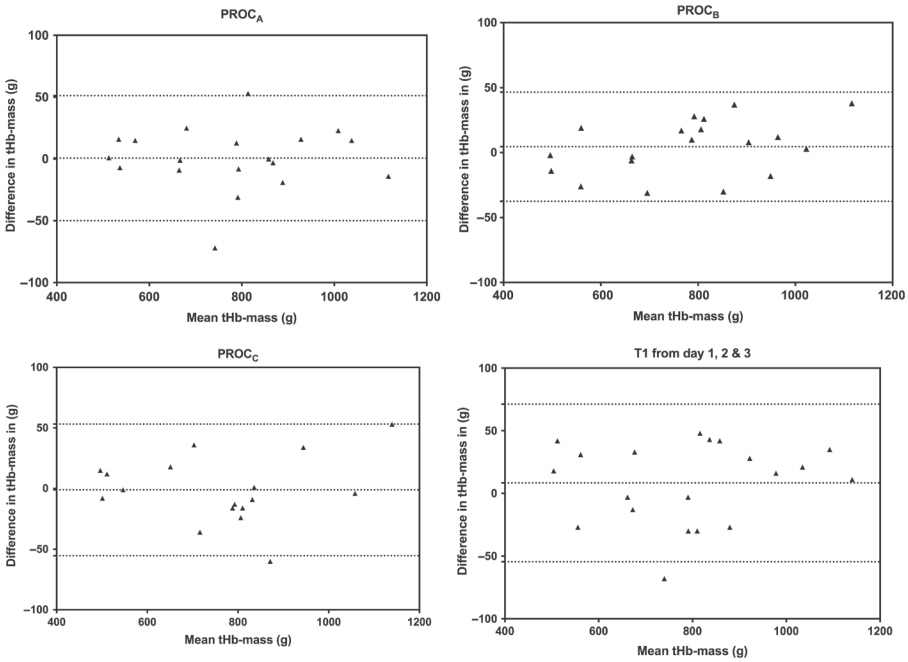

Figure 3. Bland-Altman plots comparing tHb-mass measurements between T1 and T2 for each procedure.

relatively high number of experiments that were not completed (14/136) for diverse reasons (See Appendix 3). These findings do highlight that the oCOR technique is susceptible to technical difficulties, even when performed by experienced well-trained technicians. It is possible that further refinement and the development of an automated oCOR technique in the future could improve this. Fourteen subjects were studied on consecutive days, and 10 over a period of up to 10 days. This is unlikely to have been of significance, given that tHb-mass has been shown to be stable over days to months in contrast to hemoglobin concentration and hematocrit which are not (Eastwood et al. 2008; Garvican et al. 2010a). We did not measure minute ventilation (MV) after CO inhalation and are aware that this does limit our findings due to variances in MV affecting CO removal, with ventilation playing a significant role (Zavorsky et al. 2012). Another weakness was the period of rest post each procedure for additional washout. Pragmatically this was performed for safety reasons due to the concern that the baseline levels might be too high to test immediately post the washout procedure.

One subject had a carboxyhemoglobin level exceeding the accepted safety threshold of 10% Figure 4 ($PROC_B$). They had no symptoms, and the carboxyhemoglobin level 1 hour later was safely below 10%. It should be noted that the safety limit used in this study (serum carboxyhemoglobin level of 10%) was a recommendation (Turner et al. 2014; Stewart 1975) also followed by the earlier study conducted by *Naef* and colleagues (Naef et al. 2015). We have used this level in all of our previous work and if a subject has exceeded 10% have treated them with supplemental oxygen until the level has reduced to below 10%. In fact, serum carboxyhemoglobin can be raised to 18% in healthy individuals without symptoms of toxicity developing, however the levels are lower in patients with heart disease (as one example) (Stewart 1975). As such, increasing serum carboxyhemoglobin to even 15% may also be acceptable. As most subjects had a serum

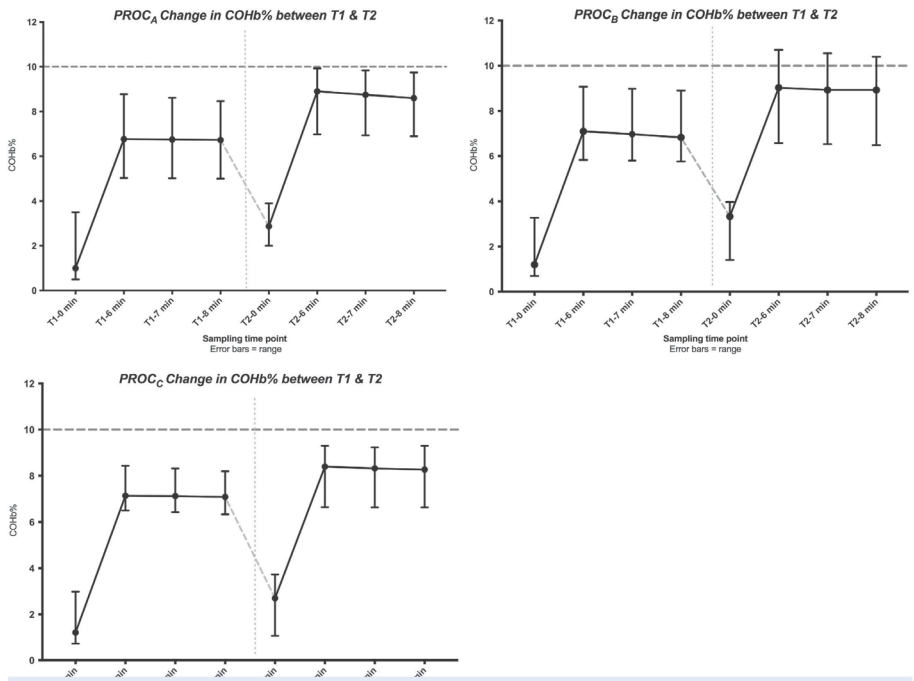

Figure 4. Change is serum carboxyhemoglobin (%) over test 1 and 2 (T1 and T2) of each procedure. Results are presented as median and range. Safety of duplicating the oCOR method is dependent on ensuring that the clearance procedure removes enough COHb after T1 to avoid excess COHb (>10%, red dotted line) during T2. Seven-minute values are calculated and not measured values but are included as they are used to calculate COHb. COHb: carboxyhemoglobin; oCOR, optimized carbon monoxide rebreathing technique; *PROC*$_{A/B/C}$, Procedure A/B/C, T1: test 1 or first measurement of the day, T2: test 2 or second measurement of the day.

carboxyhemoglobin less than 10% during all procedures, it is reasonable to believe that each of the three clearance methods was safe to use in this population. Much of this safety data are based on historical experiments and animal work (Stewart 1975; Stewart et al. 1973; Peterson and Stewart 1970). Our group alone has now conducted multiple hundreds of oCOR tests in healthy volunteer and clinical subjects without COHb levels exceeding 11% and without any adverse events being reported (Otto et al. 2017a,b). We have tested smokers in some of our previous work but do not have extensive data on levels and symptoms associated with carboxyhemoglobin levels in excess of 10%. The question of whether a top level of 10% is indeed to limit of "safety" for the oCOR method in healthy volunteers or clinical subjects was not focus of this study however we recognize that further controlled experiments examining this would be of use. This becomes relevant if multiple testing occurs within short time periods. Further work would be required to examine if the threshold of 10% could be safely exceeded.

