INVESTIGATION OF THE DOSE AND TEMPORAL EFFECTS OF INTRAVENOUS IRON ON HUMAN CARDIOPULMONARY PHYSIOLOGY



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Dedicated to my parents

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My grandmother came to Oxford after the Second World War and worked as cleaner. She never would have dreamed that her granddaughter would one day study at Oxford University. The opportunities I have had in my life are because of the hard work and love of those before me. My parents, and younger brother James have offered unending love and support during my DPhil. There will never be enough words to thank my family.

ABSTRACT:

INVESTIGATION OF THE DOSE AND TEMPORAL EFFECTS OF INTRAVENOUS IRON ON HUMAN CARDIOPULMONARY PHYSIOLOGY

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Sustained hypoxia induces a progressive rise in pulmonary artery systolic pressure (PASP). Intravenous (IV) iron administered immediately prior to the hypoxic exposure attenuates this effect, suggesting that manipulation of iron stores may modify hypoxia-induced pulmonary hypertension. Not yet understood is the duration of effect of IV iron, or the dose-response relationship between clinical indices of iron status and modest loading of iron in non-iron deficient individuals. To address this, two placebo-controlled, double-blind, sustained isocapnic hypoxia studies were conducted in healthy iron-replete participants.

In Study A, 22 participants were randomised to a single intravenous bolus dose of iron as ferric carboxymaltose (FCM), or saline on day 1 and exposed to six hours of hypoxia on days 0, 1, 8 and 43. Following the administration of FCM the concentrations of ferritin and hepcidin plus transferrin saturation rose significantly ($p < 0.001$, $p < 0.005$, $p < 0.001$ respectively), and transferrin concentration decreased significantly ($p < 0.001$); changes that persisted for the study. FCM significantly attenuated the rise in PASP in response to hypoxia ($p < 0.001$), an effect that persisted at day 43.

In Study B, 18 participants were randomised to saline or 250 mg FCM, administered monthly for six months. To avoid iron overload, FCM was only administered to participants who had a ferritin < 300 μg/L and transferrin saturation < 45%. PASP was measured during eight hours of hypoxia, before and after the six month period. Serum ferritin concentration was robustly elevated by FCM by 0.21 μg/L/mg of iron delivered (95% CI: 0.15-0.26 μg/L/mg). Both iron dose and increase in ferritin concentration correlated with an attenuation in PASP rise with hypoxia ($p < 0.05$ and $p < 0.01$, respectively).

In summary, a bolus dose of iron resulted in an increase in iron indices and altered the cardiorespiratory response to hypoxia for at least 43 days. This was also achieved by incremental doses of iron that did not cause iron overload. The persistent effect of intravenous iron suggests that it may be of therapeutic benefit in some forms of pulmonary hypertension and other hypoxic cardiorespiratory diseases.

I am nothing more than a single narrow gasping lung, floating over the mists and summits.

Reinhold Messner, 1978.

Describing climbing Mt. Everest without oxygen.

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ABBREVIATIONS USED IN THIS THESIS

AHVR	Acute hypoxic ventilatory response
AMS	Acute mountain sickness
ARNT	Aryl hydrocarbon receptor nuclear translocator
BMP	Bone morphogenic protein
BMPR2	BMP receptor-type II
HbCO	Carboxyhaemoglobin
CHF	Chronic heart failure
CMS	Chronic mountain sickness
CO	Carbon monoxide
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CP	Chuvash polycythaemia
DFO	Desferrioxamine
DMT1	Divalent metal transporter 1
ELISA	Enzyme-linked immunosorbent assays
EPAS1	Endothelial PAS (Per-Arnt-Sim) domain-containing protein 1
EGLN1	Egl nine homolog 1
EPO	Erythropoietin
ET-1	Endothelin-1
FCM	Ferric carboxymaltose
FIH	Factor inhibiting Hypoxia-inducible factor
F_{IO_2}	Fraction of inspired oxygen

GDF 15	Growth differentiation factor 15
Hct	Haematocrit
HO-1	Haeme oxygenase-1
HFE	Haemochromatosis protein gene
Hb	Haemoglobin
HAPE	High altitude pulmonary oedema
HIF	Hypoxia-inducible factor
HIF-1α	Alpha subunit of hypoxia inducible factor 1
HIF-1β	Beta subunit of hypoxia inducible factor 1
HPV	Hypoxic pulmonary vasoconstriction
HRE	Hypoxia-response element
IL-6	Interleukin 6
IPH	Idiopathic pulmonary hypertension
IRE	Iron-responsive element
IRDA	Iron refractory iron deficiency anaemia
IRP	Iron-regulatory protein
IV	Intravenous
JAK/STAT4	Janus kinase/signal transducer and activator of transcription 4
MRI	Magnetic resonance imaging
N_2	Nitrogen
NHE1	Na^+/H^+ exchanger isoform 1
NTBI	Non transferrin bound iron
NYHA	New York Heart Association class
O_2	Oxygen

P_{AO_2}	Partial pressure of alveolar oxygen
Pa_{O_2}	Partial pressure of oxygen in arterial blood
P_{ACO_2}	Partial pressure of alveolar carbon dioxide
Pa_{CO_2}	Partial pressure of carbon dioxide in arterial blood
PASMC	Pulmonary arteriolar smooth muscle cells
PAS	Per-Arnt-Sim protein group
PASP	Pulmonary arterial systolic pressure
PDK	Pyruvate dehydrogenase kinase
P_{ETCO_2}	End–tidal partial pressure of carbon dioxide
P_{ETO_2}	End–tidal partial pressure of oxygen
PHD	Prolyl-hydroxylase domain
Pmax	Maximum pressure gradient across the tricuspid valve during ventricular systole
PVR	Pulmonary vascular resistance
RAP	Right atrial pressure
ROS	Reactive oxygen species
SMAD	Small mothers against decapentaplegic
SOCC	Store operated calcium channels
STEAP3	Six-transmembrane epithelial antigen of the prostate 3
sTfR	Soluble transferrin receptor
Tf	Transferrin
TfR	Transferrin receptor
TRPC	Transient receptor potential canonical
TWSG1	Twisted gastrulation
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

CHAPTER ONE:

GENERAL INTRODUCTION

1 Introduction

Iron and oxygen are abundant on Earth and are physiologically interdependent. This chapter will explore the nature of their relationship, including a review of the mechanisms of iron homeostasis, the functional effects of both iron deficiency and iron overload, the nature of the relationship between iron and oxygen, and how their homeostasis is intimately linked at the cellular, molecular, and systemic level. The background of human experimentation both at sea level and at high altitude are discussed, as it provides a framework for understanding human hypoxia physiology and led to the method of hypoxia experimentation that is employed in this thesis. The main aim of this work is to provide additional understanding as to the effects of altering iron levels on the human hypoxic response. Furthermore, the disease states that may benefit from these studies, where hypoxia plays a role and/or iron deficiency is endemic, will be explored.

1.1 Iron Homeostasis

Like oxygen, iron is required for cellular processes in most living species. Iron can exist in two states (Fe^{2+} or Fe^{3+}), which allows it to behave as both an electron donor and acceptor. It is required for basic cell processes, from oxygen transport to electron transfer, and cellular respiration. Though iron is abundant on Earth, it is predominately found in a form that is insoluble and poorly bioavailable. Importantly, it can be toxic in excess. Therefore, iron balance is tightly regulated in order to maintain levels for physiological function, whilst preventing tissue damage due to iron overload.

Adult men have 35-45 mg of iron per kilogram of body weight. Premenopausal women have lower iron stores due to recurrent blood loss through menstruation. The distribution of iron in the body is as follows: 65% is contained in haemoglobin, where it facilitates oxygen transport, 30–35% is stored in the liver, and 1–2% is found in the form of iron–sulfur clusters or haem where it has an important function in the mitochondrial respiratory chain complexes along with numerous other enzymatic actions (Finch, 1994; Ganz & Nemeth, 2012).

Dietary iron is absorbed in the duodenum, after reduction from its toxic ferric (Fe^{3+}) to its non-reactive ferrous (Fe^{2+}) state, usually at a= rate of 1-2 mg/day (Camaschella, 2015). The process is facilitated by duodenal cytochrome b, a ferric reductase (McKie *et al.*, 2001). Most of the iron in the body is recycled from senescent red blood cells as they are destroyed, and from the liver, macrophages and other tissues. Haemoglobin, in red blood cells, contains 2-2.5 g of iron, while in contrast blood plasma contains 2-3 mg of iron bound to transferrin. Transferrin is an 80 kDa plasma glycoprotein that is able to bind one or two ferric molecules and functions as a transporter of iron.

After absorption of dietary iron, the divalent metal transporter 1 (DMT1) transports iron across the apical membrane of the enterocyte. Iron then crosses the enterocyte to the basolateral membrane where it enters the blood stream via ferroportin, which functions as the sole iron exporter (Donovan *et al.*, 2005). Iron is subsequently loaded onto transferrin, which facilitates transport throughout the circulation to different tissues. Transferrin-bound iron is then taken up into various cells via the plasma membrane spanning transferrin receptor (TfR1), which allows for controlled entry of iron. Cells with a high turnover

exhibit a larger proportion of transferrin receptors; these include erythroid precursors and activated lymphocytes. When not immediately required, iron is stored intracellularly as ferritin, a large 24-subunit protein that can store 4500 atoms of iron in its spherical cavity. It is composed of H-ferritin and L-ferritin subtypes. Ferritin and transferrin maintain iron in its non-reactive, ferrous state thereby preventing free radical formation via the reaction between iron and hydrogen peroxide, known as the Fenton reaction (Andrews, 2008).

Daily losses of iron are 1-2 mg/day through desquamation of epithelial cells in the gut and skin, menstruation and through trivial bleeding. There is no dedicated pathway for iron excretion, so iron balance is determined by regulatory control at sites of iron absorption (intestine), recycling (reticuloendothelial system) and storage (liver) (Abboud & Haile, 2000; Donovan *et al.*, 2000; Nemeth *et al.*, 2004b). Regulatory control of iron occurs via the polypeptide hormone hepcidin, which opposes the action of ferroportin. Hepcidin is a 25-amino acid protein which is synthesised mainly in the liver. Regulatory feedback of hepcidin production comes from iron levels and from erythropoietic requirements for iron. Hepcidin mRNA in mice is induced by iron loading (Pigeon *et al.*, 2001) and inhibited by anaemia and hypoxia (Nicolas *et al.*, 2002). Loading with 65 mg of oral iron in humans results in a fivefold increase in hepcidin levels on the same day (Nemeth *et al.*, 2004a).

Hepcidin is transcriptionally regulated by iron levels. Changes in iron levels are sensed by two transferrin receptors accompanied by haemojuvelin, a soluble membrane-spanning protein. These changes activate the bone morphogenic protein (BMP) pathway, which utilises cytoplasmic SMAD (small mothers against decapentaplegic) proteins to then influence the production of hepcidin by hepatocytes (Wang *et al.*, 2005; Babitt *et al.*,

2006) (Figure 1.1). BMPs are a group of cytokines initially discovered due to their ability to produce bone and cartilage, but have since been implicated in a range of signalling processes including iron homeostasis.

Iron balance in a number of disease states can be explained by disturbances of the BMP/SMAD pathway and consequently changes in hepcidin levels. For example, hepcidin levels are increased by the inflammatory cytokine interleukin-6 (IL-6) via the Janus kinase/Signal transducer and activator of transcription signalling pathway (JAK/STAT4), explaining the elevation of hepcidin in inflammation or infection (Nemeth *et al.*, 2003; Nemeth *et al.*, 2004a). This may be an evolutionarily conserved mechanism to withhold iron from invading pathogens and preventing their replication either by locking away iron within cells or by blocking its excretion from hepatocytes. Hepcidin is cleared by the kidneys, and so it accumulates with consequent iron deficiency in renal failure (Zaritsky *et al.*, 2009; Rubab *et al.*, 2015). Of interest, hypoxia has been shown to decrease hepcidin levels which may be linked to the stimulation of erythropoiesis that occurs with hypoxic exposure (Nicolas *et al.*, 2002). Erythroferrone, a hormone produced by erythroblasts, which is reduced in stress erythropoiesis (Kautz *et al.*, 2014b), may provide the mechanism behind the erythroid stimulus in iron homeostasis, however these links are still being studied.

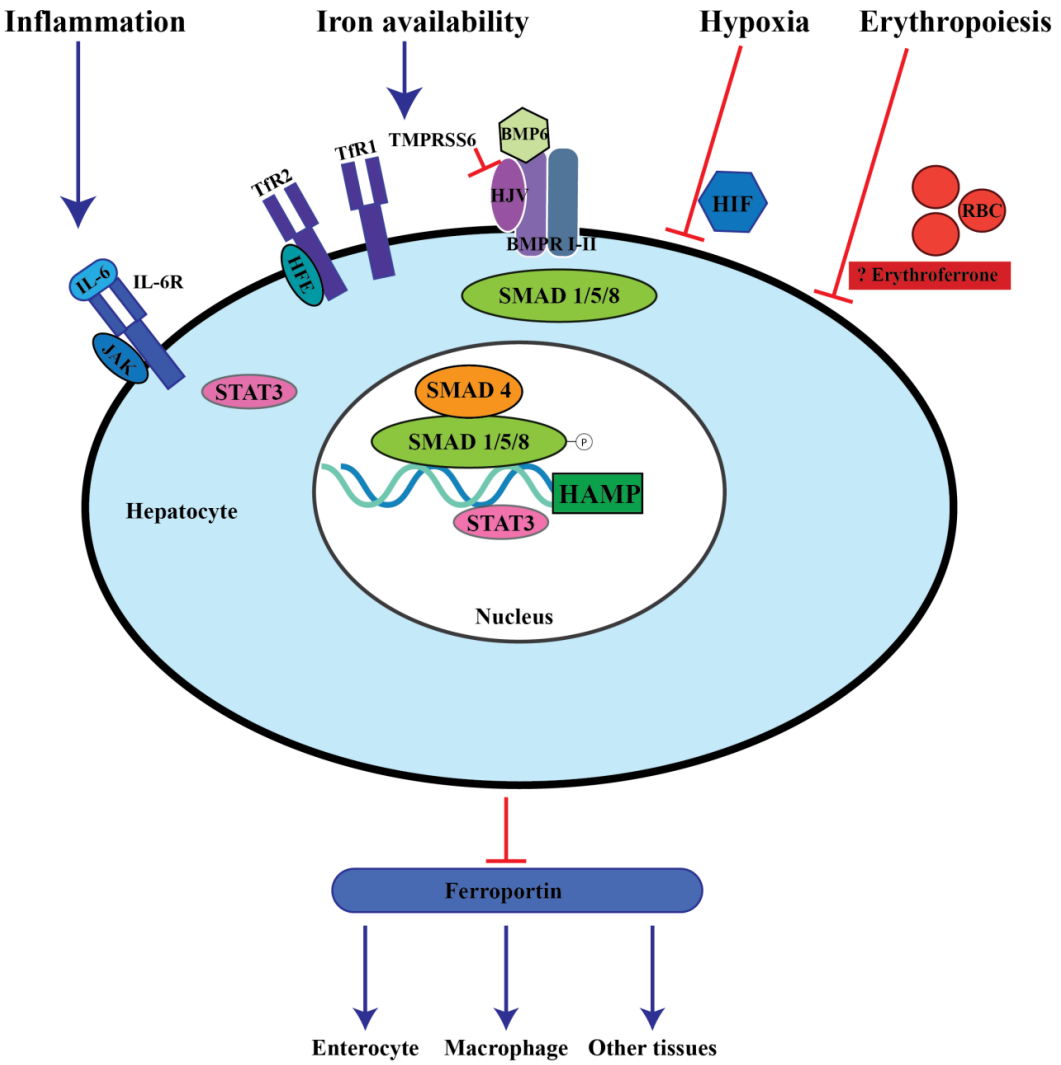

Figure 1.1 Hepcidin and iron balance. Hepcidin is transcriptionally regulated. Inflammation and iron availability increase hepcidin levels, whereas hypoxia and erythropoiesis inhibit hepcidin transcription. Changes in iron levels are sensed by two transferrin receptors accompanied by haemojuvelin. These changes activate the bone morphogenic protein (BMP) pathway, which utilises cytoplasmic SMAD (small mothers against decapentaplegic) proteins to then influence the production of hepcidin by hepatocytes. Hepcidin then opposes the action of ferroportin which functions to export iron.

BMP6, bone morphogenetic protein 6; BMPR I-II, bone morphogenetic protein receptor I-II; HFE, hemochromatosis protein; HIF, hypoxia inducible factor; HJV, haemojuvelin; IL6, interleukin 6; IL-6R, interleukin 6 receptor; JAK, Janus kinase; SMAD1/5/8, small mothers against decapentaplegic homologue 1/5/8 complex; SMAD4, small mothers against decapentaplegic homologue 4; STAT3, signal transducer and activator of transcription 3; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2. Adapted from *Rishi et al.*, (Rishi *et al.*, 2015).

1.1.1 Iron deficiency

Iron deficiency is endemic and has a significant disease burden, affecting more than two billion people worldwide (McLean *et al.*, 2009). More than a quarter of the world's population is anaemic, with one half of this attributable to iron deficiency anaemia. Iron deficiency can be the result of inadequate intake or absorption, or an increased loss of iron. Iron deficiency is associated with significant morbidity. It can cause cognitive impairment (Bruner *et al.*, 1996), worsen symptoms in heart failure (Anker *et al.*, 2009), correlate with worse outcomes in COPD (Nickol *et al.*, 2015), and is a common cause of restless leg syndrome (Connor *et al.*, 2011). There is also an increased risk of obstetric complications, including risk of preterm labour and low neonatal birth weight (Pavord *et al.*, 2012).

Iron refractory iron deficiency anaemia (IRIDA) is an autosomal recessive condition caused by mutations in the *TMPRSS6* gene resulting in iron deficiency that is unresponsive to oral iron (Du *et al.*, 2008; Finberg *et al.*, 2008; Heeney & Finberg, 2014). The expression of hepcidin is regulated by a type II transmembrane serine protease that is encoded for by *TMPRSS6,* so IRIDA patients have inappropriately elevated hepcidin levels. This is distinct from typical iron deficiency where patients have low or undetectable hepcidin levels that encourage increased uptake of iron from the gastrointestinal system. Another genetic cause of iron deficiency is a defect in the iron transporter DMT1 (Fleming *et al.*, 1997; Mims *et al.*, 2005). This genetic disease results in impaired iron absorption and utilisation. The diagnosis of either disease can be made by identification of the genetic defect in either the *TMPRSS6* gene (Finberg *et al.*, 2008) or in DMT1 (Mims *et al.*, 2005), which is less common. Some of these patients are partially responsive to oral iron, however, they often require intravenous (IV) iron to try to circumvent the effects of high

hepcidin concentration, and despite this, in some cases, they still only have partial correction of iron stores (Melis *et al.*, 2008).

Use of oral and IV iron supplementation has been shown to improve cognitive function (Bruner *et al.*, 1996; Murray-Kolb & Beard, 2007), reduce fatigue (Krayenbuehl *et al.*, 2011), increase exercise capacity and aerobic performance (Davies *et al.*, 1982) and improve heart failure symptoms (Anker *et al.*, 2009).

1.1.2 Iron overload

Iron overload states provide models for understanding defective regulatory pathways in iron balance. One example is haemochromatosis where there is accumulation of iron in tissues resulting in diabetes, cardiomyopathy, liver failure, and infertility (Bothwell & MacPhail, 1998; Tavill *et al.*, 2001). Haemochromatosis can be the result of defects at different sites in the regulatory pathway, including the classical haemochromatosis protein (HFE) gene mutation (Feder, 1999), but also mutations of haemojuvelin, transferrin receptor 2 (TFR2), and hepcidin (Andrews, 2008), which can all result in iron overload. Mouse models suggest that although the driver of the disease is both increased iron absorption in the intestine and release of iron from macrophages, the site of pathology is actually the hepatocytes themselves that fail to produce adequate hepcidin (Ahmad *et al.*, 2002; Spasic *et al.*, 2007).

Iron loading anaemias, such as thalassemia and sideroblastic anaemia, are also characterised by inappropriately low hepcidin levels. These are a spectrum of disorders in

which expansion in erythropoiesis results in a compensatory decrease in hepcidin and intestinal hyper-absorption of iron. In these disease states, iron overload is often exacerbated by multiple red blood cell transfusions. Recently, studies have implicated growth differentiation factor 15 (GDF15) and Twisted gastrulation (TWSG1) as hepcidin suppressors in thalassaemia, resulting in iron overload (Tanno *et al.*, 2007; Tanno *et al.*, 2009).