Reliability of duplicate total hemoglobin mass measurements

The overall typical error of repeat measurements within the same subject in this study of 2.0% (Table 2) is in keeping with the only published meta-analysis on the subject, which reported a value of ~2.2% (Gore et al. 2013). This meta-analysis is important as it groups together a number of small studies from different laboratories and totals 328 participants. If an individual has a tHb-mass of 1000 g, a TE of 2.0% equates to a 20 g difference between tests. As such, the study by Naef et al. had a TE of 1.4%

that would equate to only a 14 g difference. Our results were not quite as precise (Naef et al. 2015). The median total hemoglobin mass over all six oCOR tests was lower in our study than in that of Naef et al. (2015) (797 g vs. 914 g, respectively, equating to 16 g Hb vs. 13 g Hb based on a TE of 2.0 vs. 1.4) in keeping with our inclusion of four females and a less well-trained population. It is conceivable that part of the difference is also due to the technical precision of the operators and/or the accuracy of the equipment. We used a rise in carboxyhemoglobin (ΔCOHb%) of >4.5%, some authors argue that higher values are required to improve precision (Burge and Skinner 1995).

Bland–Altman plots (Fig. 3) suggest reasonable precision. The biases were low (0.7, 4.5 and −1.1 for $PROC_{A\text{-}C}$, respectively) and higher when the first test of each day was compared (8.4).

The future of the optimized carbon monoxide rebreathing method in anesthesia and perioperative care

The most promising discovery was that clearing carboxyhemoglobin through administration of oxygen alone was effective enough to ensure safe carboxyhemoglobin levels in nontrained individuals and to allow repeat testing within 3 h. This clearance method is likely to be the only option for many patients, whose co-morbidities might preclude them from performing exercise. This may allow clinically useful serial measurements to assess blood volume, plasma volume, and tHb-mass over the perioperative period, and thus the accurate quantification of blood loss. This extends the potential application of tHb-mass testing in the clinical environment. In the future it may be possible to assess rapid changes in plasma or blood volume and tHb-mass, such as might occur with surgery.

Future studies should seek to investigate the feasibility of duplicate tHb-mass testing in a single day in specific patient groups, and in the perioperative period in particular. Modification of the oCOR for use in ventilated subjects in the theatre or intensive care environment would also be of great value.

This study represents a step toward successfully integrating the use of the oCOR method as a point-of-care clinical technique. It has become increasingly apparent that [Hb] (and thus the definition of anemia) is strongly influenced by plasma volume, which can be inappropriately expanded in certain disease states (Otto et al. 2017b). Therefore, efforts to readily measure tHb-mass, as a means of deriving plasma content, are required in clinical medicine. The oCOR method is portable, minimally invasive, quick, and user-friendly.

Conclusion

The oCOR method is a safe technique that can be repeated within 3 h when carbon monoxide is suitably cleared between tests in healthy nonathletic individuals. Using oxygen therapy alone adequately achieves this. This method has minimal bias and good precision making it attractive for regular monitoring of blood volume derivatives. Clearance using oxygen alone can safely be used in healthy volunteers with a high degree of precision and safety and it is possible that this could translate to clinical subjects.

Acknowledgments

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Conflict of Interests

JP has received financial support from Siemens Healthcare Limited for consumables and hardware for research into the measurement of hemoglobin mass (2015-2018). JP was given consumables from Intersurgical UK Ltd (2015-2018); has received honoraria for speaking and/or travel expenses from Siemens and Vifor Pharma and has received unrestricted grant funding from Pharmacosmos. JP is unaware of any direct or indirect conflict of interest with the contents of this paper or its related fields. SK has no conflicts of interest. JO has no conflicts of interest. WS is a managing partner of the company "Blood tec GmbH," but he is unaware of any direct or indirect conflict of interest with the contents of this paper. TR is director of the ironclinic.com. and has received research funding from a variety of sources, including; government, charity, and industry sources for research into anemia, blood transfusion, and iron therapy, including NIHR HTA, NHMRC, Health Foundation, Gideon Richter, Vifor Pharma Ltd, and Pharmaocosmos. He has also been an invited speaker at conferences and provided consultancy to government and industry on anemia, blood transfusion, and iron therapy in the last 5 year. Please see www.ucl.ac.uk for full list of disclosures. HM consults for Google Deepmind on health technology and is on the Council of the UK Intensive Care Society but is unaware of any direct or indirect conflict of interest with the contents of this paper or its related fields. MG is vice-president of CPX International. He also serves on the medical advisor board of Sphere Medical Ltd and the board of

EBPOM Community Interest Company, Medinspire Ltd and Oxygen Control Systems Ltd. He has received honoraria for speaking for and/or travel expenses from BOC Medical (Linde Group), Edwards Lifesciences and Cortex GmBH and unrestricted research support from Sphere Medical Ltd and Pharmacosmos Ltd. He leads the Fit-4-Surgery research collaboration and the Xtreme Everest oxygen research consortium, which has received unrestricted research grant funding from BOC Medical (Linde Group), Deltex Medical and Smiths Medical. MPWG was funded in part from the British Oxygen Company Chair of the Royal College of Anaesthetists awarded by the National Institute of Academic Anaesthesia. All funding was unrestricted. The funders had no role in study design, data collection, and analysis, decision to publish or the preparation of the manuscript. This work was conducted at the Southampton NIHR Biomedical Research Centre with subjects studied within the Southampton NIHR Clinical Research Facility.

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Appendix 1: Calculation of tHb-Mass

tHb-mass was calculated using a specifically design excel spreadsheet (Microsoft Excel 2011 for Apple Macintosh) using the formula:

(1) tHb-mass (g) = K × MCO(mL) × 100 × (Δ%COHb × 1.39)−1
(2) K = barometric pressure × 760−1 × [1(0.003661 × temperature)]
(3) MCO = COadm − (COsystem + lung (after disconnection) + COexhaled (after disconnection)
(4) COadm = CO volume administered into the system
(5) COsystem + lung (after disconnection) = CO concentration in spirometer × (spirometer volume + remaining volume in the lung after disconnection)
(6) COexhaled (after disconnection) = end-tidal CO concentration × alveolar ventilation × time
(7) Δ%COHb = difference between baseline %COHb and %COHb post CO administration
(8) (average of 6- and 8-min %COHb values)
(9) 1.39 = Hufner's number (constant) (mL CO×g Hb-1)

Residual volume and alveolar ventilation were calculated according to "Standardized lung function testing. Official statement of the European Respiratory Society" (Standardized lung function testing, 1993). CO concentration is in parts per million (ppm).