1.2 The multi-layered relationship between iron and oxygen

At the most basic cellular level, oxygen is vital for life. Hypoxia, the reduction in oxygen availability, can have a number of causes including: reduction in the oxygen tension of inspired air after ascent to high altitude, inadequate gas exchange in the lungs, or a reduction in tissue perfusion. Each individual cell has the ability to sense and respond to hypoxia to avoid damage. Iron is similarly vital for life, it is important in the reduction of oxygen, and also in the transport of oxygen to tissues. When present in excess quantities, both oxygen and iron are toxic, yet physiological function is impaired with inadequate quantities of either. On a systemic level, iron is a major modulator of the hypoxia inducible factor (HIF) pathway. Furthermore, many of the downstream target genes of this pathway are implicated in iron homeostasis. At a molecular level, iron and oxygen interact via the fine tuning mechanism of the iron-regulatory protein/iron-responsive element pathway. The recent discovery of an iron-responsive element (IRE) on the 5' untranslated region of HIF-2α mRNA (Sanchez *et al.*, 2007) suggests that the relationship may be even closer than once thought.

The link between iron bioavailability and cellular hypoxia signalling is likely to occur via the HIF family of transcription factors. HIFs are a member of the basic helix-loop-helix family with an amino terminal that is part of the Pert, Arnt, Sim (PAS) protein group (Wang *et al.*, 1995). HIFs function as heterodimeric transcription factors that have been evolutionarily conserved, as even the most basic organisms have the capacity to sense and respond to changes in oxygenation (Semenza, 2004). An operational HIF system has been identified in the nematode *Caenorhabditis elegans* (Epstein *et al.*, 2001). HIF operates at a cellular level, in individual organs, and constitutes a key component of the integrative response to hypoxia.

HIF was first discovered by its binding to the 3' end of the hypoxia response element (HRE) on the erythropoietin (EPO) gene (Semenza & Wang, 1992). EPO, a 34 kDa glycoprotein hormone produced by renal interstitial fibroblasts, is the driver of red blood cell production (Jelkmann, 1992). In the same way that hypoxia has a profound effect on EPO, it was found that HIF was closely regulated by oxygen tension. What was not expected at this stage was the multitude of downstream targets of HIF, extending well beyond erythropoiesis. The HIF family of transcription factors is now considered the master regulator of oxygen homeostasis.

1.2.1 Oxygen and iron homeostasis

1.2.1.1 The HIF pathway

HIF-1 exists as a heterodimer composed of both an alpha (α) and beta (β) subunit (Wang *et al.*, 1995). Both the HIF-1α and β subunits contain similar domains. The HIF-1β subunit

previously known as the aryl hydrocarbon receptor nuclear translocator (ARNT) dimerises with other aryl hydrocarbons. There are three known isoforms of the HIF-α subunit: HIF-1α, HIF-2α, and HIF-3α. HIF-2α is very similar to HIF-1α but is more selective in its pattern of expression (Wiesener *et al.*, 1998). Both HIF-1α and HIF-2α dimerise with HIF-1β. The isomers seem to have both overlapping and distinct roles with respect to gene targets. HIF-3α exists as multiple splice variants, some of which have inhibitory effects on HIF-1α and HIF-2α (Makino *et al.*, 2001). The full role of HIF-3α is still poorly understood.

HIF-α is oxygen dependent, and is continuously expressed and rapidly destroyed by proteolytic degradation under euoxic (or normal oxygen) conditions (Figure 1.2). In euoxia, HIF-α is hydroxylated by prolyl hydroxylases (PHDs), which require oxygen as a substrate and iron as a cofactor (Figure 1.2). Hydroxylation is oxygen sensitive and is essential for the degradation of HIF (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Both HIF-1α and HIF-2α contain an N-terminal and C-terminal oxygen-dependent degradation domain. HIF-1β is constitutively expressed and functions independently of oxygen tension. The oxygen-dependent degradation domains are at the site of the prolyl residues that undergo hydroxylation by the prolyl hydroxylase-domain proteins. They allow for the binding of the von Hippel-Lindau protein (VHL), which in turn targets the complex for poly-ubiquitination and proteasomal degradation (Maxwell *et al.*, 1999). In hypoxic conditions, HIF-α dimerises with HIF-β and the complex translocates to the nucleus (Figure 1.2). The new complex goes on to interact with HREs within a range of target genes. There are several hundred HIF target genes, including those involved in the control of metabolism (Papandreou *et al.*, 2006; Zhang *et al.*, 2008b), angiogenesis (Liao & Johnson, 2007),

immune evasion (Lukashev *et al.*, 2007), cell growth and apoptosis (Carmeliet *et al.*, 1998). HIF has been implicated in embryonic development (Dunwoodie, 2009), integrative physiology (Peyssonnaux *et al.*, 2007; Kaelin & Ratcliffe, 2008) and in certain disease states (Ang *et al.*, 1999; Raval *et al.*, 2005).

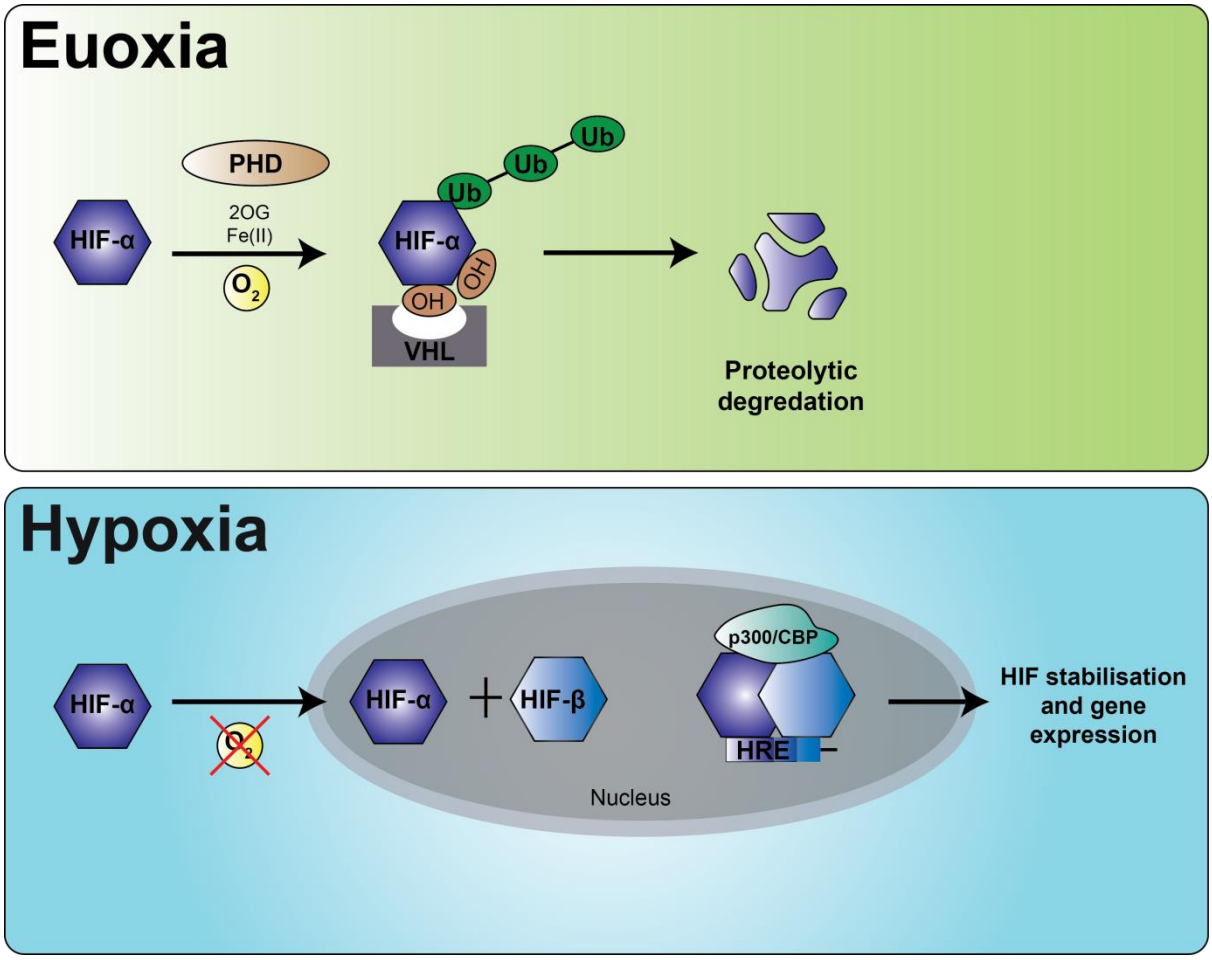

Figure 1.2 Regulation of HIF-α by hypoxia. In the euoxic state (top panel), HIF is hydroxylated by prolyl hydroxylases, which require oxygen as a substrate and iron as cofactor. Hydroxylation leads to its ubiquitination and subsequent proteolytic degradation. In hypoxia (bottom panel), HIF-α translocates to the nucleus where is dimerises with HIF-β leading to stabilisation of HIF and changes in downstream gene expression.
PHD, prolyl hydroxylase; OH, hydroxy group; 2OG, 2 oxoglutarate; VHL, Von Hippel-Lindau; p300/CBP, cAMP response element-binding protein coactivator family of proteins; Fe (II), ferrous iron; O_2, oxygen; Ub, ubiquitination; HRE, hypoxia response element.

Hypoxia is a major regulator of iron metabolism, and consistent with this is the fact that numerous transcriptional targets of the HIF axis are central to iron balance. These targets are involved in iron absorption, transport, oxidation, recycling, and export. Examples include decreased hepcidin and increased transferrin, transferrin receptor (TfR), haeme-oxygenase-1 (HO-1) and ferroportin, as shown in Figure 1.3.

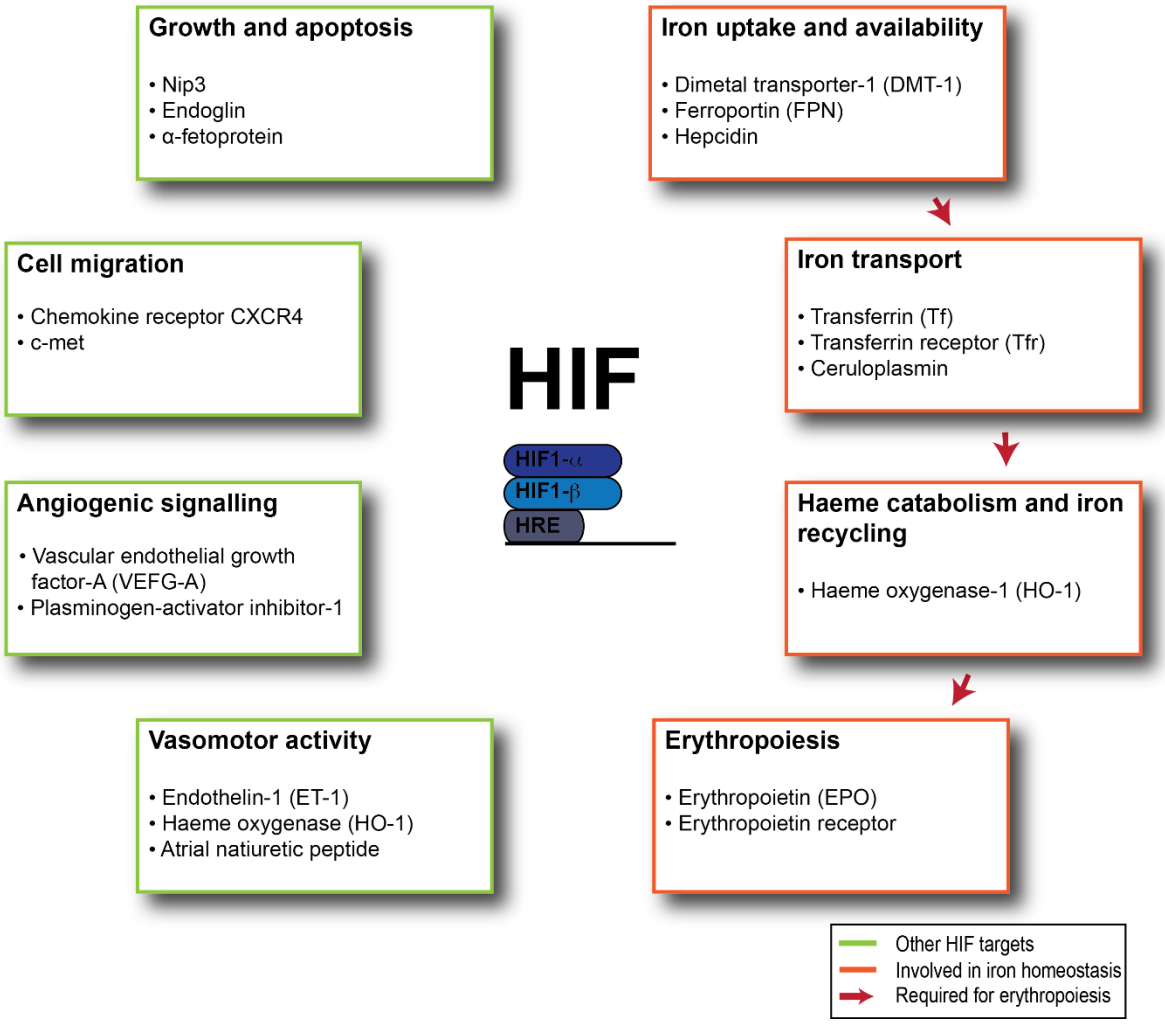

Figure 1.3 HIF target genes. HIF has a multitude of diverse transcriptional targets, such as those involved in iron homeostasis (orange boxes), those required for successful erythropoiesis (boxes linked with red arrows), and others including growth, apoptosis, and cell migration (green boxes). Adapted from Schofield and Ratcliffe (Schofield & Ratcliffe, 2004).

Three iron-dependent dioxygenases, the PHD iso-enzymes, catalyse the hydroxylation of HIF (Epstein *et al.*, 2001). The enzymes are termed PHD1, PHD2 and PHD3. They are respectively encoded by the *EGLN2* (Hs.515417), *EGLN1* (Hs.444450), and *EGLN3* (Hs.135507) genes. Activity of the PHD enzymes is dependent on oxygen, iron, ascorbate and 2-oxoglutarate. The PHD enzymes are extremely sensitive to even small changes in oxygen levels. In hypoxic conditions, HIF1-α is stabilised as the oxygen-sensitive PHD enzymes are unable to hydroxylate HIF. The PHD enzymes are also exquisitely sensitive to iron availability. On a cellular level HIF degradation is potentiated by increased iron levels (Knowles *et al.*, 2006) whilst in contrast, treatment with the chelator desferrioxamine (DFO) activates HIF-1α and its transcriptional target EPO with a time course similar to that of hypoxia (Wang & Semenza, 1993). The rise in EPO in response to DFO was also seen in human studies (Ren *et al.*, 2000). In oncogenic cells, downregulation of HIF has been demonstrated with the addition of iron or ascorbate to the cell media (Knowles *et al.*, 2003; Page *et al.*, 2008). Of note, *in vitro* studies of the PHDs may be confounded by the fact that the PHD enzyme activity varies depending on the PHD isoform (Epstein *et al.*, 2001). In addition, as these studies were done *in vitro* there may be an alternative explanation for the increase in PHD enzyme activity observed, such as free radical generation (Pan *et al.*, 2007). As yet, the mechanism for the altered activity of the PHD enzymes remains unclear.

Changes to the PHD enzymes result in an altered response in the murine model of chronic hypoxia. Mice exposed to chronic hypoxia (10% O_2) for seven days display an exaggerated hypoxic ventilatory response (Bishop *et al.*, 2013). Similarly, PHD $2^{+/-}$ mice exhibit an exaggerated ventilatory response to hypoxia with concurrent carotid body

hyperplasia, which demonstrates the importance and selectivity of PHD2 in the ventilatory response to hypoxia. Of note, pharmacological PHD inhibitors do not elicit the same response (Bishop *et al.*, 2013). This could be explained by the fact that PHD inhibitors are not isoform specific and therefore are likely to have many different, and possibly overlapping, targets. PHD inhibitors with improved selectivity for PHD subtypes are currently under development with areas of potential therapeutic benefit in mind. These include diseases where hypoxia may play a role, such as stroke, where administration could result in a reduction in ischaemic injury (Siddiq *et al.*, 2005), and improvement in left ventricular function after myocardial ischaemia (Nwogu *et al.*, 2001; Fielitz *et al.*, 2007). Another potential target is anaemia, where administration may stabilise HIF in euoxia, with a resultant increase in EPO expression (Yan *et al.*, 2010; Muchnik & Kaplan, 2011).

The expression of the HIF protein itself is determined by levels of O_2. In one study, HIF-1α and HIF-1β protein levels increased in cells exposed to 1% O_2, and decayed rapidly upon return to 20% O_2 exposure (Wang *et al.*, 1995). Correspondingly, HIF-1α and HIF-1β levels have been correlated with percentage of O_2 in human cells (Jiang *et al.*, 1996). There was a substantial increase in HIF protein concentration below 6% O_2 (42 mmHg), with half maximal induction at 1.5% and maximal levels at 0.5% O_2. Of interest, the higher concentrations of HIF-1α protein were seen at O_2 concentrations associated with ischaemic events *in vivo*. For example, O_2 concentrations can be as low as 3-4% in renal ischaemia (Schurek *et al.*, 1990; Epstein *et al.*, 1994), 2.5% in cerebral ischaemia (Whalen *et al.*, 1970) and in the heart, complete occlusion of the distal left anterior descending epicardial artery can result in near-zero O_2 concentrations (Rumsey *et al.*, 1994).

The differential expression of HIF based on oxygen tension plays a role not just in the local response but also in the systemic response to hypoxia *in vivo*. HIF-1$\alpha^{+/-}$ mice are indistinguishable from their wild type littermates under normoxic conditions (Yu *et al.*, 1999) but have significantly less marked changes on exposure to chronic hypoxia (10% O_2), including a reduction in polycythaemia, right ventricular hypertrophy and pulmonary hypertension. When compared to their wild type littermates, HIF-1$\alpha^{+/-}$ mice also lost more weight than HIF-1$\alpha^{+/+}$ mice, suggesting a role for HIF in the metabolic response to hypoxia, although glycolytic enzymes were not tested. HIF-1α dysfunction impairs ventilatory responses to both intermittent (Peng *et al.*, 2006) and chronic hypoxia, with associated defective O_2 sensing and/or signal transduction in the carotid body (Kline *et al.*, 2002). The carotid body is a peripheral chemoreceptor that functions by sensing changes in P_{O_2} predominantly via the type I, neural crest derived, glomus cells (Verna *et al.*, 1975; Whalen & Nair, 1975; Gonzalez *et al.*, 1994).

1.2.1.2 The iron-regulatory protein/iron-responsive element system

Molecular links between iron and oxygen function as additional sensing and fine tuning mechanisms to achieve homeostasis. Cellular iron homeostasis is achieved via cytoplasmic RNA-binding iron-regulatory proteins (IRPs). There are two forms of IRPs that are structurally similar to the aconitase family of enzymes, designated IRP1 and IRP2, which share sequence homology, but have different functions. The IRPs recognise and bind to iron-responsive elements (IREs) present in certain mRNAs in order to coordinate cellular iron availability (Figure 1.4). For example, in low iron conditions IRPs bind to the IREs within the 5'-untranslated region, which inhibits translation of the gene and therefore expression, as is the case with ferritin, HIF-2α and ferroportin (Rouault, 2006). In contrast,

IRPs binding to the IRE in the 3'-untranslated region of TfR and DMT-1 mRNA leads to mRNA stabilisation, thereby optimising iron uptake and reducing iron sequestration in low iron states. These interactions allow even small perturbations in iron or oxygen levels to be regulated at an individual cellular level.