Blood volume (BV), plasma volume (PV) and red cell volume (RCV) were calculated from mean corpuscular hemoglobin concentration (MCHC), [Hb] and tHb-mass, as below:

(1) BV (mL) = tHb-mass (g)/[Hb] (g dL^{-1}) × 100
(2) RCV (mL) = tHb-mass (g)/MCHC × 100
(3) PV (mL) = BV−RCV

Appendix 2: Measurement of tHb-Mass Using the Optimized Carbon Monoxide Re-Breathing Method (oCOR)

The use of CO to determine tHb-mass was first proposed in the late 1800s, with refined techniques being published 100 years later (Thomsen et al. 1991). In 2005, Schmidt and Prommer reported a simpler and faster technique (described in detail below), which also required less blood sampling (Schmidt and Prommer 2005). It was applied almost exclusively, however, in the fields of athletic physiology, and thus failed to come to the attention of the bulk of the broader clinical/medical community.

tHb-mass was determined using the validated oCOR method described in detail by Schmidt and Prommer (Schmidt and Prommer 2005). COHb concentration in blood was measured before and after 2 min rebreathing a known CO volume (0.5 to 1.0 mL kg^{-1} in this study depending on sex and [Hb]). Each participant was seated for 15 min to allow stabilization of plasma volume (PV), after which a mouthpiece connected them *via* a container of 'soda lime'~10 g (carbon dioxide scrubber) to a spirometer (Spico-CO Respirations-Applicator, Blood Tec, Germany) and a 3-L anesthetic bag pre-filled with 100% oxygen. The patient exhaled to residual volume, breathed in the CO dose *via* the spirometer, held their breath for 10s, then continued normal breathing into the closed circuit *via* the spirometer for 1 min 50 sec. The participant then exhaled to residual volume, this exhaled volume being collected and analyzed to quantify the CO not absorbed into the bloodstream. Disconnected from the mouthpiece, participants finally fully exhaled to residual volume into a CO gas analyzer (Dräger Pac 7000, Drägerwerk AG & Co. KGaA, Germany) before and at 4 min after CO rebreathing, in order to determine the end tidal CO concentration and thereby the amount of CO exhaled after disconnecting the patient from the spirometer, that will also have not been absorbed into the blood.

Appendix 3:

A total of 144 experiments were planned. Only 136 took place, in seven experiments the test was not performed due to the CO gas supply running out and in one experiment the patient's blood clotted prior to analysis. Of the 136 experiments performed on 24 subjects, 122 were suitable to be included on the final analysis. Six experiments had a ΔCOHb% of <4.5% so they were discarded as per standard laboratory practice in our laboratory. Five experiments had a significant leak identified or gas was lost on injection (one experiment) and three experiments had technical problems with the blood gas analyzer calibration and were unsuitable due to inaccurate readings.

Appendix 4:

The Hopkins method for typical error calculation is described below. "A statistic that captures the notion of random variability in a single individual's values on repeated testing is the standard deviation of the individual's values. This within-subject standard deviation is also known as the standard error of measurement. In plain language, it represents the typical error in a measurement." (Hopkins 2000)

Following this approach, the test-retest typical error is computed as follows:

(1) Computed difference in total hemoglobin mass assessed in repeated tests.
(2) Computed standard deviation of the difference in total hemoglobin mass assessed during the two tests.
(3) Mean standard deviation divided by the square root of 2.

This is the method used by Professor Schmidt's group (pers. comm., 2018). A test-retest typical error of around 2% is acceptable in the difference in tHb-mass between repeat tests as described in the tables available at www.sportsci.org, written by Hopkins (2000). It should be noted that the confidence limits for the typical error are derived from a chi-squared distribution. For small degrees of freedom, the upper limit tends to be skewed out relative to the lower limit. Tate and Klett provided an adjustment that reduces the skewness by minimizing the width of the confidence interval, although it is then not an equal-probability interval. With only slight adjustment the Tate & Klett limits can be represented conveniently by a single factor (see Table II in Tate and Klett (1959)).

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RESEARCH PAPER

A carbon monoxide 'single breath' method to measure total haemoglobin mass: a feasibility study

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Abstract

Anaemia is defined by the concentration of haemoglobin (Hb). However, this value is dependent upon both the total circulating haemoglobin mass (tHb-mass) and the plasma volume (PV) – neither of which is routinely measured. Carbon monoxide (CO)-rebreathing methods have been successfully used to determine both PV and tHb-mass in various populations. However, these methods are not yet suitable for ventilated patients. This study aimed to modify the CO-rebreathing procedure such that a single inhalation of a CO bolus would enable its use in ventilated patients. Eleven healthy volunteers performed four CO-rebreathing tests in a randomized order, inhaling an identical CO volume. In two tests, CO was rebreathed for 2 min (optimized CO rebreathing; oCOR), and in the other two tests, a single inhalation of a CO bolus was conducted with a subsequent breath hold of 15 s ($Proc_{new}$15s) or 30 s ($Proc_{new}$30s). Subsequently, the CO volume in the exhaled air was continuously determined for 20 min. The amount of CO exhaled after 7 and 20 min was respectively 3.1 ± 0.3 and 5.9 ± 1.1 ml for oCOR, 8.7 ± 3.6 and 12.0 ± 4.4 ml for $Proc_{new}$15s and 5.1 ± 2.0 and 8.4 ±2.6 ml for $Proc_{new}$30s. tHb-mass was 843 ± 293 g determined by oCOR, 821 ± 288 g determined by $Proc_{new}$15s (difference: $P < 0.05$) and 849 ± 311 g determined by $Proc_{new}$30s. Bland–Altman plots demonstrated slightly lower tHb-mass values for $Proc_{new}$15s compared with oCOR (−21.8 ± 15.3 g) and similar values for $Proc_{new}$30s. In healthy volunteers, a single inhalation of a CO bolus, preferably followed by a 30 s breath hold, can be used to determine tHb-mass. These results must now be validated for ventilated patients.

KEYWORDS
blood volume, carboxy-haemoglobin, CO rebreathing, ventilated patients

1 | INTRODUCTION

Haemoglobin (Hb) is the oxygen-carrying pigment of the circulation. Its circulating concentration ([Hb]) is routinely measured in clinical practice, and low values are used to define 'anaemia' (Beutler & Waalen, 2006). However, [Hb] is determined by the total circulating mass of Hb (tHb-mass) and the volume of plasma (PV) in which it is carried. The measurement of such independent variables has distinct advantages given that PV can change substantially with disease. The importance of tHb-mass measurement in the clinical setting and the advantages over [Hb] have been discussed previously (Otto et al., 2017a,b; Plumb et al., 2016). Indeed, [Hb] correlates poorly with tHb-mass in patients with chronic liver disease or heart failure, in whom PV may be expanded (Otto et al., 2017a). Despite this fact, [Hb] is the major trigger for the transfusion of red blood cells.

Likewise, perioperative changes in tHb-mass and PV are common (Iijima et al., 2013; Makaryus et al., 2018) due to blood loss, administration of red blood cells or haemodilution through administration of intravenous fluids or through salt/water retention due to the 'surgical stress response' (Rassam & Counsell, 2005). Fluid distribution between physiological compartments and the impact of hypo/hypervolaemia on the glycocalyx and therefore the functional integrity of the intravascular space also influence PV (Strunden et al., 2011). However, the decision to transfuse blood to a patient in clinical practice in general, and perioperative and critical care settings in particular, hinges on a variety of factors. There is a growing recognition that [Hb] may not be the best clinical indicator to guide such decisions (Plumb et al., 2016; Shander & Ferraris, 2017).

tHb-mass and derived PV can be determined by different dilution methods, in which carbon monoxide (CO) has been found to be the easiest and most precise marker to use (Gore et al., 2005). Using the inhalation of a known volume of CO (thus labelling Hb as carboxyhaemoglobin, COHb) allows the measurement of tHb-mass and, thus, the calculation of PV. In self-ventilating subjects, this is achieved using the so-called 'optimized CO rebreathing method' (oCOR). However, this protocol relies upon the participant being alert, able to follow instructions, and able to control their breathing through a closed circuit, which in turn precludes the use of oCOR in participants who are receiving mandatory ventilation from a mechanical ventilator, either under anaesthesia or when sedated in the intensive care unit. We hypothesized that delivery of a single CO bolus into the breathing circuit of a participant without the need for rebreathing could be used to reliably measure tHb-mass, so long as exhaled gas could be analysed. We thus sought to develop such a technique. Here, we describe this development and early data relating to its likely reliability. The primary aim of this study was to develop a new method for measuring tHb-mass in healthy participants simulating a procedure that might be used in participants on a mechanical ventilator ($Proc_{new}$) and to evaluate the feasibility of this novel method. We aimed to assess reliability compared to the standard oCOR method. We also repeated the oCOR test to quantify the reliability of the standard method within this experiment.

New Findings

- **What is the central question of this study?**
  Is it possible to modify the CO-rebreathing method to acquire reliable measurements of haemoglobin mass in ventilated patients?
- **What is the main finding and its importance?**
  A 'single breath' of CO with a subsequent 30 s breath hold provides almost as exact a measure of haemoglobin mass as the established optimized CO-rebreathing method when applied to healthy subjects. The modified method has now to be checked in ventilated patients before it can be used to quantify the contributions of blood loss and of dilution to the severity of anaemia.

2 | METHODS

2.1 | Ethical approval

Ethical approval was granted by the South-Central Hampshire B Research Ethics Committee (REC reference: 15/SC/0496) and from the ethics committee of the University of Bayreuth (reference: O1305/1-GB). The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. Written informed consent was obtained from all participants. The subjects volunteered to participate in the study and were free to withdraw at any time without needing to provide a reason.

2.2 | Subjects

This feasibility study took place at the University Hospital Southampton NHS Foundation Trust, UK, and at the University of Bayreuth, Germany. Eleven healthy non-smoking test subjects (five women, six men) with moderate physical training status took part in the study (for anthropometric data of these subjects see Table 1).

2.3 | Study design

In preliminary tests, we checked whether a single inhalation of a CO bolus could achieve an increase in COHb concentration ([COHb]) that would be sufficient to determine tHb-mass. For this purpose, tHb-mass was measured twice; results from the established CO-rebreathing method (Schmidt & Prommer, 2005), that is, 2 min CO inhalation within a closed spirometry system, were compared to those from a single inhalation with subsequent 10 s breath holding and 15 min collection of expired air. Because the difference in tHb-mass was lower than 50 g

TABLE 1 Anthropometric and haematological characteristics of the test subjects

	Females	Males
n	5	6
Age (years)	33.2 ± 11.6	26.8 ± 4.0
Height (cm)	165.5 ± 8.4	187.2 ± 9.8
Body mass (kg)	59.2 ± 7.0	85.9 ± 10.9
BMI (kg/m^2)	21.6 ± 1.1	24.4 ± 1.4
LBM (kg)	45.3 ± 6.9	72.2 ± 10.5
$[Hb]_{cap}$ (g/dl)	13.9 ± 0.7	15.9 ± 1.1
Hkt_{cap} (%)	41.8 ± 1.2	46.1 ± 3.0

$[Hb]_{cap}$ haemoglobin concentration determined in capillary blood, Hkt_{cap}, haematocrit determined in capillary blood.

in only six of 13 comparisons, breath holding was prolonged to 15 and 30 s and collection of the expired air to 20 min in the main study.

In the main study, at least four CO-rebreathing tests were carried out by 11 subjects in a randomized order. Two of the tests consisted of the established CO-rebreathing method over 2 min (oCOR). In the third and fourth tests, the subjects inhaled a CO bolus followed by a breath hold for 15 s ($Proc_{new}$15s) or 30 s ($Proc_{new}$30s). Afterwards, the volume and CO concentration of the expiratory air were continuously analysed for 20 min, and the whole amount of exhaled air was finally collected in a Douglas bag. tHb-mass was calculated at 2 min intervals using the prevailing [COHb] and the accumulated CO volume in the expired air. Additionally, at the end of the test, tHb-mass was obtained by using the total expiratory volume and the average [COHb] in the Douglas bag. To evaluate possible influences of the test arrangement, that is, collection and analysis of the expired air after inhaling the CO bolus, CO exhalation was determined from six subjects in a fifth test approach using the same methodology as described above after a conventional 2 min CO-rebreathing procedure ($oCOR_{+20min}$).

2.4 | Established carbon monoxide rebreathing method

tHb-mass was determined using the optimized CO-rebreathing (oCOR) method as described and modified by Schmidt and Prommer (Prommer & Schmidt, 2007; Schmidt & Prommer, 2005). Briefly, a bolus of 99.97% CO (0.8–1.0 ml CO/kg body mass, depending on the training status) was administered to subjects and rebreathed along with 3 litres of 100% O_2 for 2 min. Three arterialized capillary blood samples were taken from a hyperaemic earlobe (Finalgon, Sanofi-Aventis, Frankfurt, Germany) before the rebreathing procedure, and at minutes 6 and 8 after the rebreathing procedure, and each sample was analysed in triplicate using an OSM3 haemoximeter (Radiometer, Brønshøj, Denmark). End-tidal [CO] was assessed before and 2 min after the rebreathing procedure using a portable CO detector (Draeger Pac7000, Lübeck, Germany). tHb-mass was assessed in duplicate (test 1 and test 2) using this method, and the mean of both tests was used for comparison with the results of the modified procedures. The typical error for tHb-mass measurements determined from these duplicate tests was 1.0%.