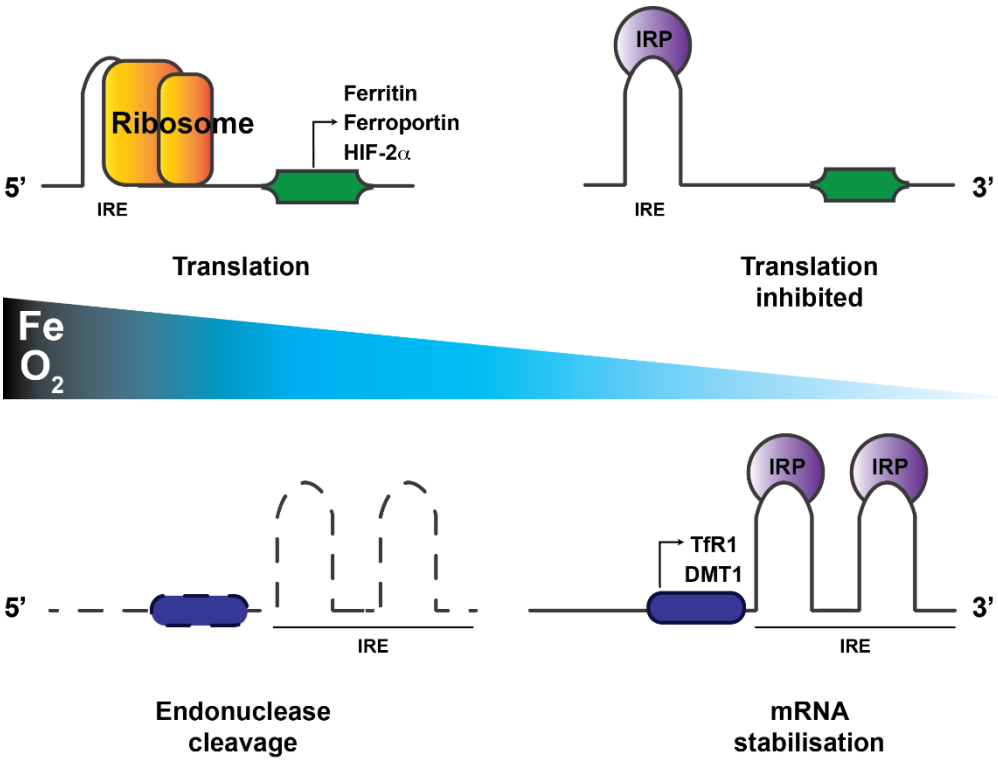

Figure 1.4 Iron-regulatory protein/iron-responsive element. In conditions of low iron, IRP binding activity increases. IRP binding to the IRE in the 5'-untranslated region inhibits translation of the gene, thereby decreasing its expression; such is the case for ferritin, ferroportin and HIF-2α. In contrast, if the IRP binds to the IRE in the 3'-untranslated region this leads to stabilisation of mRNA expression, which occurs in the cases of TfR1 and DMT1.

IRP, iron-regulatory protein; IRE, iron-responsive element; TfR1, transferrin receptor 1; DMT1, divalent metal transporter 1. Adapted from Mole (Mole, 2010).

Like iron, oxygen can influence the structure and function of the IRPs. At lower oxygen tensions, IRP2 has much greater functional activity in comparison to IRP1. Hypoxia keeps the iron-sulfur cluster of IRP1 intact, thus preventing it from interacting with IREs. In contrast, IRP2 levels are controlled by the E3 ubiquitin ligase complex containing the F-box/LRR-repeat protein 5 (FBXL5) protein, which targets IRP2 for ubiquitination and subsequent proteasomal degradation. FBXL5 has an iron and oxygen binding haemerythrin domain, and is dependent on both of these for its function (Salahudeen *et al.*, 2009), with resultant accumulation in iron and oxygen replete conditions, and degradation with iron deficiency and hypoxia.

Mice lacking copies of both IRP1 and IRP2 die at the blastocyst stage of development, thereby demonstrating the importance of the IRP/IRE system (Smith *et al.*, 2006a). IRP $2^{-/-}$ homozygous recessive mice develop anaemia and, in some studies, neurodegeneration (LaVaute *et al.*, 2001). Interestingly, mice with concomitant IRP $2^{-/-}$ and loss of one copy of IRP1 have a more profound anaemia and neurodegeneration (Smith *et al.*, 2004; Cooperman *et al.*, 2005). IRP1 does not appear to compensate for IRP2 loss thus reinforcing the importance of IRP2 in iron sensing, while IRP1 may have a vital function in erythropoiesis mediated by HIF-2α (Ghosh *et al.*, 2013).

An important discovery showed that HIF-2α, though not HIF-1α, has the classic step loop structure of an IRE on its 5'-untranslated region (Sanchez *et al.*, 2007). In iron deficiency, IRP1/IRE binding inhibits the translation of HIF-2α mRNA (Sanchez *et al.*, 2007; Zimmer *et al.*, 2008). HIF-2α plays a central role in erythropoiesis, and is selectively expressed and abundant in the kidney. When humans are exposed to hypoxia they develop an

erythropoietic response to increase the oxygen carrying capacity of the blood. One mechanism implicated in this response occurs via the stabilisation of IRP1, which leads to activation of HIF-2α and consequent erythropoietic expansion via EPO (Ghosh *et al.*, 2013). In the case of HIF-2α gain-of-function mutations, there is an increase in erythrocytosis linked to an upregulation of EPO (Scortegagna *et al.*, 2003b; Rankin *et al.*, 2007). Moreover, Tibetans demonstrate natural selection of HIF-2α to adapt to their low oxygen environments, resulting in lower haemoglobin concentrations when compared to Han Chinese (Beall *et al.*, 2010). The link between HIF-2α and iron utilisation during erythropoietic expansion has been defined (Anderson *et al.*, 2011). HIF-2α has also been implicated in the up-regulation of genes involved in duodenal iron absorption and resultant iron overload in a mouse model of haemochromatosis (Mastrogiannaki *et al.*, 2012). These findings, when taken together, show the fundamental role HIF-2α plays in erythropoiesis.

In summary, these findings suggest that the IRP/IRE system is an elegant post transcriptional fine-tuning mechanism for iron balance. This machinery allows for rapid adjustment and sensing of cytosolic iron concentrations, and appears to have a functional capacity influenced by both iron and oxygen levels.

1.3 Human hypoxia physiology

1.3.1 Oxygen and high altitude experimentation

Carl Wilhelm Scheele first discovered oxygen, but Joseph Priestley succeeded in publishing his findings first (Priestley, 1776). By placing a burning candle inside a sealed bell jar with an unfortunate rodent, Priestley clearly demonstrated the importance of

molecular oxygen for animal life in 1774. The oxygen was consumed by the candle, leading to the death of the rodent and a demonstration of the lethal effects of low oxygen levels.

The knowledge of altitude physiology dates back as early as 1644, when Torricelli proclaimed 'We live submerged at the bottom of an ocean of the element air' *'[Noi viviamo sommersi nel fondo d'un pelago d'aria elementare, la quale per esperienze indubitate si sa che pesa]'*(Loria & Vassura, 1919). French physiologist Paul Bert demonstrated that the deleterious effects of high altitude could be attributed to the low partial pressure of oxygen. Experimentation at high altitude helped to further our understanding of low oxygen states. The first scientific laboratories set up to study this were the Observatoire Vallot near the summit of Mont Blanc, France (4,350 m) and the Capanna Margherita on top of the Monte Rosa in Italy (4,550 m). The locations were not without risk, as shown by the first case of high altitude pulmonary oedema and the corresponding autopsy report being described at the Observatoire Vallot (Mosso, 1898).

The Pikes Peak laboratory outside Colorado Springs, United States (4,300 m) set a new standard for high altitude research in 1911. The expedition team was E.C. Schneider, Y. Henderson from the United States, J.S. Haldane, C.G. Douglas, from the University of Oxford, as well as M.P. Fitzgerald, who attended physiology lectures in Oxford despite the university being closed to women. The Pikes Peak laboratory was on a 'nice comfortable mountain' [Haldane 1911, quoted from (West, 2015)], in that it could be reached by railway, and was located in a hotel at the summit. The laboratory established many protocols for the future of high altitude experimentation. These protocols were used in

subsequent expeditions to Cerro de Pasco, Peru (1921-1922) and to Mt. Everest, Nepal (1960-1961).

The high altitude environment is an excellent model for understanding the human response to hypoxia. Regardless of altitude, the O_2 concentration, namely fraction of inspired O_2 (F_{IO_2}), remains constant; but it is actually the reduction in barometric pressure that results in a reduction in the partial pressure of oxygen (PO_2). Lowland residents ascending to altitude experience profound physiological effects in response to the reduction in PO_2.

A key limitation of the altitude model is that humans hyperventilate in response to a reduction in atmospheric PO_2, resulting in hypocapnia, or reduced carbon dioxide (CO_2) in the blood (Douglas *et al.*, 1913). The fall in P_{ACO_2} (alveolar CO_2 partial pressure) results in an increase in P_{AO_2} (alveolar O_2 partial pressure) as calculated by the alveolar gas equation (Fenn *et al.*, 1946; Curran-Everett, 2005). Changes in CO_2 can affect the pulmonary vascular response to hypoxia, with higher levels of CO_2 having a constrictor effect and lower levels causing a vasodilator affect (Balanos *et al.*, 2003). CO_2 levels can also affect ventilatory control via chemoreceptors. Human systemic responses to hypoxia can be induced in a specially designed hypoxic chamber, in which the partial pressure of individual gases can be controlled. Two such hypoxic chambers were an important part of the experimental methods employed in this thesis, which facilitated the delivery of isocapnic hypoxia, where the same level of CO_2 was maintained despite changes in ventilation, rather than the poikilocapnic hypoxia that would normally be seen at altitude. This is described further in Chapter Two.

1.3.2 Physiological responses to hypoxia

There are four main physiological responses to systemic hypoxia. These include changes in ventilation and cardiac output, a pulmonary vascular response and erythropoiesis. Initially, increases in ventilation and cardiovascular changes allow for improved oxygen delivery to tissues. Over a longer time course, haematological changes contribute to improve the transport of oxygen to the tissues.

Ventilation is an integral part of gas exchange, providing oxygen for metabolism and removing the by-product, CO_2. Ventilation is affected by the inspired partial pressure of both of these gases, and this control is mediated by both central and peripheral chemoreceptors. Central chemoreceptors are located within the medulla oblongata and respond to the changes in acid-base state of the cerebrospinal fluid (Mulkey *et al.*, 2004). Peripheral chemoreceptors are located in the carotid and aortic bodies, and are predominantly sensitive to changes in arterial oxygen tension, but also the partial pressure of CO_2 and acid-base state (Prabhakar & Semenza, 2012b). Acute hypoxia, (e.g. $Pa_{O_2} < 60$ mmHg) causes a rapid increase in minute ventilation, which is mediated by the peripheral chemoreceptors (Lenfant & Sullivan, 1971; Powell *et al.*, 1998). Ventilation increases in seconds, reaches a peak within minutes, termed the acute hypoxic ventilatory response (AHVR), and then declines towards pre-hypoxic levels over about 20 minutes. With sustained hypoxia, ventilation begins to rise again after an hour and then increases progressively for many days (Weil & Zwillich, 1976; Powell *et al.*, 1998).

Cardiac output is defined as heart rate multiplied by stroke volume. During hypoxia, cardiac output initially increases due to a rise in heart rate. After a period of days, cardiac

output then returns to normal values with heart rate remaining high, but with a reduced stroke volume (Vogel & Harris, 1967). Acute hypoxia leads to an increase in heart rate and peripheral vasodilation. Of note, there is no effect on systemic blood pressure. At altitude this increase in heart rate can persist for a period of several days (Vogel & Harris, 1967), and has been observed over a period of eight hours of hypoxia in chamber experiments (Dorrington *et al.*, 1997).

Oxygen transport is dependent on the erythropoietic system, so changes in oxygen levels can have profound effects on system components. EPO rises within 90 minutes of exposure to low oxygen (Eckardt *et al.*, 1989), peaks within two days and then declines over a period of one to two weeks (Abbrecht & Littell, 1972). Polycythaemia is a recognised feature of high altitude exposure. The concentration of haemoglobin, a metalloprotein composed of four iron-containing haem groups attached to a globin chain that is responsible for oxygen transport, and the associated haematocrit, the percentage by volume of red blood cells in blood, are both elevated within days of ascent to altitude (Richalet *et al.*, 1994). Moreover, red cell mass, a more accurate reflection of the total number of red cells in blood, can continue to rise for several months after hypoxic exposure and increase by up to 50% at 4,500 m (Reynafarje *et al.*, 1959).

The vascular bed of the human lung is unique, in that it constricts in response to hypoxia, rather than dilates. This hypoxic pulmonary vasoconstriction (HPV) results in a rise in pulmonary arterial systolic pressure (PASP). Although the exact mechanism is still debated, it is generally accepted that HPV is mediated by the pulmonary arteriolar smooth

muscle cells (PASMCs), which function to both detect hypoxia and mediate changes (Sylvester *et al.*, 2012).

1.3.3 The pulmonary vasculature

In the foetal circulation, the pulmonary vessels remain in a state of vasoconstriction to prevent blood flow through the non-ventilated lungs. At birth, lung inflation and associated oxygenation lead to vessel dilation. From this ontogenic beginning, it is interesting that the lungs are the only vascular bed in the body to constrict in response to hypoxia; most vascular beds dilate to increase perfusion of blood and therefore provide oxygen to tissues. The pulmonary vessels use vasoconstriction as a way of matching ventilation to perfusion in hypoxia. This function is evolutionarily preserved and is seen in mammals and to a lesser extent in reptiles (Skovgaard *et al.*, 2005), whereas fish have a parallel system of gills that mount a hypoxic response intrinsic to their arteries (Smith *et al.*, 2001).

As early as 1894, it was observed that there is an increase in pulmonary pressure in response to asphyxia (Bradford & Dean, 1894). Half a century later, von Euler and Liljestrand used hypoxic (10% O_2) and hypercapnic gas mixtures (von Euler & Liljestrand, 1946) on anaesthetised cats to describe the mechanics of HPV. A study in humans exposed to sustained hypoxia (eight hours) characterised the time course of the rise in pulmonary pressure and its consequent plateau during this period of sustained hypoxia as well as demonstrating a period of latency before the pressure returned to normal in euoxia (Dorrington *et al.*, 1997). This study also characterised the hypoxic priming of the pulmonary vessels where, with repeated exposure to hypoxia, there is a more substantial increase in HPV.

Oxygen sensing and consequent constriction has been attributed to the PASMC. Individual PASMCs have the capacity to constrict in response to hypoxia. In contrast, smooth muscle cells from other vessels such as the carotid arteries or even conduit pulmonary arteries do not constrict in response to a hypoxic stimulus (Madden *et al.*, 1992). Given that even one PASMC can contract, it follows that local areas of the lung can constrict in response to an isolated hypoxic stimulus, shunting blood to well-ventilated areas (Carlsson *et al.*, 1985).

For HPV to occur, a cascade of oxygen sensing, signal transduction and smooth muscle contraction takes place (Moudgil *et al.*, 2005; Sylvester *et al.*, 2012). The exact mechanisms behind this process are still poorly understood and are a subject of much research. It is known that HPV occurs as a consequence of an increase in intracellular calcium concentration via hypoxic regulation of transmembrane ion channels (Figure 1.5). It is believed that oxygen-sensitive voltage-gated potassium channels establish the resting membrane potential in PASMCs (Moudgil *et al.*, 2006). Low oxygen leads to inhibition of these channels, membrane depolarisation and an influx of calcium through voltage-gated calcium channels. In the PASMCs, as within other types of muscle cells, when intracellular calcium increases, the calcium binds to calmodulin, which activates the myosin light chain kinase and in turn causes a conformational change in myosin resulting in muscle contraction. Unlike other cells, PASMCs have variable sensitivity to calcium during hypoxia, due to post-translational modification of calcium sensors (Borman *et al.*, 2002).

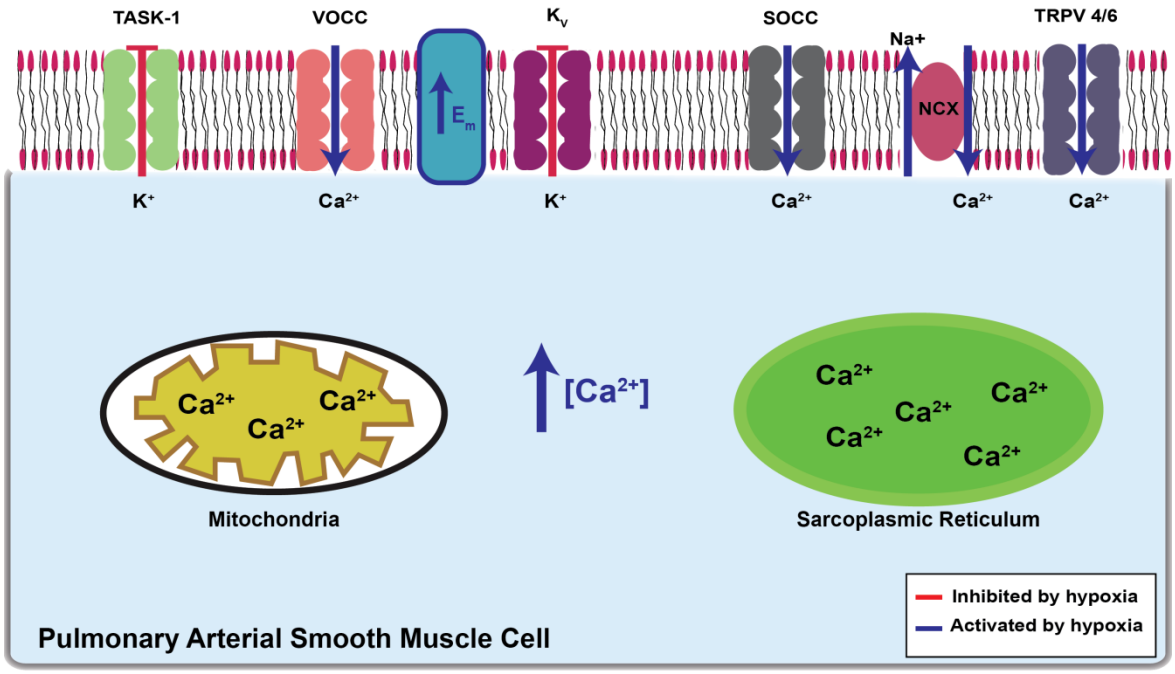

Figure 1.5 Suspected mechanism of hypoxic pulmonary vasoconstriction. When the PASMC is exposed to hypoxia, potassium channels such as K_v and TASK-1 are inhibited leading to depolarisation of the membrane (E_m), which in turn activates voltage-operated calcium channels such as VOCC. Many other channels including TRPV4/5 and NCX affect the membrane potential and facilitate influx of calcium. The opening of store operated calcium channels (SOCC) increases the concentration of intracellular calcium. PASMC contraction is triggered by the rise in [Ca^{2+}]. Red lines indicate channels inhibited by hypoxia, and blue lines those activated by hypoxia.
TASK-1, TWIK-related acid-sensitive channel-1; VOCC, voltage-operated Ca_2 channel; K_v, voltage-dependent K channel; NCX, Na-Ca exchanger.

PASMCs have the capacity to function as both oxygen sensors and effectors (Madden *et al.*, 1992; Liu *et al.*, 2003). The exact nature of the 'oxygen sensor' for HPV is a source of much debate, although the mitochondria are a likely candidate, as the site of cellular respiration and determination of the redox state of the cell. Intact endothelium plays an important role in the effector function of PASMCs; removal of endothelium results in submaximal HPV with sustained hypoxia (Ward & Robertson, 1995; Dipp *et al.*, 2001).

The PASMC responds to hypoxia with an initial rapid increase in vascular tone, which is endothelium-independent, with a second, slower sustained increase in tone that is endothelium-dependent (Ward & Robertson, 1995).

The global HPV time course is biphasic like that of the individual PASMC. This has been demonstrated invasively using pulmonary artery catheterisation (Dorrington *et al.*, 1997) and non-invasively with Doppler echocardiography (Talbot *et al.*, 2005). There is an initial phase with an acute rise in pressure that plateaus at five minutes, and a second, latent phase which begins at around 40 minutes and consists of a progressive rise in pulmonary pressure. It has been postulated that the second phase is mediated by changes in gene expression that could occur via the HIF transcription factor pathway (Talbot *et al.*, 2005).

The duration of hypoxic exposure is important for HPV. When the pulmonary vasculature is exposed to a short hypoxic stimulus, PASP quickly returns to pre-hypoxic levels in euoxia. However, if the hypoxic exposure lasts more than ~40 minutes (the first phase of HPV), it takes longer for PASP to return to baseline (Dorrington *et al.*, 1997; Balanos *et al.*, 2002; Smith *et al.*, 2008). Exposure that lasts for days to weeks results in subsequent pulmonary vascular remodelling, such that supplemental oxygen will not reduce the rise in PASP (Groves *et al.*, 1987).

Innervation of the pulmonary circulation is via both adrenergic and cholinergic fibres (Sylvester *et al.*, 2012). Despite this, neither the acute nor sustained hypoxic pulmonary vascular response is affected by complete autonomic blockade, as seen in a study where

participants were exposed to hypoxia after the ganglion blocker trimetaphan was administered (Liu *et al.*, 2007). It follows, therefore, that HPV is determined by intrinsic properties of the pulmonary vasculature.