2.5 | Modified method

To adapt the method so that it can be used in everyday clinical practice, several modifications were necessary (see Figure 1). Since a patient frequently cannot put the spirometer into their mouth by themselves, the gas supply was replaced by a mask (Hans Rudolph, Inc., Shawnee, KS, USA) with an access port for the CO supply. This access port is designed in such a way that the manually administered CO from a syringe passes the mask via a small tube directly into the back of the mouth and thus into the test person's inhalation path. In contrast to the established method, in which rebreathing occurs in a closed system, this modification presents an open system in which ambient air is inhaled via a three-way valve. The inhaled and expired air passes a volume flow sensor (breath-by-breath registration, Metalyzer 3B, Cortex Biophysics GmbH, Leipzig, Germany) and subsequently a small mixing chamber, which is equipped with a CO sensor (Draeger Pak 7000, Liebefeld, Switzerland), and is finally collected in a Douglas bag (Cranlea Human Performance Ltd, Birmingham, UK).

After connecting and accustoming the subject to the equipment for at least 10 min in the sitting position, the subject exhaled normally, and the three-way valve was turned. Subsequently, the subject deeply inhaled, and CO was administered by the investigator via the access port into the subject's inhalation path. The subject held their breath for 15 s (test 3, $Proc_{new}$15s) or 30 s (test 4, $Proc_{new}$30s) and breathed normally thereafter for the following 20 min into the Douglas bag.

Until the fifth minute after starting the test, the CO concentration and the volume of the exhaled air were monitored at 30 s intervals and thereafter at 1 min intervals until disconnecting the subject from the equipment after 20 min. In the same way as in test 1 and test 2, three capillary blood samples were taken before the test, one sample each was collected after 1 and 2 min, and then further samples were taken every 2 min until min 20.

2.6 | tHb-mass calculation

For the established method (tests 1 and 2), tHb-mass was calculated as described previously (Schmidt & Prommer, 2005):

$$\text{tHb} - \text{mass (g)} = K \times \text{MCO} \times 100 / (\Delta\text{COHb\%} \times 1.39) \quad (1)$$

where K = (current barometric pressure/760) × [1+ (0.003661 × current temperature)],

$\text{MCO} = \text{CO}_{adm} - (\text{CO}_{system+lung(AfterDisconnection)} + \text{CO}_{exhaled(AfterDisconnection)} - \text{M}_{Hb}\,\text{CO}_{adm}$

= CO volume administered into the system $\text{CO}_{system+lung(AfterDisconnection)}$

= CO concentration in the spirometer × (spirometer volume + lung residual volume)

M_{Hb} = CO diffusing to myoglobin $\text{CO}_{exhaled(AfterDisconnection)}$

= Δ end-tidal CO concentration × alveolar ventilation × time

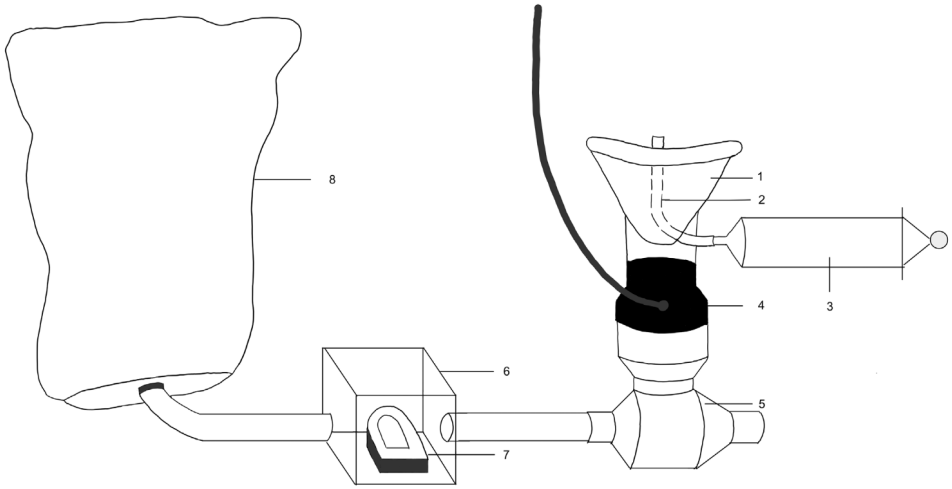

FIGURE 1 Experimental set-up and equipment for the single-breath method. 1: breathing mask; 2: tube inserted into the subject's inhalation path; 3: syringe for CO administration; 4: flow meter; 5: 3-way valve; 6: mixing chamber; 7: CO sensor; 8: Douglas bag

ΔCOHb% is the difference between basal COHb% and COHb% in the blood samples after CO administration. 1.39 = Hüfners number (ml CO × g Hb^{-1}) (e.g. Gorelov, 2004).

For tests 3 and 4 ($Proc_{new}$15s and $Proc_{new}$30s), tHb-mass was calculated using formula (1) in two modified ways: (i) for each time point of taking blood after CO inhalation using the corresponding COHb concentrations and accumulated values for CO exhalation ($MCO = CO_{adm} - CO_{exhaled} - M_{Hb}$), and (ii) using the COHb concentration at min 20 and the totally exhaled CO volume collected in the Douglas bag. To compare the results of the new methods with those of oCOR, tHb-mass was calculated for min 7 ($tHb\text{-}mass_{min7}$) as well as using data from the whole test, that is, the mean from the plateau between min 6 and 20 ($tHb\text{-}mass_{plateau}$) and for min 20 using the data from the air collected in the Douglas bag ($tHb\text{-}mass_{DouglasBag}$).

2.7 | Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 25 for Windows, IBM Corp., Armonk, NY, USA). Values are presented as the mean ± standard deviation (SD), unless otherwise stated. Categorical variables are presented as frequencies (%).

This was a feasibility study, and a formal power calculation was therefore not required. Test–retest data (repeated measures from the same patient with different analytical methods) are presented using Bland–Altman plots with limits of agreement (Bland & Altman, 1986). Additionally, a specific approach to compute reliability statistics to compare test–retest performance expressed as the typical error of measurement (TE) was used (see Hopkins, 2000).

Student's paired *t*-test was used to compare mean values from both tests at identical time points, and a paired *t*-test was also used to compare the mean values at different time points of the identical test. All tests were two-sided, and statistical significance was set at $P < 0.05$. To minimize the risk for type I errors, a correction for multiple measurements according to Benjamini & Hochberg (1995) was performed.

3 | RESULTS

All of the tests were conducted without complications or adverse events. All participants inhaled the identical CO volume during the four tests (63.3 ± 23.1 ml; males 82.1 ± 16.8 ml, females 44.5 ± 6.3 ml). The exhaled CO volume was highest in the first minute of $Proc_{new}$15s (6.3 ± 3.5 ml; $Proc_{new}$30s 2.9 ± 1.6 ml). This initial phase was followed by an almost linear and parallel increase in both new procedures, showing an accumulation of 8.7 ± 3.6 and 5.1 ± 2.0 ml in min 7 and 12.0 ± 4.4 and 8.4 ± 2.6 ml in min 20, respectively. When the expired air was collected after $oCOR_{+20min}$, the values (3.1 ± 0.3 and 5.9 ± 1.1 ml) were clearly below those of $Proc_{new}$15s and $Proc_{new}$30s (Figure 2) and not different from the volume exhaled 7 min after oCOR.

[COHb] exhibited well-known time-dependent changes, with a fast increase in the first minute followed by a rapid and then decelerating decrease during the rest of the observation period (Figure 3). The values of the new procedures were clearly below those of $oCOR_{+20min}$. In min 7, the CO volume ligated to Hb was 59.5 ± 22.5 ml (oCOR), 57.2 ± 21.5 ml ($Proc_{new}$30s) and 53.9 ± 21.6 ml ($Proc_{new}$15s),

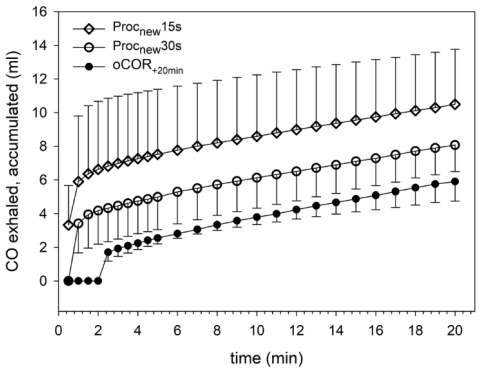

FIGURE 2 Cumulative CO volume exhaled after the three different methods of CO application. $n = 6$; Proc$_{new}$15s: single CO bolus inhalation with 15 s breath holding; Proc$_{new}$30s: single CO bolus inhalation with 30 s breath holding; oCOR$_{+20min}$: 2 min CO rebreathing followed by an 18 min analysis of exhaled air

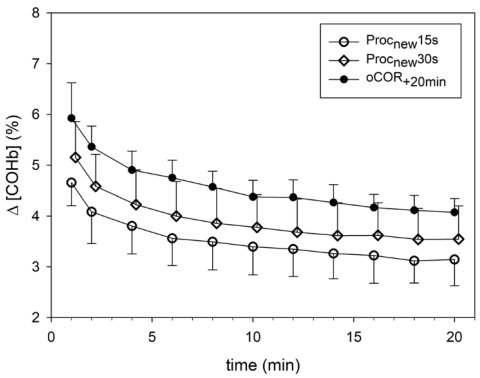

FIGURE 3 Changes in carboxy-haemoglobin ([COHb]) concentration after the inhalation of CO by three different application methods. $n = 6$; Proc$_{new}$15s: single CO bolus inhalation with 15 s breath holding; Proc$_{new}$30s: single CO bolus inhalation with 30 s breath holding; oCOR$_{+20min}$: 2 min CO rebreathing followed by an 18 min analysis of exhaled air

corresponding to 94.0 ± 2.1%, 90.4 ± 4.7% and 85.2 ± 6.0% of the inhaled CO volume, respectively.

tHb-mass was calculated for min 1 and 2 and then further measured in 2 min steps until min 20. We found increasing tHb-mass values for Proc$_{new}$15s until min 6 (Figure 4a) and for Proc$_{new}$30s until min 8 (Figure 4b) followed by a plateau after both procedures until min 20. Comparing the tHb-mass determined at min 7 yielded similar results for the three methods (Table 2). When tHb-mass from oCOR was compared with tHb-mass$_{plateau}$ and tHb-mass$_{DouglasBag}$, we found slightly lower values for Proc$_{new}$15s and very similar values for Proc$_{new}$30s (Table 2). Comparison of tHb-mass values obtained with the established method (oCOR) and with oCOR$_{+20min}$, that is, with collection of the expired air as in Proc$_{news}$, yielded almost identical results (Table 2).

Bland–Altman plots comparing oCOR and the new procedures demonstrate slightly lower tHb-mass values for Proc$_{new}$15s (tHb-mass$_{plateau}$: −21.8 ± 15.3 g; tHb-mass$_{DouglasBag}$: −12.2 ± 24.2 g) and very similar values for Proc$_{new}$30s over a large range of tHb-mass values between 450 and 1300 g (Figure 5).

4 | DISCUSSION

We wished to identify a way to measure tHb-mass in mechanically ventilated patients. We thus explored whether it is possible to reliably measure tHb-mass using a single-breath inhalation (with a 15 or 30 s breath hold) of CO gas and showed for the first time that it is feasible (Proc$_{new}$). Exhaled gas was collected and measured for the duration of the testing period (20 min). Our data suggest that Proc$_{new}$ with 15 and 30 s breath holds was closely related to the established CO-rebreathing method.

The principle of the CO-rebreathing method is to administer a defined amount of CO by breathing to determine the resulting COHb concentration in the completely mixed blood and to take into account the CO not bound to the Hb, that is CO exhaled and CO diffused to myoglobin. When these conditions are fulfilled, different procedures of the CO method can be applied.

Modifications to the CO rebreathing technique for the measurement of tHb-mass have therefore been made many times since the technique was revived by Fogh-Andersen et al. (1990), notably in 1995 when Burge and Skinner achieved improved precision of the measurement (Burge & Skinner, 1995). The current technique described by Schmidt and Prommer reduced the rebreathing period to only 2 min to improve convenience for participants (Otto et al., 2017a,b; Plumb et al., 2020; Schmidt & Prommer, 2005). The finding that a bolus of CO gas inhaled with a single breath and only rebreathed for 2 min led to valid and reliable results characterized by a typical error between 1% and 2% allowed the method to be used in a variety of different settings. Initially, these were primarily focused on elite sports physiology and performance, but more recently, oCOR has also been used to answer clinical questions (Otto et al., 2017a,b). The high reliability is confirmed in this study with a TE of 1.0% for the standard oCOR method.

When CO is administered for tHb-mass determination in an open spirometry system as we did in this study for the first time, an exact determination of the exhaled CO is mandatory. We determined the exhaled CO volume twice, that is, first by continuously monitoring the volume and CO concentration of the exhaled air, and second by collecting the whole amount of expired air in a Douglas bag and measuring the exhaled CO volume after the test.