Sustained HPV is modulated by a number of vasoactive substances. Some of these are vasodilatory, including nitric oxide, which is important in the resting vascular tone in a neonate (Nelin & Dawson, 1993; Domkowski *et al.*, 1995), and prostacyclin. It is believed however, that greater influence is exerted by endothelium-derived contracting factors (Sylvester *et al.*, 2012). Of these, endothelin-1 (ET-1) is important, as its blockade has been shown to prevent or attenuate HPV (Jones & Morice, 1998; Goirand *et al.*, 2003). It is believed that it is the action of these factors in concert, which mediates the acute rise in pulmonary arterial pressure, as well as longer term pulmonary vascular remodelling with hypoxic exposure (Barbera *et al.*, 2003). Evidence of the role of these mediators is seen in the lungs of patients with pulmonary hypertension, where there are elevated levels of ET-1 (Giaid *et al.*, 1993), and reduced levels of endothelial nitric oxide synthase and prostacyclin (Christman *et al.*, 1992).

HIF-1α and HIF-2α may both play a role in HPV (Figure 1.6), where HIF-1α has many downstream targets and HIF-2α is more selective (Loboda *et al.*, 2010). For example, when the lungs are exposed to hypoxia, HIF-1α protein is expressed in all cell types (Yu *et al.*, 1998), whereas HIF-2α appears to only be expressed in the type II pneumocytes and vascular endothelium (Wiesener *et al.*, 2003). HIF-2α is widely expressed in the lungs

and has a non-redundant and complementary role to that of HIF-1α (Wiesener *et al.*, 2003).

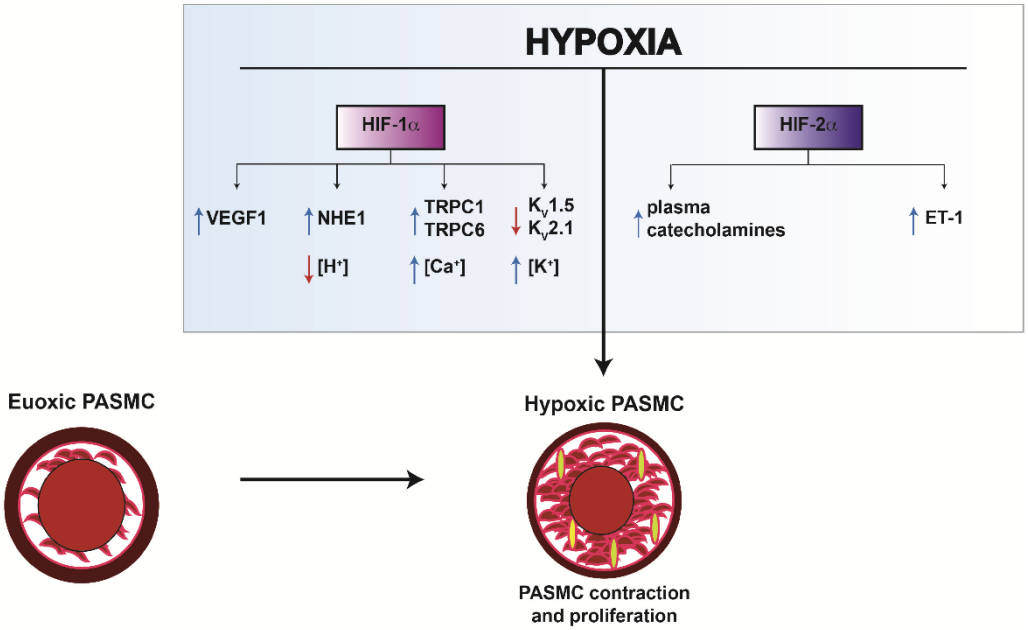

Figure 1.6 HIF targets involved in hypoxic pulmonary vasoconstriction. Pulmonary vasculature constricts in response to hypoxia as a way of matching ventilation with perfusion (top of figure). Exposure to a hypoxic stimulus stabilises HIF-1α and HIF-2α resulting in changes in a number of genes including those that play a role in HPV such as ion channels (NHE, TPRC, K_v), endothelin derived contracting factors (ET-1), and angiogenic signalling factors (VEGF), which facilitate PASMC contraction and proliferation.
NHE, Na^+/H^+ exchanger; TRPC1 and 6, transient receptor potential canonical 1 and 6; K_v, voltage-dependent potassium channel; ET-1, endothelin-1; VEGF, vascular endothelial growth factor.

HIF-1α may utilise feed-forward mechanisms to sustain HPV by incorporating vasoactive mediators (Pisarcik *et al.*, 2013). In a study where rat PASMCs were treated with ET-1 and exposed to prolonged hypoxia, ET-1 administration resulted in an increase in the levels of HIF-1α via both the upregulated synthesis of HIF-1α mRNA and downregulation of PHD2 mRNA and protein expression (which is known to degrade HIF-1α). Interestingly,

there were increased levels of pulmonary ET-1 and plasma catecholamines present in wild type mice, but not in HIF-2$\alpha^{+/-}$ mice, suggesting that these may be targets of HIF-2α (Brusselmans *et al.*, 2003). Despite this, HIF-1α has been shown to play a role in the pathogenesis of pulmonary hypertension via altered expression of genes known to affect the PASMC resting potential, such as increased expression of Na^+/H^+ exchanger isoform 1 (NHE1)(Shimoda *et al.*, 2006), genes affecting the voltage-gated potassium channels (Whitman *et al.*, 2008), and the transient receptor potential canonical (TRPC) family, including both TRPC1 and TRPC6 (Wang *et al.*, 2006).

A reduction in the degree of right ventricular hypertrophy and pulmonary hypertension is seen in HIF-1$\alpha^{+/-}$ mice compared with wild type mice upon exposure to hypoxia (Yu *et al.*, 1999). By way of contrast, loss of IRP1 results in translational de-repression of HIF-2α, which manifests as splenomegaly, extra-medullary haematopoiesis, and pulmonary hypertension (Ghosh *et al.*, 2013; Wilkinson & Pantopoulos, 2013). In addition, IRP1$^{-/-}$ mice fed a low iron diet develop more severe pulmonary hypertension and polycythaemia than controls (Ghosh *et al.*, 2013). In contrast, HIF-2$\alpha^{+/-}$ mice were found to be immune to the development of pulmonary hypertension, as well as right ventricular dysfunction, upon exposure to sustained hypoxia (Brusselmans *et al.*, 2003).

1.3.3.1 HPV is modulated by iron status

Intravenous iron has a role in modulating HPV, and this may be HIF-related. Our laboratory has previously shown that prior infusion of IV iron abrogates the rise in PASP that is typically seen upon exposure to eight hours of hypoxia (Smith *et al.*, 2008).

Moreover, prior iron infusion appears to affect the second, latent phase of HPV that occurs after 40 minutes, rather than the initial rise. This finding may well be due to the HIF transcriptional family, with increased iron availability resulting in HIF degradation via the iron and oxygen dependent HIF hydroxylases. In keeping with this, infusion of DFO appears to increase PASP with a similar time course to that of hypoxia (Balanos *et al.*, 2002), while EPO is induced by DFO on exposure to hypoxia, again with a similar time course (Ren *et al.*, 2000). There are, however, several alternative explanations for this. These include an increased labile iron pool or an increase in reactive oxygen species (ROS) generation as a consequence of iron infusion.

Scurvy, a disease of severe ascorbate deficiency, causes spontaneous pulmonary hypertension that is reversible after correction of ascorbate levels (Kupari & Rapola, 2012). Ascorbate, a known modulator of HIF, was studied further to better elucidate the role of the HIF axis in HPV (Talbot *et al.*, 2014), the hypothesis being that loading with ascorbic acid may produce similar affects to iron loading. Iron infusion resulted in the abrogation of PASP rise by a magnitude of ~50%, reproducing the earlier work from the same laboratory, but there was no significant difference seen in PASP rise with prior ascorbate loading (Talbot *et al.*, 2014). The possible explanations for this are that either the HIF pathway is not responsible for the damping down of HPV with iron, or ascorbate itself does not contribute to this mechanism via the pathway.

At altitude, IV iron infusion reduces the increase in pulmonary pressure that occurs during hypobaric hypoxia, whereas reduction in iron levels (using progressive venesection) causes an increase in pulmonary artery pressure (Smith *et al.*, 2009). This could produce an

improvement in Acute Mountain Sickness (AMS) symptoms, with individuals who have been pre-treated with iron reporting lower Lake Louise scores, a measure of AMS-related symptoms, than control participants (Talbot *et al.*, 2011).

1.4 Disturbances of the HIF/PHD/VHL oxygen sensing pathway

Genetic variations in the HIF axis provide an insight into the activation of HIF and its downstream targets. Through comparison of the different diseases and models, the effect of activating or silencing various components of the pathway can be better elucidated. Examples of this include arrested embryonic development, Chuvash polycythaemia, HIF-2α gain of function, classic von Hippel-Lindau disease, and whole-genome studies of high altitude populations. One question that remains is whether biological factors, such as iron bioavailability, may influence the relationship between oxygen tension and HIF activation.

1.4.1.1 HIF and embryonic development

Oxygen functions as a morphogen during development via the HIF pathway as embryogenesis occurs at oxygen concentrations between 1-5% (Dunwoodie, 2009). Complete deficiency of HIF-1α in embryos results in cardiac and vascular malformations and eventual lethality on embryonic day 10.5 (Iyer *et al.*, 1998; Yoon *et al.*, 2011). Deletion of HIF-1α within individual organ systems also causes distinct pathology. For example, deletion of HIF-1α can selectively impair cartilage development (Schipani *et al.*, 2001), bone development (Wang *et al.*, 2007) and innate immunity (Zinkernagel *et al.*, 2007). It is no surprise then that certain disease states, such as impaired wound healing, infection and malignancy, may involve dysfunction of the HIF axis.

Disruption of HIF-2α leads to embryonic lethality generally between embryonic day 9.5 and 13.5. However, unlike HIF-1$\alpha^{-/-}$ mice, HIF-2α disruptions are associated with a diverse range of phenotypic pathology. Of note, disruption of HIF-2α often leads to cardiopulmonary manifestations. Some HIF-2$\alpha^{-/-}$ mice die of bradycardia and a reduction of catecholamine function (Tian *et al.*, 1998), whereas others die as neonates due to impaired surfactant production and lung maturation (Compernolle *et al.*, 2002), or die several months after birth due to multi-organ failure (Scortegagna *et al.*, 2003a).

1.4.1.2 Chuvash Polycythaemia

Chuvash polycythaemia (CP) is an autosomal recessive disorder characterised by homozygosity for a C598T mutation in the VHL gene, resulting in reduced affinity of VHL for hydroxylated HIF. This leads to a modest stabilisation of HIF under euoxic conditions and subsequent upregulation of HIF target genes (Ang *et al.*, 2002). The same genetic mutation is present in both humans and mice with CP. Characterised as a primary polycythaemic disorder, it follows that mortality is often associated with thrombotic complications. The phenotypic abnormalities of the IRP1$^{-/-}$ mice (Ghosh *et al.*, 2013; Wilkinson & Pantopoulos, 2013), with associated activation of HIF-2α, are reminiscent of those of CP where HIF is stabilised in euoxic conditions (see 1.2.1), suggesting that this model carries through to human integrative pathology. The purported link in mammalian biology, however, has yet to be elucidated. To date, it has been shown that the polycythaemia is driven by inappropriately elevated EPO levels (Hickey *et al.*, 2007), and HIF target genes including HIF-2α, vascular endothelial growth factor (VEGF), glucose uptake transporter 1, ET-1, and aldolase C, which are all upregulated in CP (Gordeuk *et al.*, 2004; Bushuev *et al.*, 2006; Hickey *et al.*, 2007).

The cardiorespiratory manifestations of stabilisation of HIF in CP are of particular interest for this project. Mice with CP demonstrate elevated basal ventilation in euoxia and an exaggerated AHVR when exposed to hypoxia (Slingo *et al.*, 2014). This response is the initial increase in ventilation upon exposure to hypoxia, and is mediated by glomus (type I) cells in the carotid body. Interestingly, when carotid body immunohistochemistry was performed in mice with CP, marked hyperplasia of type I cells was seen (Slingo *et al.*, 2014).

In human studies, CP patients had a lower baseline Pa_{CO_2} suggesting an altered set point for respiratory drive and increased basal ventilation (Smith *et al.*, 2006b). These patients are known to have a mild degree of pulmonary hypertension at rest (Bushuev *et al.*, 2006). Such findings are likely to be related to the activation of HIF even in euoxia. With a functioning HIF/VHL axis, HIF is normally rapidly degraded in euoxia. With even mild hypoxia, CP patients developed a marked increase in both PASP and ventilation. These effects become more substantial with increasing levels of hypoxia (Smith *et al.*, 2006b). This may correlate with cellular studies where it was shown that there was differential HIF expression based on oxygen concentration (Jiang *et al.*, 1996). Together, these studies suggest that the phenotypic manifestations of CP demonstrate that HIF activation, even in euoxia, produces an altered physiological threshold for a cardiorespiratory response, promoting pulmonary vasoconstriction and hyperventilation.

1.4.1.3 HIF-2α gain of function

Whilst HIF-1α and HIF-2α are conserved at the protein level, and both dimerise with HIF-β, they appear to have different targets. This is elegantly demonstrated in the subtle differences in the HIF-2α gain of function mutation in comparison to CP. Mice with HIF-2α gain of function mutations develop pulmonary hypertension (Gale *et al.*, 2008; Tan *et al.*, 2013). The genetic mutation for HIF-2α gain of function is the same in mice and humans. In human studies, HIF-2α gain of function results in increased erythrocytosis linked to an upregulation of EPO (Scortegagna *et al.*, 2003b; Rankin *et al.*, 2007). A small group of patients, each possessing a HIF-2α gain of function mutation, were found to have high baseline PASP measurements and an exaggerated pulmonary vasoconstriction in response to moderate hypoxia (Formenti *et al.*, 2011). Patients with HIF-2α gain of function mutations exhibited similar baseline ventilation and a similar increase in ventilation in mild and moderate hypoxia when compared to healthy controls (Formenti *et al.*, 2011). Unlike the CP patients, they demonstrated less sensitive ventilatory and pulmonary vascular responses to hypoxic stimulus. Finally, they were observed to have less marked metabolic responses to exercise than those seen in CP patients. This is of interest because it shows the specific function of HIF-2α in the pulmonary vascular response to hypoxia. Moreover, it suggests that CP is a more global model of HIF dysregulation and that this may also involve HIF-2α.

1.4.1.4 Von Hippel-Lindau disease

Von Hippel–Lindau disease is a hereditary cancer syndrome characterised by both benign and malignant tumours. Tumours form only after a second somatic mutation leading to two defective copies of VHL (Kim & Kaelin, 2004; Kaelin, 2007). The most common tumours

are retinal and central nervous system haemangioblastomas, renal cell carcinomas, renal cysts and phaeochromocytomas.

Malignancy is not the main manifestation of CP or HIF-2α gain of function mutations although cases with malignancy have been reported (Favier *et al.*, 2012). In contrast, in classic VHL disease the striking polycythaemia and cardiorespiratory changes seen in other disruptions of the axis do not seem to occur (Formenti *et al.*, 2011) (discussed in 1.4.1.2, 1.4.1.3).

1.4.1.5 Genetic adaptation studies

High altitude populations provide insights into adaptive and maladaptive changes that occur in response to living long-term in a hostile environment. Tibetans have the longest history of living at altitude, with approximately 25,000 years at an average barometric pressure of 500 mmHg (Aldenderfer, 2011), where barometric pressure at sea level is 760 mmHg. On the Tibetan Plateau, at this average barometric pressure, the PO_2 is ~ 66% that of sea level, and despite the challenges of hypobaric hypoxia the Tibetan population seem to have adapted in a way that allows them to thrive. Compared to Andean or Han Chinese counterparts, Tibetans have increased ventilation (Hackett *et al.*, 1980), lower haemoglobin concentrations (Beall *et al.*, 2010) and lower prevalence of chronic mountain sickness (Moore *et al.*, 2000). Tibetans have also been shown to have a lesser degree of resting pulmonary hypertension and hypoxic vasoconstriction (Groves *et al.*, 1993; Petousi *et al.*, 2014) as well as minimal long term changes seen in the lungs including pulmonary vascular remodelling and endothelial changes (Gupta *et al.*, 1992). Advances in genetic studies have provided some explanation for this phenotype and have implicated HIF in the

process. Genome-wide studies have shown that in Tibetans there is an increased natural selection for *EPAS*1 (which encodes for HIF-2α) (Beall *et al.*, 2010), and *EGLN*1 (which encodes for PHD2) (Simonson *et al.*, 2010).

There is a great deal of phenotypic variation in the human response to hypoxia. This is demonstrated both in individual organ systems and whole systems biology. Examples include substantial individual variation in EPO (Richalet *et al.*, 1994), ventilation (Hirshman *et al.*, 1975) and pulmonary vascular response (Groves *et al.*, 1993), which is of particular note in the case of individuals susceptible to high altitude pulmonary oedema, as will be discussed later (Dehnert *et al.*, 2006) (see 1.5.1.2). Moreover, there seems to be an absence of correlation between measures of ventilatory acclimatisation and pulmonary vascular response (Wood *et al.*, 2008; Fatemian *et al.*, 2016). There is currently no reliable indicator for how a subject will handle ascent to altitude (Bartsch & Swenson, 2013), nor to determine which patients may fare better with an equivalent hypoxic insult.

1.5 Clinical applications of iron supplementation in hypoxia

Tissue hypoxia is a common end point of a myriad of disease processes. For example, in the case of chronic lung disease long-term hypoxia leads to pulmonary vascular remodelling and endothelial changes resulting in pulmonary hypertension (Barbera *et al.*, 2003), which is associated with significant morbidity and mortality. There are many potential therapeutic opportunities that could be gained by finding the link between oxygen, iron and the pulmonary vascular response. A number of trials are currently investigating whether improving an individual's iron status might be a way of modulating pulmonary hypertension of various aetiologies (Rhodes *et al.*, 2011b; Howard *et al.*, 2013). Of particular interest to this thesis are: chronic obstructive pulmonary disease, pulmonary hypertension, heart failure and high altitude pulmonary oedema, which are conditions where both iron deficiency and hypoxia are important in the disease process and clinical outcomes.

Iron deficiency is prevalent in chronic obstructive pulmonary disease (COPD), and this disease carries a significant world-wide health burden. Patients with iron deficiency were found to be more hypoxaemic than iron-replete individuals (median resting SpO_2 92% vs 95%, $p < 0.001$); interestingly this result was independent of haemoglobin concentration (Nickol *et al.*, 2015). This study also showed that patients with iron deficiency had a trend towards having both more frequent exacerbations and poorer exercise tolerance. Pulmonary hypertension often occurs in COPD patients, and is associated with a worse outcome (Chaouat *et al.*, 2008), and this is closely linked with hypoxaemia (Kent *et al.*, 2011).

Pulmonary hypertension has been classified into five main groups by the World Health Organisation according to aetiology: pulmonary arterial hypertension, pulmonary hypertension due to left heart disease, pulmonary hypertension due to chronic lung disease, chronic thromboembolic pulmonary hypertension, and pulmonary hypertension of unclear mechanism (Simonneau *et al.*, 2013). Idiopathic pulmonary hypertension (IPH) is rapidly progressive and often fatal. Iron deficiency affects up to 30-70% of patients with IPH (Ruiter *et al.*, 2011). Therefore, IPH may provide some mechanistic insights into the link between iron balance and pulmonary hypertension overall.

Patients with IPH and iron deficiency showed no improvement in iron status with oral iron supplementation, determined by small changes to serum iron levels, and with some patients showing no alteration to ferritin levels (Ruiter *et al.*, 2011), while intravenous iron may be of benefit (Howard *et al.*, 2013). This is likely to be due to a link between inflammation and hepcidin. BMP/SMAD signalling is a mechanism responsible for iron sensing and feedback for hepcidin expression. Interestingly, the expression of BMP receptor-type II (BMPR2), which is a receptor kinase that binds BMP, is reduced in all IPH patients (Atkinson *et al.*, 2002), loss-of-function mutations in BMPR2 have been reported in up to 10-20% of IPH cases. BMP6 is considered the most important BMP for hepcidin signalling (Andriopoulos *et al.*, 2009). One study of a cohort of patients with IPH showed increased BMPR2 knockdown in cells with associated increased BMP6–stimulated hepcidin expression (Rhodes *et al.*, 2011a), with most patients in the group having abnormally high hepcidin levels even in the presence of iron deficiency. However, some patients in the cohort had negligible or absent hepcidin levels, suggesting that other factors may also be involved in this process. The exact role of BMP in the pathogenesis of IPH, and whether it drives iron deficiency in this cohort, is yet to be elucidated. The above

findings suggest that iron deficiency is common in IPH patients and appears to impair functional status, with IV iron being a reasonable treatment strategy to restore iron levels, and potentially attenuate pulmonary pressures.