To check whether the breathing procedure after the test exerts any unexpected influence on CO exhalation, we compared a 2-min inhalation period with subsequent collection of exhaled air

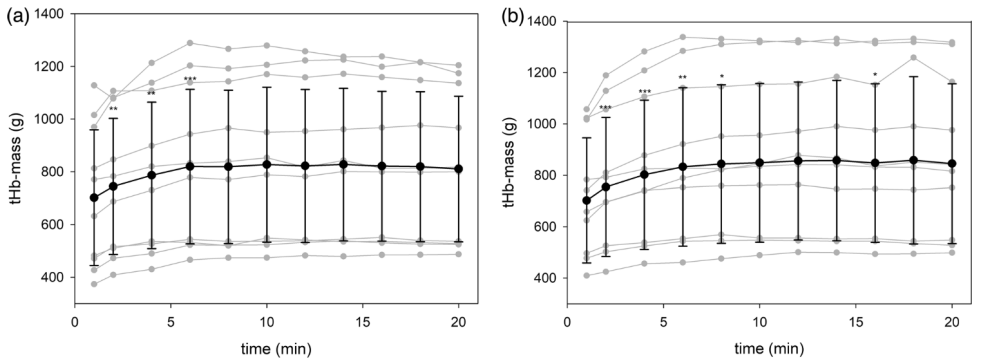

FIGURE 4 Time course of the calculated tHb-mass after CO breathing and subsequent breath holding for 15 s (a; $Proc_{new}15s$) and for 30 s (b; $Proc_{new}30s$). Presented are mean values and individual data of the tHb-mass calculated for different time points of blood sampling. Significant differences from previous values: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

TABLE 2 tHb-mass calculated from the three CO application methods

	oCOR($n = 11$)	$Proc_{new}15s$($n = 11$)	$Proc_{new}30s$($n = 11$)	oCOR($n = 6$)	$oCOR_{+20min}$($n = 6$)
$tHb\text{-}mass_{min7}$ (g)	843 ± 293	819 ± 285	838 ± 301	705 ± 209	665 ± 194
$tHb\text{-}mass_{plateau}$ (g)		821 ± 288*	849 ± 311		686 ± 200
$tHb\text{-}mass_{DouglasBag}$ (g)		830 ± 276	846 ± 309		711 ± 196

oCOR: established CO-rebreathing method; $Proc_{new}15s$: single CO bolus inhalation with 15 s breath holding; $Proc_{new}30s$: single CO bolus inhalation with 30 s breath holding; $oCOR_{+20min}$: 2 min CO rebreathing followed by an 18 min analysis of exhaled air; $tHb\text{-}mass_{min7}$: tHb-mass calculated with [COHb] determined 7 min after inhalation, $tHb\text{-}mass_{plateau}$: Hb-mass calculated with [COHb] determined between min 6 and min 20, $tHb\text{-}mass_{DouglasBag}$: tHb-mass calculated with [COHb] determined at min 20 and using the CO volume exhaled into the Douglas bag. Significant difference from oCOR: $^{*}P < 0.05$.

($oCOR_{+20min}$) with the established method (oCOR). As we did not find any difference in the resulting tHb-mass, we conclude that the exhalation protocol does not affect the precision of the new procedures.

When breath was held for 15 s, the initial CO exhalation after 1 min was twice as high as that in $Proc_{new}30s$, indicating that ~10% ($Proc_{new}30s$ ~4%) of the inhaled CO did not diffuse into the blood. After 7 min, that is, when the CO mixing in the blood was completed and therefore used in the established oCOR for blood sampling after the test, the loss of CO was ~13% in $Proc_{new}15s$ and only ~8% in $Proc_{new}30s$. Although this loss in CO clearly exceeded the CO volume exhaled after oCOR (~4%), these data demonstrate the rapid diffusion of CO from the lungs into the blood, which is also a precondition for the determination of the lung diffusion capacity by means of a deep inhalation of a 0.3% CO-containing gas followed by a 10-s breath hold (Modi & Cascella, 2020). We therefore suggest that tHb-mass might be easily and exactly calculated after a single breath when considering sufficient mixing time of the inhaled CO bolus.

These considerations are supported by the calculated tHb-mass over time. In the new procedures, tHb-mass reached a plateau in min 6 or in min 8 indicating complete mixing (Bruce & Bruce, 2003), that is, that any time point beyond can be used for tHb-mass and blood volume determination (Wachsmuth et al., 2019). In this study, we used the plateau value between min 6 and 20 and compared its mean with the time point usually used in the oCOR (min 7) and with the results obtained in min 20 from the exhaled air collected in the Douglas bag. As shown in Table 2, there is no obvious difference between tHb-mass obtained from the oCOR and that obtained from the new procedures at the time points mentioned above. In the Bland–Altman plot, a small but systematic underestimation by approximately 25 g compared with the reference method becomes obvious in $Proc_{new}15s$. As such a deviation does not occur between $Proc_{new}30s$ and oCOR, we suggest that the smaller CO volume taken up from the blood during $Proc_{new}15s$ may be the cause. Additionally, the very low limits of agreement in the comparison of both new methods with oCOR indicate that $Proc_{new}15s$ already presents a promising tool for tHb-mass and blood volume determination, and $Proc_{new}30s$ seems to be as exact as the established oCOR.

In future studies, the administration of higher CO volumes than those used for the oCOR may be taken into consideration to compensate for the lower CO uptake during the single-breath application. On the one hand, this procedure increases the [COHb], reducing the measurement error of the CO oximeter (Alexander et al., 2011) but also increases the volume of exhaled CO and thereby

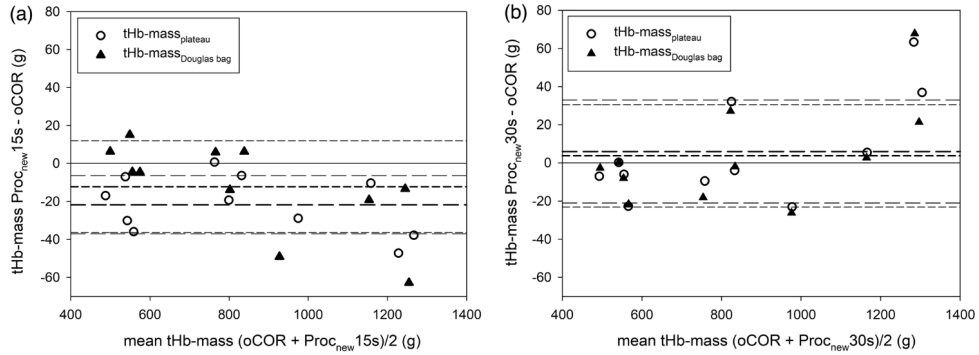

FIGURE 5 Bland–Altman plots for the comparison of tHb-mass values achieved from the new procedures (a, $Proc_{new}$15s; b, $Proc_{new}$30s) with the established CO-rebreathing method (cCOR). Long dashed lines represent the mean and SD of $tHb\text{-}mass_{plateau}$ (Hb-mass calculated with [COHb] 6–20 min after inhalation); short dashed lines represent the mean and SD of $tHb\text{-}mass_{DouglasBag}$ (tHb-mass calculated with [COHb] 20 min after inhalation and the CO volume collected in the Douglas bag)

introduces another source of inaccuracy. In the literature, there has also been extensive debate about the merits of having a higher [ΔCOHb%] versus the increased toxicity risk (Alexander et al., 2011; Garvican et al., 2010; Turner et al., 2014). [COHb] of up to 10% has been described without remarkable side effects in healthy subjects (Schmidt et al., 2020), but to our knowledge, it has never been studied in seriously ill patients. Because CO is endogenously produced and is actually considered for the treatment of various diseases (Motterlini & Otterbein, 2010), we are convinced that the increase in COHb by 4–5%, as achieved in our study, represents a reliable compromise balancing sources of error with minimal patient risk.