1.5.1.1 Heart failure

Iron deficiency is common in heart failure and, independent of anaemia, may result in worse symptoms and increased rates of hospitalisation (Anker *et al.*, 2009). The FAIR-HF trial undertaken by Anker and colleagues used modest values to determine iron deficiency, these being a ferritin level of < 100 μg/L, or between 100 and 299 μg/L if the transferrin saturation was < 20%. This definition is based on evidence that there is a poor correlation between serum ferritin concentration and iron stores seen in bone marrow aspirates in heart failure patients (Nanas *et al.*, 2006). It is likely that inflammatory mediators present in these patients result in higher ferritin levels despite them being functionally iron deficient (Nanas *et al.*, 2006). FAIR-HF patients were given infusions of ferric carboxymaltose until they were iron-replete. Patients randomised to the iron group showed an improvement in their New York Heart Association class (47% NYHA class I and II at week 24 compared with 30% for placebo 95% CI, 1.55 to 3.71), improved symptoms as measured by the Patient Global Assessment and an increased distance achieved during the six minute walk test. The limitation of this study is that it focused on symptoms whereas physiological end-points such as ejection fraction or PASP were not measured. Another study demonstrated that correction of iron deficiency lead to improvements in left ventricular systolic and diastolic diameters, as well as left ventricular function as measured by echocardiography (Toblli *et al.*, 2015b).

1.5.1.2 High Altitude Pulmonary Oedema

High altitude pulmonary oedema (HAPE) is non-cardiogenic and is associated with dyspnoea, fatigue, pink frothy sputum and poor gas exchange. If left untreated HAPE carries a mortality of 50% (Bartsch & Swenson, 2013). Individuals susceptible to HAPE possess an exaggerated pulmonary vascular response to acute hypoxia (Maggiorini *et al.*, 2001). Iron status may also be linked to the pathogenesis of HAPE. As discussed earlier, prior iron loading reduces the rise in PASP seen with ascent to altitude (Smith *et al.*, 2009). Whether iron status itself is contributory to the development of HAPE is unclear. A retrospective study of a high altitude cohort did not link iron status to pulmonary pressure, but that HAPE susceptible individuals have an unexpected increase in hepcidin on the second night after ascending to an elevation of 4559 m, but not the first (Altamura *et al.*, 2015). The heterogeneity in the hepcidin response is reminiscent of that of IPH. In this study, HAPE susceptible individuals with high hepcidin levels had a correspondingly high level of IL-6, a known mediator of inflammation.

Other studies have shown that inflammation may not be linked to the pathogenesis of HAPE. In one such study, bronchoalveolar lavage fluid showed no elevations in pro-inflammatory cytokines, eicosanoids or nitric oxide metabolites in individuals prior to, or at the onset of HAPE symptoms (Swenson *et al.*, 2002). However, inflammatory cells may be present in patients with well-established HAPE (Schoene *et al.*, 1986). The link between hepcidin, iron and PASP may be confounded by other factors. A previous study from our laboratory showed that the known hypoxia-induced decrease in hepcidin was not linked to iron status (Talbot *et al.*, 2012). It can be surmised that iron status may be

involved in the development or prevention of HAPE, but hepcidin may not be a good marker for this as it is influenced by numerous factors.

1.6 Summary

Iron and oxygen homeostasis are intimately linked. Both iron deficiency and hypoxia result in similar patterns of altered gene expression and cellular function (Peyssonnaux *et al.*, 2008; Mole, 2010). Changes in iron status can alter the human physiological response to low oxygen (Smith *et al.*, 2008) and there is the potential to use this therapeutically. Hypoxia is involved in many disease processes, such as heart failure and pulmonary disease, and is seen in a number of diverse settings, including chronic illness, high altitude, and critical care. To date, there has been sparse literature to answer pragmatic questions such as defining the dose(s) of iron necessary for a therapeutic response, or to determine the dose(s) that should be given for maximum effect, and the duration of such effect. The two studies conducted for this thesis aimed to answer these questions.

1.6.1 Aims of this thesis:

Study A:

1) To determine the time course, in healthy humans, of the effect of IV iron on the pulmonary vascular response to hypoxia over six weeks.
2) To determine the time course, in healthy humans, of the effects of IV iron on haematological parameters and iron status over six weeks.

Study B:

1) To determine a dose for IV administration of iron that will cause a mild elevation of iron indices and is therefore practical in a health care setting.
2) To determine the effects of supra-therapeutic iron levels on haematological parameters, iron haematinics, and a downstream marker of hypoxia inducible factor i.e. EPO.
3) To determine whether smaller, incremental iron doses influence the pulmonary vascular response after a period of sustained hypoxia.

CHAPTER TWO:

METHODOLOGY

2 Experimental Methods

The experiments were conducted with the purpose of measuring the human systemic and pulmonary vascular responses to a hypoxic environment. The principal experimental techniques used to control respiratory gases and record cardiopulmonary responses during hypoxic challenges are described in this methodology. The ideal experiments would employ continuous control and measurement of Pa_{O_2}, but even with invasive techniques this is not possible. In addition, hypoxia stimulates an increase in respiratory rate with resultant hypocapnia. The combined hyperventilation and hypocapnia are variables which make results difficult to interpret. In order to examine the specific response to hypoxia, without confounding by other gases, both Pa_{O_2} and Pa_{CO_2} need to be actively controlled.

In healthy humans, alveolar partial pressure of oxygen ($P_{A_{O_2}}$) can be calculated using the alveolar gas equation (Fenn *et al.*, 1946; Curran-Everett, 2005). The $P_{A_{O_2}}$ is assumed to be in equilibrium with the arterial partial pressure of oxygen (Pa_{O_2}), depending on the A-a gradient. The A-a gradient in a young adult non-smoker is between 5-10 mmHg. A further assumption is that end-tidal partial pressures of gases can be a representation of alveolar partial pressures. There are some confounders in this assumption including anatomical and alveolar dead space. Despite this, using these assumptions, the end-tidal partial pressures of oxygen ($P_{ET_{O_2}}$) and carbon dioxide ($P_{ET_{CO_2}}$) can be used as indirect measures of Pa_{O_2} and Pa_{CO_2}. Gas control can be achieved in humans through manipulation of $P_{ET_{O_2}}$ and $P_{ET_{CO_2}}$ while at the same time monitoring the composition of inspired gas (Howard *et al.*, 1995).

2.1 Sustained hypoxic exposure

2.1.1 Techniques for sustained hypoxic exposures in humans

Two identical custom-built chambers (Bassaire, Southampton, United Kingdom) were used to expose human participants to an extended period of normobaric hypoxia. These chambers have been used to control gas composition and achieve sustained hypoxic exposure in many previous studies in the laboratory and the technique is well validated (Howard *et al.*, 1995; Howard & Robbins, 1995). The chamber used in the experiment with associated gas supply and control, as well as safety mechanisms, can be seen in Figure 2.1 and will be discussed in detail in this chapter.

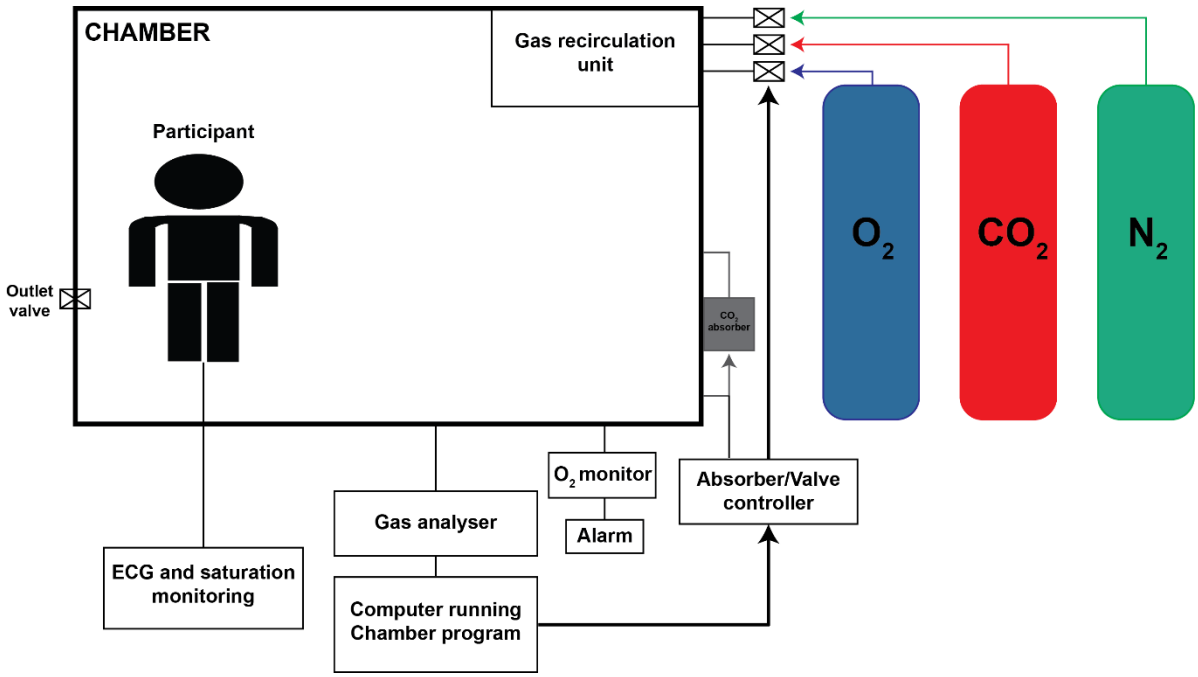

Figure 2.1 Chamber used for sustained hypoxic exposure. Two purpose-built chambers were used to expose participants to sustained hypoxia (six hours in Study A, eight hours in Study B). Control over gas composition was achieved by measuring and adjusting gases every five minutes, resulting in desired $P_{ET}O_2$ and $P_{ET}CO_2$. O_2, CO_2 and N_2 were all supplied externally to the chamber.

The chambers are airtight rooms measuring 8.6 m^3 (length: 2.3 m, width 1.7 m, and height 2.2 m). Participants were permitted to move freely inside and were allowed brief toilet breaks outside the chambers. During their period in the chamber, participants would read, watch television and study. Participants were prevented from sleeping during the experiments due to associated changes in ventilation. In Study A the participants underwent six hours of sustained hypoxia, and in Study B they underwent eight hours. For each participant the $P_{ET}O_2$ was set at 55 mmHg and the $P_{ET}CO_2$ was clamped at the participant's baseline for the duration of the hypoxic exposure.

Desired $P_{ET}O_2$ and $P_{ET}CO_2$ were achieved through manipulation of the composition of the gas using the specially designed system (Howard *et al.*, 1995). Gas control within the chamber was tightly regulated with gas composition being measured and adjusted every five minutes. At each interval, the average end-tidal values for $P_{ET}O_2$ and $P_{ET}CO_2$ over the prior three minutes were compared with the desired values. Accordingly, inspired gas composition was adjusted by opening valves for oxygen (O_2), carbon dioxide (CO_2) and nitrogen (N_2) that allowed a calculated duration of flow of each gas at a known rate (O_2: 105.5 $1.min^{-1}$, CO_2: 85.5 $1.min^{-1}$ and N_2: 882.1 $1.min^{-1}$).

2.1.1.1 Gas supply and control

The O_2, CO_2, N_2 (BOC Manchester, United Kingdom) and air were all supplied and installed externally to the chamber in cylinders. CO_2 and N_2 were supplied as liquids. O_2 was supplied as a compressed gas. CO_2 and N_2 were vaporised by passive warming. Measurements of inspired and end-tidal gases were sampled breath-by-breath, with

participants wearing nasal cannulae connected to a gas analyser (Datex Normocap 200 Oxy, GE Healthcare, United Kingdom). A heated humidifier (Paykel HC150, Fisher, Loughborough, United Kingdom) was used for the humidification of each gas. When the CO_2 concentration needed to be reduced in the chamber, it was removed from the chamber via a soda lime absorber at a flow rate of 1200 l.min^{-1}.

2.1.1.2 Monitoring and manipulation of gas composition

Chamber is a custom-written program used for gas control and recording data that was run on a desktop computer alongside the chamber. This program was written using National Instruments Labview 5.1 (National Instruments Corp. Ltd., Newbury, Berkshire, United Kingdom), and uses a National Instruments PCI-6713 data acquisition card, a National Instruments PCI-6712 analogue output card, external instruments and interface writing. It was used for the sampling and recording of breath-to-breath inspired and expired values. It contains a forcing function to maintain desired end-tidal values that are determined by the experimental protocol. Adjustments of chamber gas composition are determined from the Lloyd-Cunningham equation, which models the ventilatory response of a single participant in response to changing compositions of O_2 and CO_2 (Lloyd *et al.*, 1958).

Adjustments of chamber gas composition are determined by first calculating ventilation associated with the current (VE_{old}) and desired (VE_{new}) $P_{ET}O_2$ and $P_{ET}CO_2$.This equation looks at the particular ventilatory response of a single participant in response to changing levels of both $P_{ET}O_2$ and $P_{ET}CO_2$ (Howard *et al.*, 1995). The new inspired partial pressures were then calculated and the corresponding gases subsequently injected or removed from the chamber as appropriate.

Reductions in CO_2 were made from the calculation of the volume that needed to pass through the soda lime absorber ($V_{absorber}$). This is calculated from the following equation:

$$V_{absorber} = \frac{V_{chamber}}{0.9997} \log e \frac{(1-F_{ICO2\ (old)})}{(1-F_{ICO2\ (new)})} \quad (1)$$

The constant 0.9997 is derived from the amount of CO_2 in the air. $V_{chamber}$ is the volume of the chamber. F_{ICO_2} is the fraction of inspired CO_2. Reductions in O_2 are calculated from the appropriate amount of N_2 that needs to be injected into the chamber (VN_2). This is calculated from the following equation:

$$VN_2 = V_{chamber} \log e \frac{(1-F_{IO2\ (old)})}{(1-F_{IO2\ (new)})} \quad (2)$$

2.1.1.3 Monitoring oxyhaemoglobin saturation and heart rate

A pulse oximeter was used to measure arterial oxyhaemoglobin saturation, (Ohmeda Biox 3740, Hartfield, United Kingdom) with a finger probe (OXY-F4-H finger sensor, GE Healthcare). The oximeter contains a light source and photodetector capable of transmitting to a monitor (Mannheimer, 2007). The probe contains light emitting diodes which shine two alternating lights of different wavelengths through the tissue at 660 nm and 940 nm. The principle behind the apparatus is that oxyhaemoglobin absorbs more light at 940 nm (infrared), but less light at 660 nm (visible red light) than deoxyhaemoglobin. There is also a phase where no light is emitted before the light emittance cycle continues again. The photodetector readings during the zero light emittance phase are used as a baseline to subtract from maximum absorption readings, thus correcting for non-pulsatile tissues and natural light. This provides the pulsatile nature of the pulse oximeter, and the

saturation readings can therefore be assumed to be arterial. By the same process the pulse rate is also monitored through detecting the attenuation of light caused by pulsatile arterial blood flow. Heart rate was measured using a three lead electrocardiogram (Micromon 7142 B ECG, Kontron Medical).

2.1.2 Safety during sustained hypoxic exposures

At all times participants were closely monitored, and were able to communicate any problems to a medical professional outside the chamber. The chamber system was calibrated before each experiment. An example of a participant taking part in the experiment and lying within the chamber is seen in Figure 2.2. As mentioned, arterial oxyhaemoglobin saturation and pulse rate were monitored via pulse oximetry, and audio-visual alarms sounded if arterial oxyhaemoglobin saturation fell below 85%, or pulse rate exceeded 100 beats per minute. Respiratory rate, inspired and expired breath volumes, $P_{ET}O_2$ and $P_{ET}CO_2$ were monitored via a combination of the Chamber program and specialised nasal cannulae. Audio-visual alarms would also sound if there was a significant pause or slowing of respiratory rate, or if inspired or expired breath volumes exceeded pre-set safety limits. At least one medically trained individual was in the room at all times. If two chambers were run simultaneously, two medical officers were present. A fully stocked resuscitation trolley, equipped to the United Kingdom Resuscitation Council minimal equipment guidelines, as well as an automated external defibrillator was kept within the laboratory. A source of 100% O_2 was available in the experimental area in the event of any complication, and both the resuscitation trolley and the O_2 source were routinely checked.

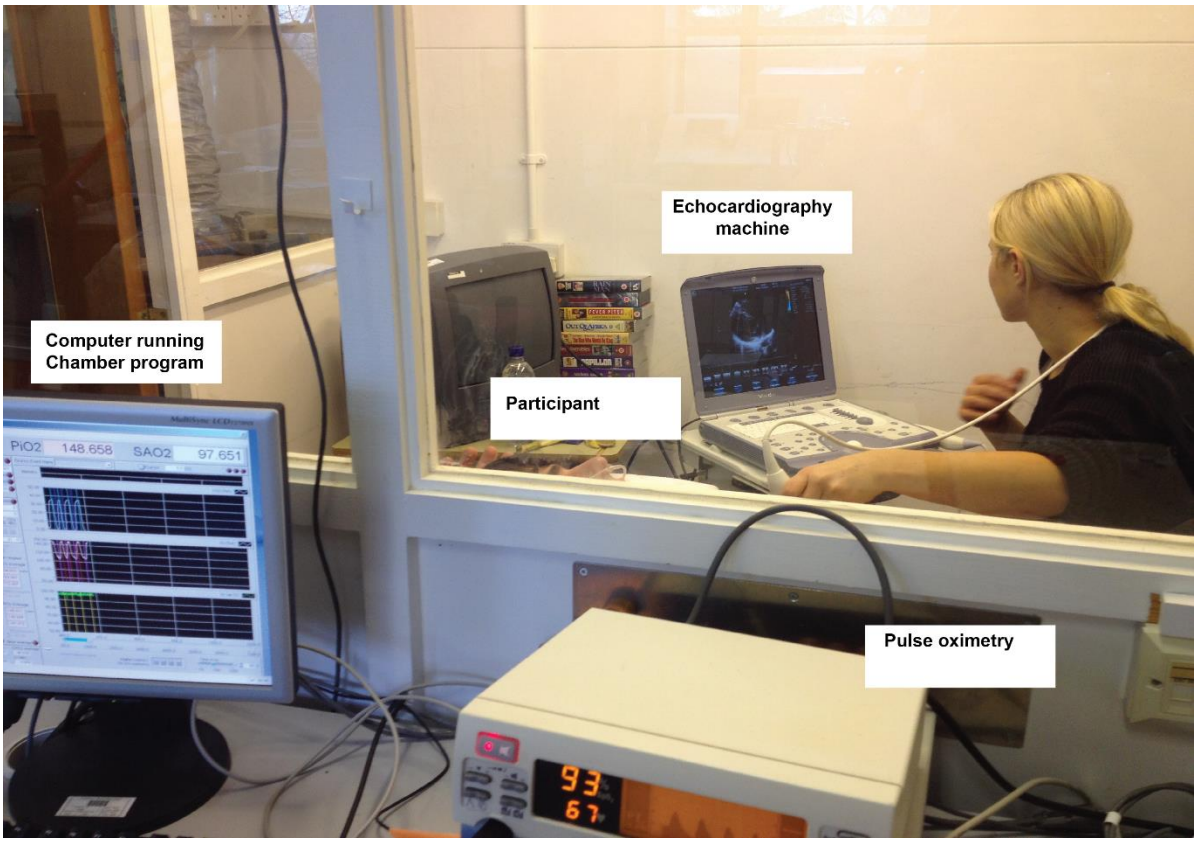

Figure 2.2 Example of a participant inside the hypoxic chamber. The participant is sitting comfortably inside the chamber while an investigator performs an echocardiogram. All participants were monitored with continuous electrocardiography and pulse oximetry.

2.2 Echocardiography

Echocardiography, the technique of cardiac ultrasound, is one of the most widely used cardiac investigations for the diagnosis and management of valvular heart disease, congestive cardiac failure and cardiomyopathies (Douglas *et al.*, 2011). It is a non-invasive, widely practiced means of assessing cardiac structure and function. Ultrasound is based on the concept of acoustic impedance, representing the passage of sound waves from one material to another.

Doppler echocardiography is an extension of the principle of ultrasound, and depends on fundamental principles relating to frequency and motion. In 1843, Christian Johann Doppler proposed that the frequency of light emitted by stars in motion would vary depending on their movement relative to Earth. This principle of 'frequency' can be applied to direction of the flow of a fluid, for example flow of blood through valves in the heart. Blood flowing towards a transducer has a higher frequency, or positive frequency shift, and conversely blood flowing away is regarded as having a negative frequency shift.

The Bernoulli principle is derived from the law of conservation of energy and looks at the relationship between velocity and pressure of inviscid flow of a fluid between two points. This concept of pressure and flow across a narrowing led to the development of non-invasive cardiac imaging for valvular lesions using the Doppler principle (Lindstrom & Edler, 1969a, b). The modified Bernoulli equation calculates the pressure gradient across an orifice. The equation uses three assumptions: that the velocity before the orifice is low, the orifice is short in length, and the ultrasound beam is well aligned with the flow. The original Bernoulli equation is:

$$\Delta P_{Pa} = \frac{1}{2}\rho v^2 \quad (3)$$

Where ΔP_{Pa} is the pressure across the orifice in Pascals, ρ is the density of blood in $kg.m^{-3}$ and υ is the flow velocity in $m.s^{-1}$. In order to apply this equation to clinical practice, the equation is modified to assume that the density of blood is ρ =1067 $kg.m^{-3}$ and the pressure units are converted from pascals to mmHg to facilitate clinical interpretation where 1 mmHg = 133.32 Pa. The resultant equation is known as the modified Bernoulli

equation and used commonly in echocardiography (Skjaerpe & Hatle, 1981; Yock & Popp, 1984). The modified Bernoulli equation is given as:

$$\Delta P_{max} = 4v^2 \quad (4)$$

With the modified Bernoulli equation, pulmonary arterial systolic pressure can be determined by measuring the velocity of a regurgitant jet across the closed tricuspid valve. A trivial degree of tricuspid regurgitation is common in healthy individuals (Sahn & Maciel, 1988; Berger *et al.*, 1989) and can be used to assess pulmonary arterial pressure in human physiology studies.

The ultrasound machines used for the purpose of this study were Vivid I and Vivid q (GE Healthcare, Buckinghamshire, United Kingdom) with an S4 two-dimensional transducer (2-4 MHz). A three-lead electrocardiograph monitor was used to record heart rate. Data obtained were recorded onto a computer disc and analysed retrospectively in a systematic, blinded manner. To determine the degree of tricuspid regurgitation, flow across the tricuspid valve was visualised in the apical four-chamber view. The probe was placed over the apex of the heart, at the fifth intercostal space in the mid-clavicular line. Individuals without a clear jet of tricuspid regurgitation were excluded from the study. The Doppler beam was then correctly aligned and a continuous-wave spectral analysis with a sweep speed of 50 $mm.s^{-1}$ was recorded. This can be seen Figure 2.3. One blinded investigator obtained the echocardiographic images used for PASP measurements and analysed the data. A second blinded investigator separately analysed a sample of the images to check consistency of the analysis.

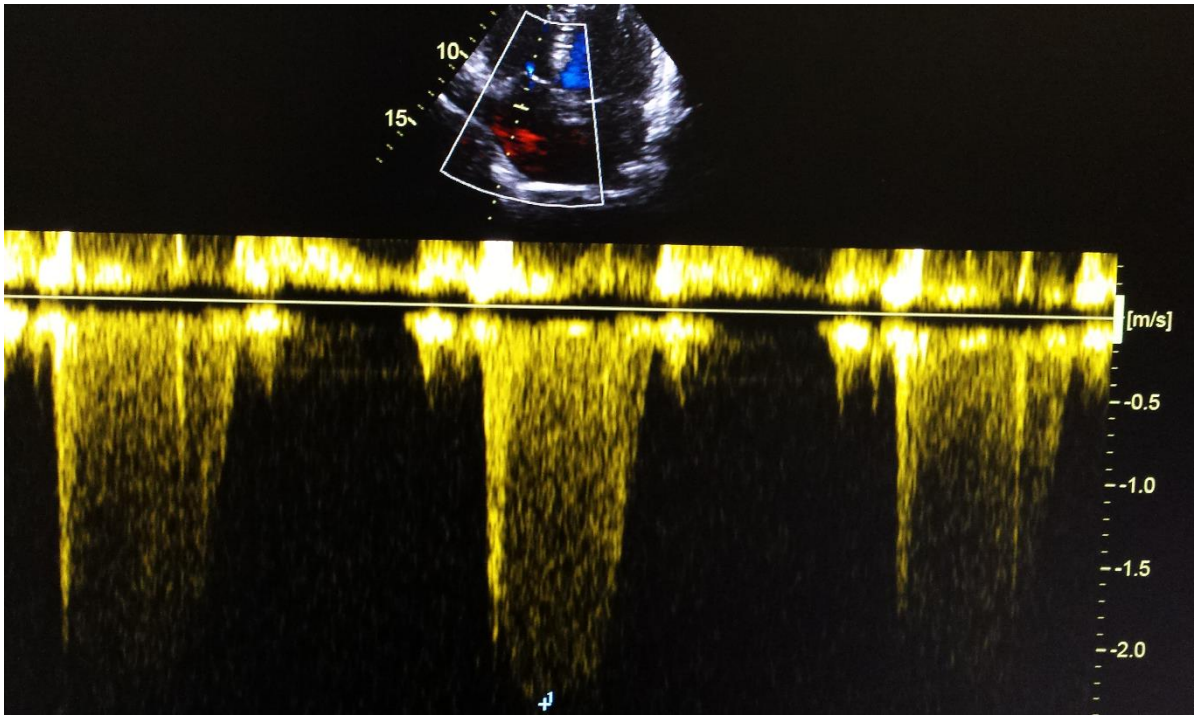

Figure 2.3 Doppler echocardiography for tricuspid regurgitation. A standard apical four-chamber view was used to view the right atrium, right ventricle and tricuspid valve. The jet across the tricuspid valve was used to measure the peak pressure difference (ΔP_{max}) from the right ventricle to the right atrium.

The peak pressure difference (ΔP_{max}) across the tricuspid valve was measured and converted to the peak difference between the right ventricular pressure (RVP) and the right atrial pressure (RAP) using the modified Bernoulli equation. Approximately five measurements for ΔP_{max} were taken and the average calculated. For the purpose of determining pulmonary arterial pressure, the following modified Bernoulli equation was applied:

$$\Delta P_{max} = RVP - RAP \quad (5)$$

ΔP_{max} represents the maximal systolic pressure gradient across the tricuspid valve. RVP is equivalent to pulmonary arterial systolic pressure (PASP), so:

$$\Delta P_{max} = PASP - RAP \quad (6)$$

As RAP is assumed to be constant and unaffected by hypoxia (Groves *et al.*, 1987), changes in ΔP_{max} reflect changes in PASP. In this work, RAP was assumed to be ~ 5 mmHg, so PASP was calculated using the following:

$$PASP = \Delta P_{max} + 5 \text{ mmHg} \quad (7)$$

This technique has been extensively validated (Yock & Popp, 1984; Allemann *et al.*, 2000), and has been used in previous studies in our laboratory (Balanos *et al.*, 2003; Smith *et al.*, 2008; Smith *et al.*, 2009) leading up to the work described in this thesis.

2.3 Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) can be either used to detect the presence of antigens that are recognised by an antibody, or to test for antibodies that recognise an antigen. ELISAs were performed in duplicate on serum to measure EPO and interleukin 6 (IL-6) (Quantikine® IVD human EPO and Quantikine® high sensitivity human IL-6, R&D Systems, Abingdon, United Kingdom) and on plasma to measure soluble transferrin receptor (sTfR) (Quantikine® IVD human sTfR, R&D Systems, Abingdon, United Kingdom) and hepcidin (Bachem, Peninsula Laboratories, Torrance, USA) according to the manufacturer's instructions. As can be seen in Figure 2.4, serum and plasma samples were analysed for EPO, sTfR and IL-6 by using a double sandwich technique, and hepcidin by using competitive binding.

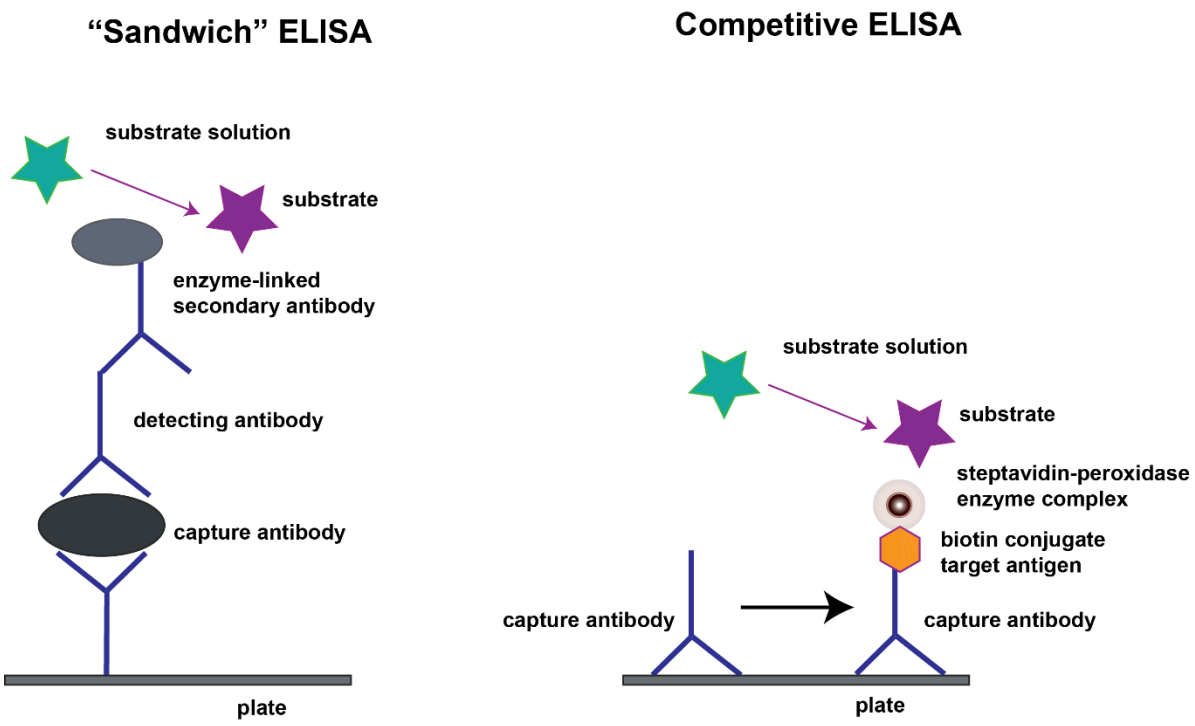

Figure 2.4 Enzyme-linked immunosorbent assay technique. Serum and plasma samples were analysed for erythropoietin, soluble transferrin receptor, interleukin-6 by using a double sandwich technique, and hepcidin by using competitive binding. The sandwich ELISA is able to quantify antigens between two layers of antibodies. In the competitive ELISA, the hepcidin competes against the biotin conjugate contained within the antibody on the plate. The amount of colour generated is proportional to the amount of conjugate bound substrate in the sandwich ELISA, whereas the reverse is true for the competitive ELISA. Adapted from O'Kennedy *et al*, and Abnova (O'Kennedy *et al.*, 1990; Abnova, 2010).

2.3.1 Venous blood sampling and ELISA analysis

All blood samples were taken using standard aseptic technique. They were drawn from the antecubital vein in the participant's left or right arm into a 4.5 mL tube pre-treated with anticoagulant potassium EDTA (K3E 15% 0.054 mL, BD Vacutainer®) and a 5 mL tube coated with silicone and micronised silica particles (BD Vacutainer® SST™). Blood was centrifuged at the time of venesection for fifteen minutes at 3000 rpm and separated into

red blood cells and plasma or serum. The plasma or serum were then separately pipetted into 5 mL Eppendorf tubes and stored at -60 °C in a locked laboratory until the time of analysis by ELISA. Blood samples were numbered with the participant's code so that investigations were performed in a blinded manner.

2.3.2 Erythropoietin

The human EPO assay is based on the double-antibody sandwich method. The EPO in the sample is immobilised by pre-coated antibody within the well and, after adding the sample and control, the wells were washed. A solution containing a rabbit anti-EPO polyclonal antibody, bound to a horseradish peroxidase, was then added to the wells. This technique required a second period of incubation, which allowed the antibody-enzyme complex to bind to the available EPO. A second wash was subsequently conducted. A chromogen was added, which was oxidised by the enzyme reaction to form a coloured complex. The reaction was then stopped by the addition of acid, which caused a colour change from blue to yellow. The amount of colour generated was directly proportional to the amount of conjugate bound to the EPO antibody complex (i.e. proportional to the EPO present in the serum or control of known concentrations). The plate was then read by a microplate reader at 450 nm with a reference wavelength of 600 nm. The EPO concentration of the unknown specimen was determined by comparing the optical density of the specimen to the standard curve.

2.3.3 Interleukin-6

Interleukin-6 (IL-6) was measured using the Quantikine Human IL-6 Immunoassay, a solid phase immunoassay that has a 4.5 hour protocol. Like EPO, the protocol employed a

double sandwich technique using monoclonal antibodies. It contained *Escherichia coli*-expressed recombinant human IL-6, and antibodies raised against the recombinant protein. This test was performed as per the manufacturer's instructions.

2.3.4 Soluble transferrin receptor

Soluble transferrin receptor (sTfR) was measured using a solid phase sandwich ELISA assay employing two different monoclonal antibodies, in a 96-well plate with a sensitivity of 0.5 nmol/L. Plasma samples and standards were manually pipetted into the plate wells in duplicates. Each well came pre-coated with a monoclonal antibody, which captured the sTfR, rendering it immobile within the well. The wells were washed, following which a second monoclonal antibody conjugated to horseradish peroxidase was added. A second washing was conducted using wash solution. After washing, the remaining conjugate in the well was proportionate to the amount of sTfR, which was initially immobilised. The quantity of conjugated enzyme in the well was measured by incubation with a chromogenic substrate. The results were read on a microplate reader at a wavelength of 450 nm with a correction at 570 nm. For calculation of results, an average of the duplicate readings for each standard, control and sample was used with a subtraction of the average zero standard optical density.

2.3.5 Hepcidin

Hepcidin (Bachem, Peninsula Laboratories, San Carlos, CA) was analysed using an ELISA method based on competitive binding, unlike the assays above, which use a double sandwich technique. Plate wells were pre-coated with monoclonal mouse antibody

directed against hepcidin. The endogenous hepcidin competed with the biotin conjugate contained within the antibody in the well. After incubation, the unbound conjugate was washed off and a streptavidin-peroxidase enzyme complex was added to each well. This was then allowed to incubate. The unbound complex was washed off and a substrate solution was added. Similar to the EPO protocol there was a colour change from blue to yellow with the addition of a stopper. The intensity of the colour was then read by using a microplate reader, being inversely proportional to the amount of hepcidin in the sample. The microplate reader was set at 450 nm.

2.4 Determination of red cell volume using carbon monoxide

Blood consists of red cells and plasma. Venous haematocrit, represented as a percentage, may not correctly reflect the absolute volume of red blood cells (Jacob *et al.*, 2012). One third of each red blood cell's volume is made up of haemoglobin which is vital in the transport of O_2. An accurate measure of red cell mass is therefore important in terms of monitoring changes in O_2 transport in the blood (Balga *et al.*, 2000). The gold standard for the measurement of red cell mass employs radioisotope labelling of red cells according to the International Committee for Standardization in Haematology (Pearson *et al.*, 1995). However, due to both cost and radiation this method is less attractive in a research setting for healthy participants. Red cell mass has been studied using radiolabelled techniques in haematology patients (Fairbanks, 2000), after EPO administration (Ekblom, 1996), during space flight (Alfrey *et al.*, 1996), and using re-breathing techniques during exercise (Ashenden *et al.*, 1999).

The carbon monoxide rebreathing techniques are based on the binding affinity of carbon monoxide for haemoglobin. By injecting a known dose of carbon monoxide and assuming it binds tightly with the haemoglobin, the red cell mass can be calculated for a known temperature and pressure. A large meta-analysis demonstrated that a closed circuit breathing technique, using a mixture of O_2 and carbon monoxide, had a similar error rate to that of radiolabel techniques (Gore *et al.*, 2005). Two well validated protocols have been described (Burge & Skinner, 1995; Schmidt & Prommer, 2005).The protocol used in this study was close to a modified Prommer protocol that aimed to minimise the errors of the original protocols (Prommer & Schmidt, 2007).

2.4.1 Carbon monoxide rebreathing circuit

A closed circuit rebreathing apparatus was designed in the laboratory based on previous designs. As seen in Figure 2.5, a standard mouthpiece was used and was cleaned between participants. A standard anaesthetic antimicrobial heat moisture exchange filter (Intersurgical, Wokingham, Berkshire United Kingdom) was attached to the mouthpiece. This was attached to a two-way valve system. Beyond this, soda lime was added to absorb carbon dioxide. This was connected to a four litre standard anaesthetic reservoir (Intersurgical, Wokingham, Berkshire, United Kingdom). The individual parts were tightly joined in the workshop to minimise air leaks. The apparatus was tested for air leaks between uses and adjusted if any deficits were found.

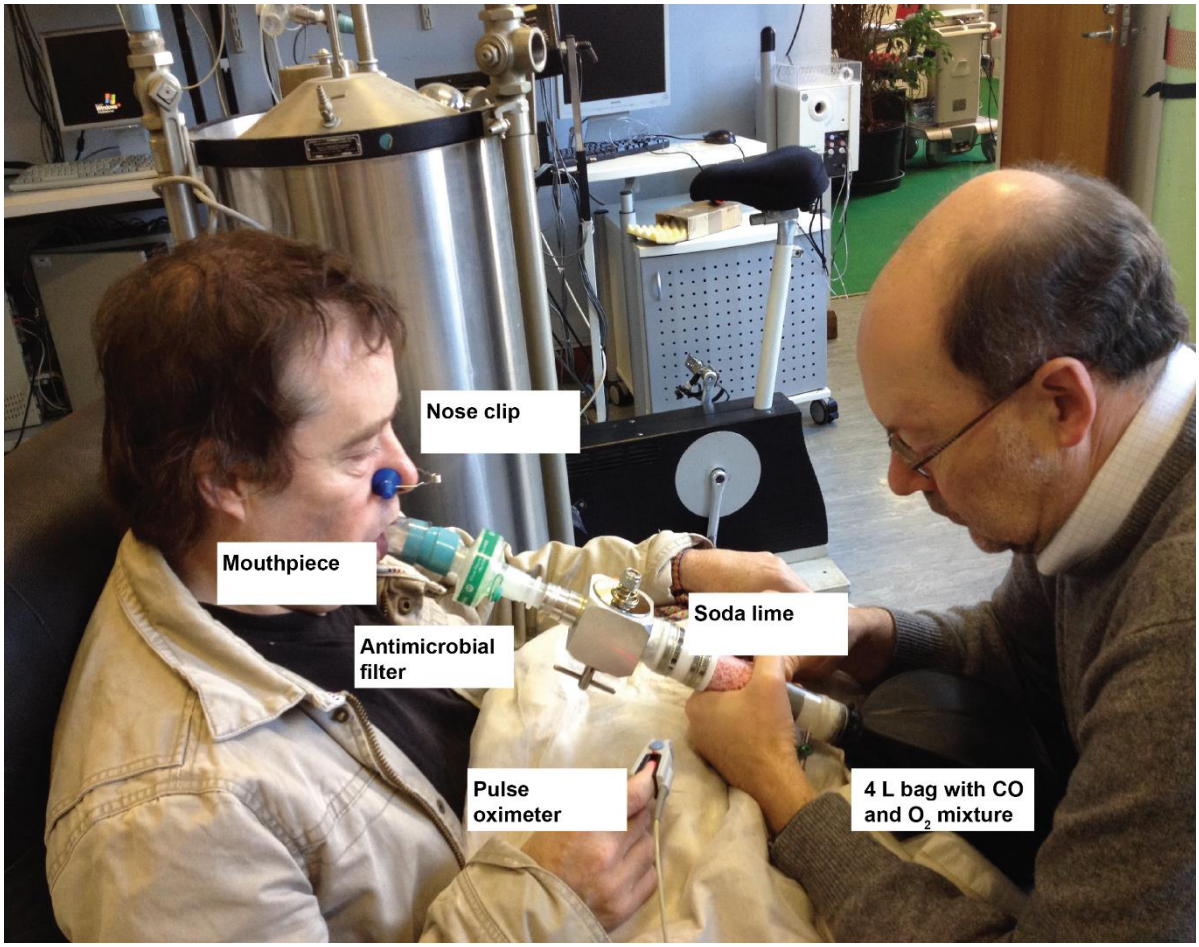

Figure 2.5 Carbon monoxide rebreathing circuit. The circuit consisted of a standard anaesthetic antimicrobial filter attached to a mouthpiece, connected to a two-way valve system and then a four litre anaesthetic bag. Soda lime was used to absorb carbon dioxide. All participants had a practice run with the apparatus to check their technique, and wore a nose clip to ensure the smooth running of the protocol and to minimise air leaks.