4.1 | Practical application

We hypothesize that this method might be used to diagnose and provide more information on the origin of anaemia in intensive care (Magee & Zbrozek, 2013) and the amount of blood loss during surgery (Shoemaker et al., 1996), as well as for distinguishing between dilutional anaemia and genuine anaemia in patients with heart failure (Miller & Mullan, 2015) and liver failure (Plumb et al., 2020). Here, it is of critical importance that the modified method has sufficient accuracy to reveal clinically relevant changes in tHb-mass and their contribution to changes in [Hb]. Although the reliability of the modified method was not explicitly determined in this feasibility study, the methodological error (typical error, TE; Hopkins, 2000) compared to the established method is 3.2% ($Proc_{new}$30s) or 3.5% ($Proc_{new}$15s). This is higher than the TE of the established CO rebreathing methods (TE 2.2%) but close to the TE of the gold standard methods using radioactive markers (^{51}Cr, 2.8%; Gore et al., 2005). Since even mild real anaemic states ([Hb] 11.2 g/dl) are associated with a reduction of at least ~15% tHb-mass (Wachsmuth et al, 2015), the accuracy of the method should be sufficient to distinguish anaemia due to reduced tHb-mass from that due to dilution. This contention is supported by Otto et al. (2017a) who describe two patients with liver disease who had identical tHb-mass (9.2 g/kg body mass), but with a normal [Hb] (16.1 g/dl) in one and dilutional anaemia ([Hb] 10.7 g/dl) in the other. They also describe two cases of heart failure in which the presence of a severe reduction in tHb-mass (5.2 g/kg) was reflected in a low [Hb] in one (6.9 g/dl), but masked by a contracted PV in another ([Hb] 10.7 g/dl) (Allsop et al., 1998; Otto et al. (2017a). In addition to the frequently occurring dilution anaemia, decompensated heart failure can also be associated with proportional increases in both tHb-mass and PV (Miller, 2016). In all these cases, the determination of the tHb-mass, also when using the modified method, enables a much more precise diagnosis.

The modification described here may permit the CO method to be used in ventilated patients. CO is applied to the inhalation path, and breathing is interrupted in the inhalation position for 15 or 30 s. The CO volume not absorbed by the patient can be determined either by collecting the entire expiratory air for a period of approximately 20 min or by continuous monitoring of the volume of the expired air and its CO concentration. These measurements are carried out until the CO is completely mixed in the blood and a blood sample for the determination of COHb is drawn.

The application of our approach to ventilated patients would offer possible clinical advantages. In most patients treated in an intensive care unit, the [Hb] drops significantly within a few days and transfusions are recommended when [Hb] reaches a threshold of 70 g/l (Watson & Kendrick, 2014) without excluding dilution. Indeed, there is no routine way in which to assess intravascular volume, with central venous pressure being a very poor guide indeed (De Backer & Vincent, 2018). In addition, blood and fluid loss during surgery are imprecisely measured and this, together with altered cardiovascular tone, and the variable administration of packed red blood cells and crystalloid/colloid solutions, makes determination of

intravascular volume (and of true Hb deficit) difficult. Our method may find application in all such situations. Nonetheless, the applicability and validity of this 'single breath method' remains to be validated in clinical circumstances.

In such clinical studies CO mixing time must be considered. It is prolonged in patients with polycythaemia (Wachsmuth et al., 2019) and heart failure (Ahlgrim et al., 2018), and perhaps also in other patient groups. This should, however, not be a major problem as the exhaled CO is collected for 20 min and a significant increase in mixing time can be tolerated if the individual COHb plateau is determined for each patient after inhalation of the CO. The use of the new method in patients with pulmonary diffusion disorders could be more problematic if sufficient CO cannot diffuse from the alveoli into the blood within 30 s. Higher CO doses may have to be used in such circumstances, but this can have an adverse effect on the accuracy of the test.

In healthy subjects, there is no risk of interrupting breathing for 30 s, and the risk can be classified as very low also in ventilated patients. Since the oxygen consumption during the 30-s breath interruption is only approximately 150 ml, the arterial O_2 saturation does not change during this period (Parkes et al., 2016); but in any case, it must be checked during and after the test. In severely anaemic patients, the test might be used with great caution after extensive validation.

5 | CONCLUSION

Using the single-breath method, tHb-mass and blood volume can be determined with approximately the same accuracy as that with established CO-rebreathing methods. We recommend that this method be developed further for use in ventilated patients, that is, patients in intensive care, patients undergoing major surgery, and patients with heart and liver failure.

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COMPETING INTERESTS

W.F.J.S. is a managing partner of the company 'Blood tec GmbH', but he is unaware of any direct or indirect conflict of interest with the contents of this paper. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

L.M.K., J.O.M.P., H.E.M., N.B.W. and W.F.J.S. were involved in the conception and design of the study, the acquisition of data, the analysis and interpretation of the data, and the drafting of the manuscript. S.H., J.S., S.B.K., J.M.O. and M.P.W.G. were involved in the acquisition of data, analysis and interpretation of data for the work, as well as in the critical revision of the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Glossary of Terms

ABP	Athlete's biological passport
ATP	Adenosine triphosphate
BV	Blood volume (ml)
CarO	Cardiac output
CBV	Circulating blood volume
CO	Carbon monoxide gas
COHb%	Carboxyhaemoglobin percentage
CKD	Chronic kidney disease
CLD	Chronic liver disease
CPET	Cardiopulmonary exercise test
EV	Erythrocyte volume
[Hb]	Haemoglobin concentration
tHb-mass	Total haemoglobin mass
HF	Heart failure
IBD	Inflammatory bowel disease
IBW	Ideal body weight
ID	Iron deficiency
IDA	Iron deficiency anaemia
IQR	Interquartile range
NHS	National health Service
NO	Nitric oxide
oCOR	Optimized Carbon Monoxide Rebreathing Method
oCOR1	Optimized carbon monoxide re-breathing method- Test 1
oCOR2	Optimized carbon monoxide re-breathing method- Test 2 (at least 24 hours from test 1)
PBM	Patient blood management
PCV	Packed cell volume
PolV	Polycythaemia vera
PROCnew	New modified procedure for measuring tHb-mass in ventilated participants
PV	Plasma volume
RCV	Red cell volume
T1	Test 1- first test of the day
T2	Test 2 (second test of the day after carbon monoxide clearance procedure)
UHS	University Hospital Southampton
WHO	World Health Organization
ΔCOHb%	Change in carboxyhaemoglobin percentage

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