2.4.2 Protocol for administration of carbon monoxide

The protocol for this study involved the closed circuit apparatus described above (Figure 2.6). Participants were given a practice run to ensure a good technique and a good seal with the apparatus. A standard 20 gauge IV cannula was inserted into a vein and two baseline carboxyhaemoglobin (HbCO) levels were taken. Following this, the standard anaesthetic bag was filled with 100% O_2 and injected with 1 mL/kg of body weight of

carbon monoxide. Participants were asked to breathe from the mixture for 3.5 minutes while wearing a nose-piece, which minimised any potential air leaks. Blood samples were then taken in duplicate at 10, 12 and 15 minutes to measure HbCO. The blood samples were analysed in real time using a blood gas analyser. This protocol was conducted prior to the start of the experiment, at month three, and then at month six, to assess percentage change in HbCO (ΔHbCO) and then calculate red cell mass.

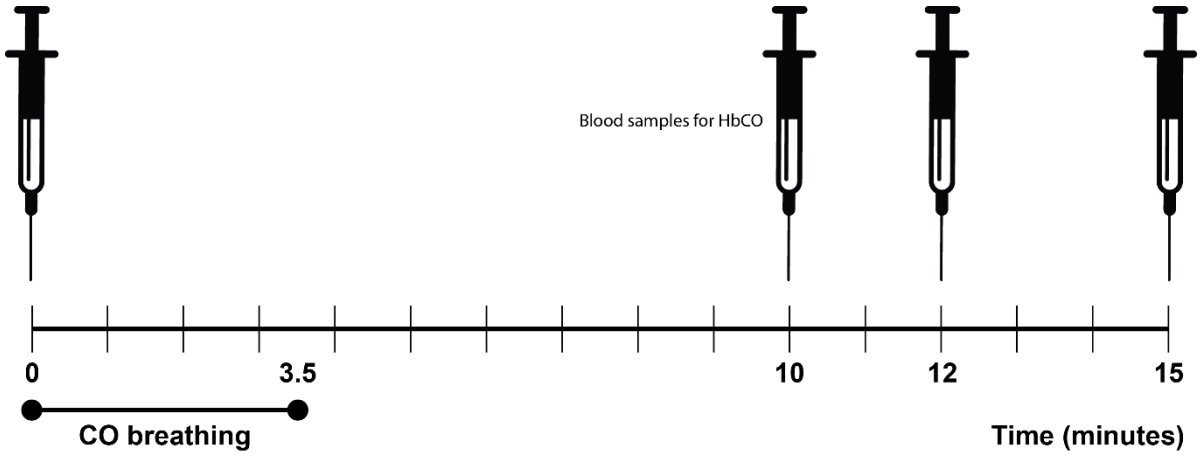

Figure 2.6 Carbon monoxide protocol. This diagram represents the protocol for measurement of HbCO. Participants breathed from a standard anaesthetic bag that contained 1 mL/kg of carbon monoxide mixed with 100% O_2. Blood samples were taken in duplicate from a venous cannula in the arm at 10, 12 and 15 minutes to measure HbCO.

2.4.3 Limitations of the closed circuit technique

The main predicted sources of error of the rebreathing technique include gas leaks due to the mouthpiece, nose clip or rebreathing system. Inadequate dosing, and using a reservoir bag with excessive volume are also issues (Gore *et al.*, 2005). The protocol was designed to minimise these issues. One problem which could not be accounted for in this technique is loss of CO from the intravascular space. CO is known to diffuse into the myoglobin

after a relatively short time (Gore *et al.*, 2005). Note that myoglobin is the main compartment for diffusion with cytochrome oxidase, cytochrome p450 and hydroperoxidase accounting for negligible losses. Diffusion of CO can result in an overestimation of red cell mass.

2.4.4 Side effects and safety with the use of carbon monoxide

Side effects associated with carbon monoxide toxicity occur at levels of 10% and above (Smollin & Olson, 2010). These side effects include headaches, malaise and nausea. At levels of 50-70%, seizures, coma or even death can occur. As part of the protocol of the study, the rebreathing circuit was checked and calibrated prior to each use. Participant levels did not exceed a 6.5% rise in carbon monoxide. Individuals who are non-smokers are estimated to have HbCO levels of 1.5-2%. Smokers were excluded from the study. To measure the red cell volume, a dose of carbon monoxide which induced no more than a 6.5% rise in HbCO was given, to allow a large margin of safety.

The carbon monoxide was stored outside the building in a locked cage. Only the syringe with the correct amount of carbon monoxide needed for the participant was carried into the building. Administration of carbon monoxide was conducted by two fully qualified medical officers. The participant was monitored throughout the experimental period and for fifteen minutes after rebreathing the gas mixture. A source of 100% O_2, which is used to treat carbon monoxide toxicity through competition at haemoglobin binding sites, and has also been shown to reduce the half-life of carbon monoxide (Weaver *et al.*, 2000), was kept readily available. Carbon monoxide was only administered 24 hours after exposure to

hypoxia, and participants were not exposed to hypoxia until two days after administration of carbon monoxide to allow a wide safety margin.

2.4.5 Calculation of red cell mass

Red cell mass could be calculated using the relationship between CO and haemoglobin, knowing that one molecule of haemoglobin (Hb) has four binding sites for O_2, (and therefore carbon monoxide). The molecular weight of Hb is 64,458 $gmol^{-1}$ (van Beekvelt *et al.*, 2001). Based on this the following can be derived:

$$1g\ Hb = \frac{1}{64{,}458}\ mol\ of\ Hb \tag{8}$$

Therefore, since each haemoglobin molecule presents four binding sites for oxygen or carbon monoxide, the number of binding sites for carbon monoxide for each gram of haemoglobin can be shown as follows:

$$1g\ Hb = \frac{4}{64{,}458} gmol\ CO\ binding\ sites = 6.206x10^{-5} gmol\ CO\ binding\ sites \tag{9}$$

The number of carbon monoxide molecules delivered to each participant can be calculated using the ideal gas law expressed below, where p represents pressure (Pa), V is volume (m^3), n is the amount (mols), R_o is the universal gas constant (8.3145 $Jmol^{-1}K^{-1}$), and T is temperature (Kelvin).

$$n = \frac{pV}{R_o T} \tag{10}$$

On each experimental day, the temperature in Celcius, the pressure in mmHg, and ml of CO administered to the participant were recorded. Participants were given 1 ml/kg of CO. Percentage change (%Δ HbCO) of carboxyhaemoglobin was measured against the baseline. Therefore, these calculations can be combined into a single equation, accounting for the conversion of units from those used experimentally, to the international system of units of the universal gas equation. Together, the mass of haemoglobin can be calculated from the values measured in the study as follows:

$$\mathrm{Hb} = \frac{100}{\%\Delta\mathrm{HbCO}} \times \frac{1}{6.206\times10^{-5}} \times \frac{(133.3p)(V\times10^{-6})}{8.3145\times(273.15+T)} \tag{11}$$

In this equation, Hb is the total g of haemoglobin, %ΔHbCO is the percentage change in haemoglobin, 6.206 x 10^{-5} is the gmol of CO per 1 g of Hb, P_{atm} atmospheric pressure (mmHg), V is mL of CO given and T is temperature in degrees Celsius.

The final equation is:

$$\mathrm{Hb} = \frac{25.847}{\%\Delta\mathrm{HbCO}} \times \frac{PV}{273.15+T} \tag{12}$$

2.5 Properties and administration of intravenous iron

Oral and parenteral forms of iron have been available for many decades to treat the symptoms of iron deficiency anaemia. Formulations of IV iron consist of an iron-carbohydrate complex, which is made up of an iron core surrounded by a carbohydrate ligand, which stabilises the complex and allows it act like a pro-drug (Toblli & Angerosa, 2014). Iron preparations are classified based on the stability of the complex, with type I (ferric carboxymaltose, high and low molecular weight iron dextran), being strong and robust, meaning that only small amounts of ionic iron are released into the blood, while

type II (iron sucrose) and type III (sodium ferric gluconate complex) are progressively less stable.

The carbohydrate ligand itself is important because its stability will determine when it dissociates from the iron complex, which will also affect plasma clearance (Toblli & Angerosa, 2014). Weaker iron carbohydrate complexes such as iron sucrose and ferric gluconate are more dissociated before entering the macrophage. Depending on the dose of iron administered and the proportion of labile iron, non-transferrin bound iron (NTBI) can be produced which in turn, leads to oxidative stress and potential tissue damage.

2.5.1 Ferric carboxymaltose

For the studies described in this thesis, ferric carboxymaltose (FCM) was chosen on the basis of its safety and administration profile. FCM is a newer formulation of iron, and is water-soluble, and brown coloured. It has a relative molecular weight of 150,000 Da, containing approximately 1,000 iron atoms (Toblli & Angerosa, 2014). It is a complex containing a polynuclear iron-hydroxide core combined with a carbohydrate ligand. This carbohydrate shell facilitates the slow release of the ferric hydroxide with minimal risk of release of large quantities of ionic iron into the serum (Lyseng-Williamson & Keating, 2009). The iron in FCM is in the ferric state, which is stable. After administration, FCM is cleared rapidly from the circulation, as it is taken up primarily by the reticuloendothelial system, particularly the bone marrow (~80%) but also the liver and spleen (Lyseng-Williamson & Keating, 2009) rather than being available as NTBI.

As with other iron carbohydrate complexes, the exact mechanism of metabolism by the macrophage remains unclear. It is believed that after administration the carbohydrate shell of FCM is partially broken down by the α-amylase enzyme, following which it is taken up by endocytosis into the macrophage. The carbohydrate shell is then completely broken down in the endolysosome and Fe^{3+} is released. Fe^{3+} is subsequently reduced to Fe^{2+} by the six-transmembrane epithelial antigen of the prostate 3 (STEAP3) and transported out of the endolysosome by DMT1. It then goes into the labile iron pool and is stored as ferritin, or is transported out of the macrophage via ferroportin where it can be loaded onto transferrin and transported for use in the bone marrow, liver and other tissues. This proposed mechanism of FCM incorporation to tissues is seen in Figure 2.7.

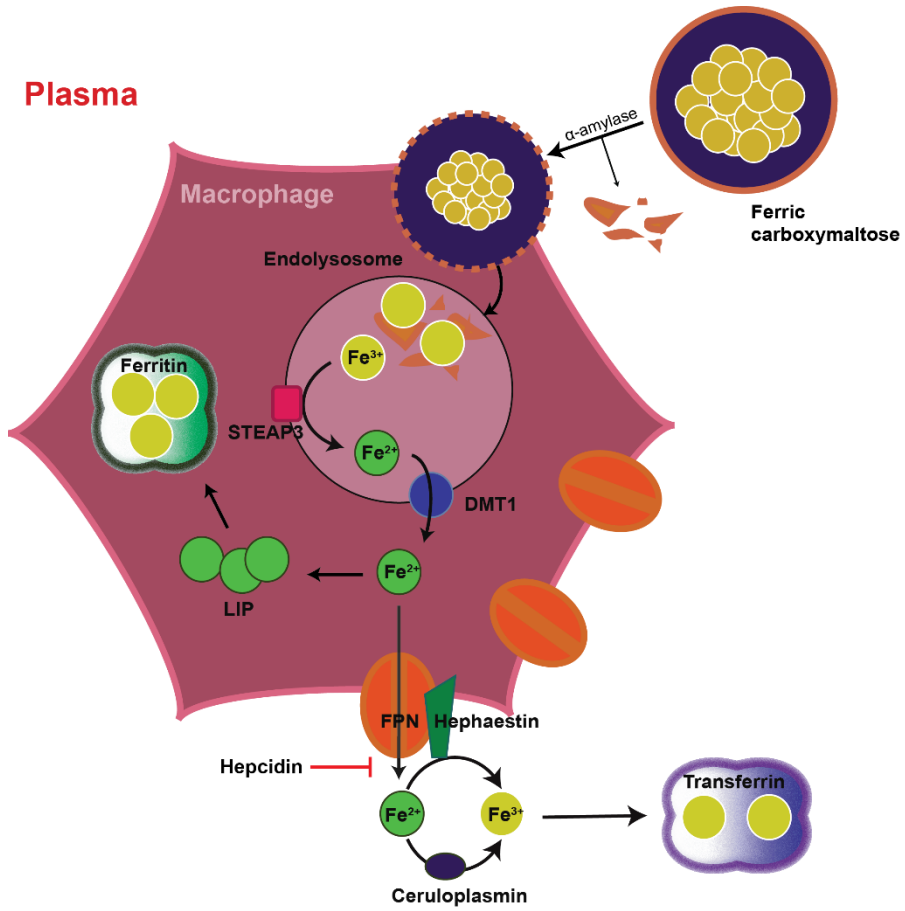

Figure 2.7 Mechanism by which ferric carboxymaltose is incorporated into the reticuloendothelial system. Following IV administration, FCM is taken up by the macrophage and the α-amylase enzyme partially breaks down its carbohydrate shell. It then goes into the endolysosome such that the shell and the polynuclear iron core can be completely broken down to release Fe^{3+}. Fe^{3+} is reduced to Fe^{2+} via STEAP3. Fe^{2+} exits the endolysosome via DMT1. It can then go into the LIP and be stored as ferritin, or be transported out of the macrophage by FPN where it is then transported by transferrin to the liver, bone marrow and other tissues.
DMT1, divalent metal transporter 1; FPN, ferroportin; STEAP3, six-transmembrane epithelial antigen of the prostate 3; LIP, labile iron pool. Image adapted from Toblli 2014 (Toblli & Angerosa, 2014).

Infusion of FCM was over fifteen minutes and was used safely without a test dose (Lyseng-Williamson & Keating, 2009). This is advantageous over other iron preparations that can take up to four hours to administer. In addition, as FCM is not a dextran

containing preparation, there were fewer hypersensitivity reactions reported and currently no documented case reports of anaphylaxis with this preparation of iron (Lyseng-Williamson & Keating, 2009).

In a study of 200 patients with inflammatory bowel disease, infusion of 1 g of FCM over fifteen minutes was shown to be safe and non-inferior to oral iron (Kulnigg *et al.*, 2007). Furthermore, FCM administration resulted in a rapid increase in haemoglobin in patients with iron deficiency anaemia. In haemodialysis patients, there was correction of haemoglobin with a regime of 100-200 mg of FCM on the day of dialysis. The infusion was safe and well tolerated in this patient cohort (Covic & Mircescu, 2010).

After infusion of FCM, there is a rapid dose-dependent increase in serum iron concentration. Maximum concentrations range from 36.9 μg/ml with 100 mg to 317.9 μg/ml after a 1000 mg dose (Geisser & Banke-Bochita, 2010). Ferritin concentration has a dose-dependent, but not dose-linear, relationship with FCM in iron deficient patients (Geisser & Banke-Bochita, 2010). In the same study, ferritin levels rose between 6-12 hours after FCM administration with peak concentrations (23-210 fold increase from baseline) at 120 hours after 1000 mg.

After IV administration, FCM is cleared from serum with a terminal half-life of approximately 7.4–12.1 hours (Geisser & Banke-Bochita, 2010). The percentage of urinary excretion is believed to be negligible (0.0005%). Most FCM is broken down

within 24 hours of a 100 mg dose and within 72 hours of a 500-1000 mg dose (Geisser & Banke-Bochita, 2010).

2.5.2 Iron dosing

Discussions with Vifor Pharma Ltd (manufacturer of iron sucrose and FCM) revealed that they were unable to supply any information on the administration of iron to individuals who are iron-replete on conventional criteria. Moreover, it is currently difficult to identify any studies that demonstrate the effect of administration of increasing amounts of IV iron on ferritin concentration and transferrin saturation in healthy iron-replete humans.

In current practice, one method of calculating the dose of iron to administer to anaemic patients is the Ganzoni formula, as given below (Ganzoni, 1970).

Ganzoni formula: Body weight (kg) × 2.3 × haemoglobin deficiency (target haemoglobin level (g/dL)−patient haemoglobin level(g/dL)) + 500 to 1000 mg iron for the repletion of iron stores

There are a number of limitations to this method including inconsistent usage in clinical practice, frequent errors, and inadequate iron store replenishment (Kulnigg *et al.*, 2008; Camaschella, 2015). Some studies have started to address this, for example one study of inflammatory bowel disease patients guided dosing on absolute haemoglobin value and body weight using doses of either 1000 mg or 1500 mg of FCM (Evstatiev *et al.*, 2011). In this study patients received a maximum of three fixed doses of FCM versus up to eleven

doses of iron sucrose (as calculated by the Ganzoni formula), resulting in better efficacy and fewer deviations from scheduled iron doses in the FCM group. Another study has shown that a dose of 1 g of IV FCM was safe in mildly iron deficient participants defined as serum ferritin < 20 μg/L and transferrin saturation < 16% (Geisser & Banke-Bochita, 2010). In both studies, participants were iron-replete and not anaemic, and so the current dosing guidelines did not apply. The relevant dosing methodologies for each study are discussed in Chapters Three and Four respectively.

2.5.3 Intravenous iron administration

Parenteral iron was the chosen preparation as oral iron administration may be ineffective in manipulating iron status in iron-replete individuals. There are tight regulatory mechanisms in place for the absorption of iron across the duodenal enterocyte. In iron-replete individuals, hepcidin and other factors block the uptake of iron across the basolateral membrane of the enterocyte. Intramuscular administration of iron is equally efficacious as IV administration. It is however, associated with permanent skin staining and is no more safe than IV infusion. Its use is therefore discouraged in clinical settings unless there are specific issues with IV access or administration (Solomons & Schumann, 2004).

Individuals were randomised to either the iron or placebo group. On the day(s) of the infusion, a standard IV cannula was inserted into the cubital fossa. Participants were blindfolded so they could not see the infusion, and the blinded investigator remained outside the laboratory for the duration of the infusion and monitoring.

On the day(s) of infusion, participants received either 50 mL of sterile 0.9% m/V NaCl (placebo group) or a dose of FCM (Ferinject®,Vifor Pharma Ltd, half-life 7.4-12.1 h) (Geisser & Banke-Bochita, 2010) diluted in sterile 0.9% m/V NaCl (iron group) given over 15 minutes by a syringe infusion pump (Graseby™ 3100 Syringe Pump, Smith Medical International Ltd, United Kingdom). Following administration, the cannula was flushed with 10 mL of normal saline to avoid de-pigmentation of the overlying skin. The participant was observed for 15 minutes following the infusion, during which time heart rate, blood pressure and pulse oximetry were recorded.

2.5.4 Side effects and safety for FCM administration

The definition of 'common' side effects refers to symptoms that occur in more than 1 in 100 individuals receiving FCM. Common side effects of FCM include constipation or diarrhoea, dizziness, headaches and injection site problems including skin colour changes or irritation if the infusion is given incorrectly (Lyseng-Williamson & Keating, 2009).

'Uncommon' side effects are symptoms that occur in more than 1 in 1000 individuals receiving FCM. Uncommon side effects include muscle or joint pain, fevers or rigors, general malaise or lethargy, nausea or vomiting, chest discomfort, oedema or paraesthesia of the extremities, unusual taste in the mouth, and hypersensitivity reactions including anaphylaxis. Of the various parenteral forms, iron dextran is the agent responsible for the lingering reputation of IV iron as a cause of potentially fatal anaphylaxis (Geisser & Banke-Bochita, 2010).

Few studies exist on the effects of long-term administration of IV iron. The most closely observed sub population in this regard are patients with chronic renal disease. A retrospective analysis of all-cause mortality in a cohort of 32,566 haemodialysis patients from 1996 to mid-1998 showed no adverse effect on two year survival with doses of up to 1000 mg given over a six month period, but increased mortality for doses ranging from 1000 to 1800 mg (Feldman *et al.*, 2004). However, using multivariate models, there was no statistically significant association between any level of iron administration and mortality.

Two qualified medical officers monitored the participant during the administration of iron. A full resuscitation kit was available in the laboratory, including adrenaline in the event of an anaphylactic reaction. Participants were monitored for symptoms of an adverse reaction for at least 15 minutes after completion of the infusion.

CHAPTER THREE:

STUDY A

3 Study A: Iron blunts the hypoxic pulmonary vascular response in humans.

3.1 Introduction

Sustained hypoxia leads to a progressive rise in PASP (Dorrington *et al.*, 1997). Most vascular beds dilate in response to hypoxia in order to deliver oxygen to tissues. In contrast to this, hypoxia results in constriction of the low-pressure pulmonary system. In the long term this can lead to permanent pulmonary vascular remodelling and pulmonary hypertension (Barbera *et al.*, 2003).

Intravenous iron administered immediately prior to a hypoxic exposure reduces pulmonary vasoconstriction (Smith *et al.*, 2008). In iron-replete individuals with no medical comorbidities, iron infusion blunts the rise in PASP in response to sustained hypoxia whereas iron chelation with DFO augments pulmonary vasoconstriction (Smith *et al.*, 2008). Smith and colleagues demonstrated an attenuation in the rise in PASP by ~50% on day one post iron infusion with acute hypoxia and a similar attenuation over eight hours of sustained hypoxia (Smith *et al.*, 2008). At high altitude, the effect of iron on the pulmonary vascular response is known to last for several days (Smith *et al.*, 2009). This study of 22 healthy sea-level resident men followed over one week of hypoxia at an altitude of 4340 m showed that 40% of the rise in PASP was attenuated by a single bolus dose of 200 mg of Fe(III)-hydroxide sucrose. The change in PASP began by four hours post iron infusion, and the effect persisted for up to four days post iron infusion with an associated rise in serum ferritin concentration that remained elevated throughout the measurement period.

Hypoxia-induced pulmonary hypertension is seen in many cardiorespiratory diseases, and therefore the manipulation of iron stores could be a useful approach in these disease states. Iron deficiency is common in COPD (Nickol *et al.*, 2015) and pulmonary hypertension (Ruiter *et al.*, 2011). IV iron replacement has been shown to be of benefit in a range of cardiovascular diseases, including coronary artery disease (Ponikowska *et al.*, 2013; Grammer *et al.*, 2014; Ponikowski *et al.*, 2015) and left ventricular systolic failure (Anker *et al.*, 2009; Jankowska *et al.*, 2011). Iron deficiency is prevalent in these patient groups, and correction of iron stores was of benefit independent of the presence of anaemia (Anker *et al.*, 2009).

3.1.1 Downstream markers of iron homeostasis and HIF

Given that a single bolus dose of IV iron was administered in this study, the hypothesis was that this may lead to an acute rise in downstream markers of iron homeostasis. Therefore, the conventionally measured clinical biochemistry parameters of serum ferritin concentration, transferrin saturation and transferrin concentration were of interest. While HIF itself cannot be directly measured, ELISA analysis was employed to measure markers of iron homeostasis (hepcidin, sTfR), a measure of inflammation (IL-6), and an indicator of erythropoiesis, as well as a downstream marker of HIF (EPO).

Standard biochemical iron studies include ferritin concentration, transferrin saturation, and transferrin concentration and these are interpreted together to assess iron status. Serum ferritin concentration reflects the amount of iron stored in the body. Importantly, it also functions as an acute phase reactant, and can be influenced by inflammatory states (Cavill, 1999), and therefore may be more difficult to interpret in patients with chronic disease.

Serum iron can fluctuate with diet as well as diurnal variation and therefore is not a reliable marker. A much more dependable measure is the transferrin saturation which represents the ratio of serum iron to total iron binding capacity. Serum transferrin is the transport protein for iron and is expected to increase in iron deficiency states.

Hepcidin is considered the master regulator of iron homeostasis. Hepcidin is produced in the liver encoded for by the *HAMP* gene (Ganz, 2003). The bioactive peptide hepcidin-25 is produced from pro-hepcidin in the liver by proteolytic cleavage, and subsequent processing produces smaller peptides, which then accumulate in the urine (Park *et al.*, 2001; Pigeon *et al.*, 2001). Hepcidin is expected to increase after a bolus dose of FCM, along with ferritin concentration and transferrin saturation. In contrast, administration of EPO causes a significant and prolonged decrease in hepcidin (Pilon *et al.*, 1981). Hepcidin was examined in this study to assess changes after the acute administration of IV iron, and then at different time points after the iron had been incorporated into the body stores.

Transferrin receptors are transmembrane proteins involved in iron transport, uptake and storage. They are the principal means by which cells acquire iron, and play an indispensable role in erythroid precursors for haemoglobin synthesis (Skikne, 2008). sTfR is the extracellular portion of transferrin receptor 1, which is released into the serum after proteolysis (Cook *et al.*, 1993). It is a 190,000 Dalton dimer protein linked by two pairs of disulphide bonds. The amount of sTfR is regulated by intracellular iron concentration and sTfR has a high affinity for transferrin (Beguin, 1992). sTfR is a reflection of total transferrin receptor expression and is a measure of unmet iron requirements (Skikne *et al.*, 1990; Allen *et al.*, 1998). Inflammation does not significantly affect sTfR levels, making it

a more reliable marker of iron status than serum ferritin concentration. Clinically however, sTfR and serum ferritin concentration are invaluable tools for measurements of bone marrow iron status in the majority of patients compared to haemoglobin levels, which may only decrease with severe iron deficiency. Though sTfR may provide a more reliable marker of iron stores in the body, excluding inflammation was important in this study to ensure that the results were not confounded by inflammation or infection in the participants.

Interleukin-6 is a pleiotropic 22-28 kDa phosphorylated cytokine (Heinrich *et al.*, 2003). It has a role in inflammation as an acute phase reactant, and is secreted by T cells and macrophages (Kopf *et al.*, 1994). IL-6 works on the hypothalamus, resulting in a febrile reaction to infection or inflammation (Evans *et al.*, 2015).

HIF cannot be measured directly in humans, although in one animal study HIF measurement was used to demonstrate the relationship between HIF and hypoxia. Isolated ferret lungs were exposed to eight hours of sustained hypoxia and HIF was measured at intervals. At 0 hours, HIF-1α was not detected, but increased at 1-2 hours with a peak at 4 hours and remained elevated at 8 hours (Yu *et al.*, 1998). A previous human study demonstrated that DFO administration produced a rise in EPO, likely via a HIF-related mechanism (Ren *et al.*, 2000), showing that EPO can be measured as a downstream marker of HIF. EPO is a glycoprotein hormone stimulated by hypoxia that controls erythropoiesis (Eckardt *et al.*, 1989; Jelkmann, 1992). As such, EPO was selected as a surrogate marker for HIF in this current study.

3.1.2 Duration of effect of a bolus dose of intravenous iron on blood and hypoxic pulmonary vascular responses

Healthy humans have a total body iron content of 4 g, most of which is in haemoglobin (Mole, 2010). Humans have no way of actively excreting iron and, as a consequence, disturbance of iron regulatory pathways can lead to iron overload. Administration of 1 g of FCM has been shown to be safe in iron-replete individuals (Geisser & Banke-Bochita, 2010). Assuming total body iron content of 4 g, the addition of 1 g of IV iron was predicted to increase total iron levels by 25% and remain present for a prolonged period due to the lack of active iron excretion (Mole, 2010). The choice of a single bolus dose of iron was hypothesised to be able to significantly elevate iron stores within a safe range without causing iron overload. It is also a practical dose to be given in a health setting.

The plasma half-life of FCM is 7.4-12.1 hours (Geisser & Banke-Bochita, 2010). Anker and colleagues have shown that infusion with FCM improves iron related parameters with a corresponding improvement in symptoms and quality of life over a period of weeks to months in heart failure patients (Anker *et al.*, 2009). The study was notable, however, for its absence of mechanistic links to explain improvement in symptoms. One potential mechanism could be the effect of IV iron on the pulmonary vasculature.

In general, recent experiments have focused on the response of the PASP response to hypoxia on the same day as the iron administration. An exception to this is a study where IV iron was administered to participants who had already been at high altitude (4,340 m) for a period of three days. In this study, the reduction in PASP with iron was shown to last

for a subsequent four days (Smith *et al.*, 2009). However, participants in both the saline and iron groups will have had a cellular iron demand that was greatly increased through the enhanced rate of haematopoiesis associated with high altitude exposure. Overall, it remains unclear whether the effects of iron on the pulmonary vasculature are arising as a relatively transient phenomenon, perhaps associated with the presence of an artificial iron-sugar complex in the blood, or whether they are effects that persist long after the elimination of the iron-sugar complex when the iron has been fully assimilated into the body's stores. Thus, while the close interaction between iron and the pulmonary vasculature has been well documented, the duration of effect of iron is unknown. Clinically, this is important as it will determine whether or not the effects of IV iron administration on the pulmonary vasculature will persist over time.

Aims of Study A:

1) To determine the time course, in healthy humans, of the effect of IV iron on the pulmonary vascular response to hypoxia over six weeks.
2) To determine the time course, in healthy humans, of the effects of IV iron on haematological parameters and iron status over six weeks.

3.2 Methods

3.2.1 Sample size calculation

The primary outcome measure was to determine whether IV iron resulted in a clinically significant attenuation of ΔPASP defined as the change in PASP with hypoxia, and whether this was maintained at days 1, 8 and 43. Previous data from our group showed a mean ΔPASP in response to hypoxic exposure of 3.4 mmHg on day 1 in those post iron infusion, and of 12.6 mmHg day 1 post saline infusion, with changes and variance as recorded by previous investigators using Doppler echocardiography (unpublished data). To detect a clinically significant ΔPASP between IV iron and saline of 4 mmHg at day 1 post IV iron, with a power of 80% and two-tailed significance at the level of $p < 0.05$, a total of 22 participants were studied.

3.2.2 Protocol

This study received ethical approval from NRES Committee South Central Oxford A (Ref: 12/SC/0586) and was performed in accordance with the *Declaration of Helsinki*. The study protocol is detailed in Figure 3.1. This is a double-blind placebo controlled study. Twenty-two healthy participants were recruited from the community by way of advertisement. Participants were required to be non-smokers and iron-replete. Individuals with a ferritin concentration of > 300 μg/L with a normal C-reactive protein (CRP), or with a transferrin saturation of > 45% were excluded from the study. Individuals with no detectable tricuspid regurgitation, as assessed by Doppler echocardiography, were also excluded from the study, as this was required to measure PASP.

Participants were block randomised 1:1 to an infusion of either IV iron or saline (see Table 3.1 and Table 3.2). There were four full study days (days 0, 1, 8, 43) in each arm of the study. Day 0 was the day on which baseline measurements for haematology variables, iron indices and PASP were conducted. There were at least three days separating 'day 0' measurements from 'day 1'. On day 1, participants received either iron or placebo by IV infusion. Immediately following the infusion, participants were exposed to six hours of sustained isocapnic hypoxia. Echocardiographic measurements were conducted hourly, and three blood tests were taken over the six hour period. These measurements were repeated one week post infusion (day 8) and six weeks post infusion (day 43). A protocol for each experimental day is shown in Figure 3.2.

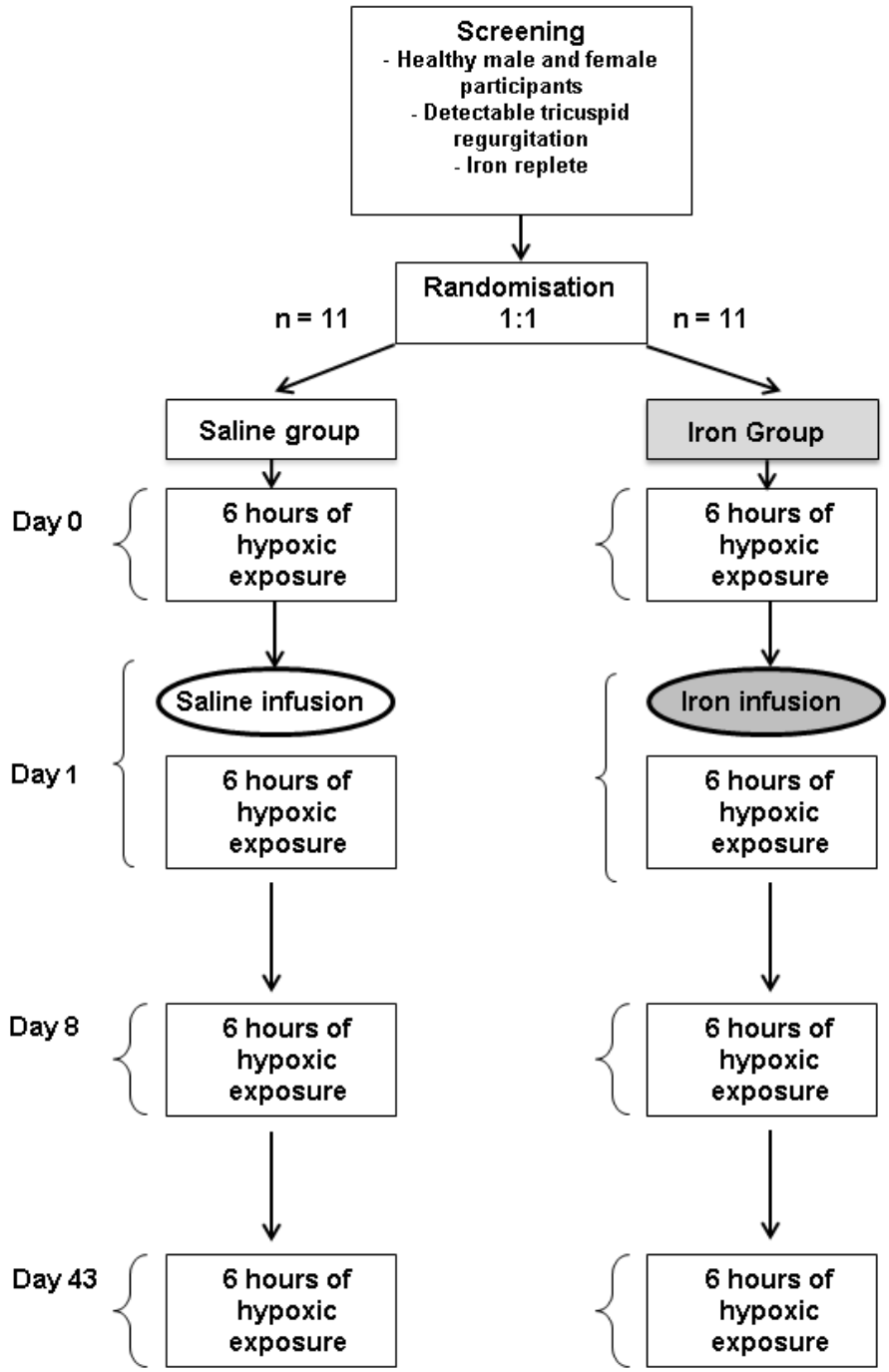

Figure 3.1 Protocol for Study A. Twenty-two participants were recruited. Participants were randomised 1:1 to iron or saline. Participants were exposed to sustained isocapnic hypoxia for six hours on days 0, 1, 8 and 43. Day 0 was a baseline measurement day that was undertaken at least three days before day 1. An infusion of iron or saline was given on day 1.

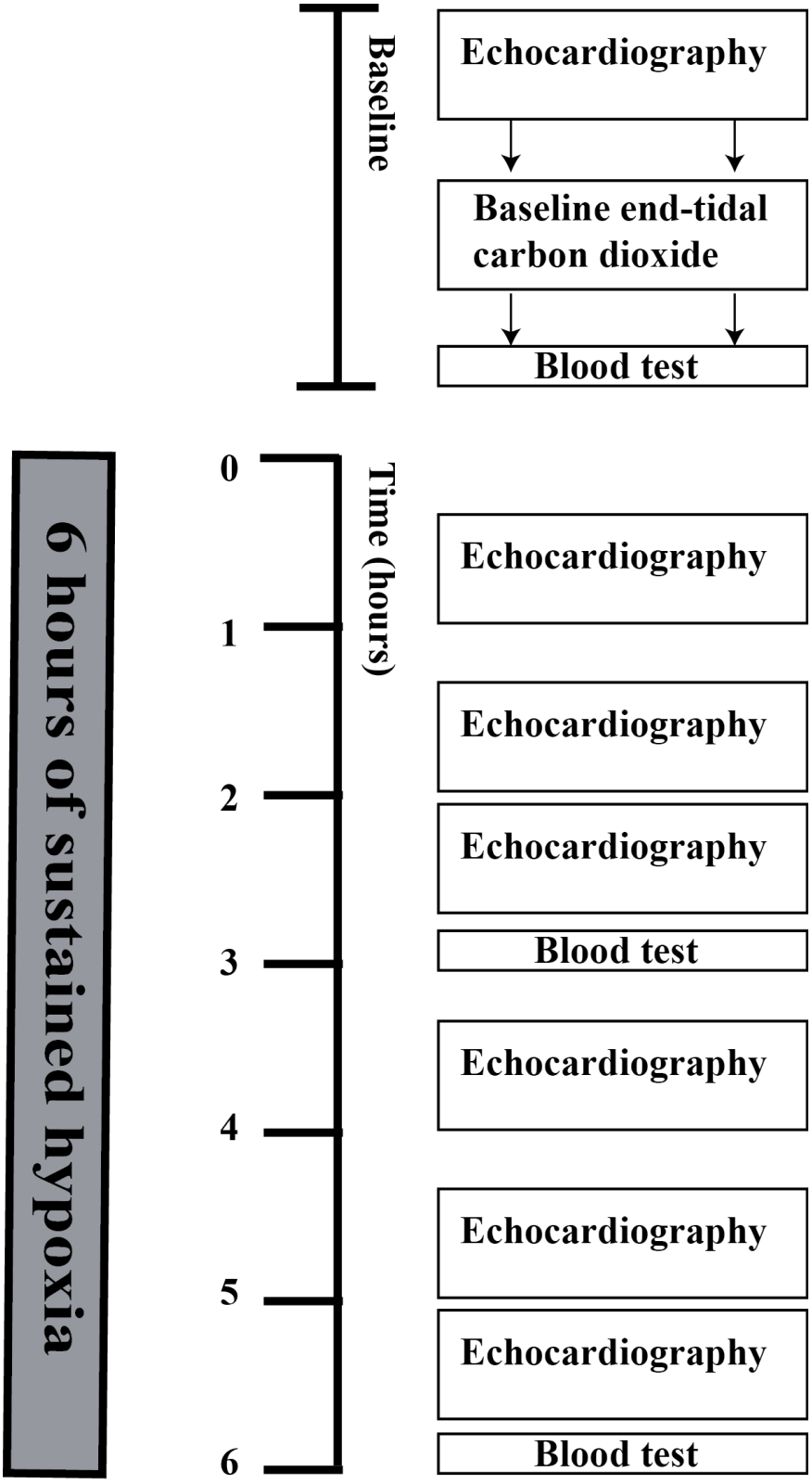

Figure 3.2 Protocol for each experimental day. Participants had baseline echocardiography, ventilation and blood measurements taken at rest. Following this, they were exposed to six hours of sustained isocapnic hypoxia. Echocardiography measurements were taken hourly, and blood tests after three and six hours.

3.2.3 Randomisation

Participants were block randomised 1:1 to receive iron or saline. A permuted block system was used by an unblinded investigator to randomise participants between groups. Male and female participants were randomised separately. To randomise participants, three letter (A to E) and number (1-20) combinations were randomly chosen. The number corresponding to this was read from the table shown below (Table 3.1). For example, 20D would equal 8, as seen in bold in the Table 3.1. A list with two iron allocations and two saline allocations was created from the number in Table 3.1 to determine the order of infusions in each block (Table 3.2). In this example the infusion schedule would be: iron, placebo, iron, placebo (IPIP).

	A	B	C	D	E
1	7	8	2	8	9
2	6	1	5	6	6
3	9	9	7	9	1
4	5	1	6	7	3
5	6	5	4	4	1
6	8	1	4	8	9
7	1	4	4	8	6
8	7	1	5	6	9
9	5	5	7	1	2
10	9	9	4	1	8
11	5	1	5	3	5
12	5	3	9	2	2
13	6	4	8	3	7
14	4	8	9	1	1
15	6	9	9	6	3
16	2	8	4	5	3
17	9	6	6	5	8
18	5	3	2	1	2
19	8	2	1	8	2
20	5	1	2	**8**	8

Table 3.1 Randomisation of participants. To randomise participants, three letter (A to E) and number (1 to 20) combinations were chosen at random. The number corresponding to this was read from the table above. For example, 20D would equal 8, as highlighted in bold.

Numbers	Order of block
0-1	PPII
2-3	PIPI
4-5	PIIP
6-7	IIPP
8	IPIP
9	IPPI

Table 3.2 Permuted blocks for randomisation. Permuted blocks were used to determine the order of infusions. P indicates placebo, I indicates iron.

3.2.4 Iron infusion and venous blood sampling

On day 1, participants received either 50 mL of sterile 0.9% m/V NaCl (placebo group) or 1 g of iron as FCM (Ferinject®,Vifor Pharma Ltd) diluted in 50 mL sterile 0.9% m/V NaCl (iron group), administered over 15 minutes via a syringe infusion pump (Graseby™ 3100 Syringe Pump, Smith Medical International Ltd, UK). Heart rate and blood pressure were measured before and after the infusion, and the participant was directly observed during the infusion. Participants were asked to report any side-effects experienced to the unblinded investigator giving the infusion.

On each experimental day, venous blood samples were taken from each participant before any other intervention took place. Haematological parameters including a full blood count, haematocrit and mean cell volume were processed through the laboratory at the John Radcliffe Hospital NHS Foundation Trust in Oxford. Similarly, iron markers including

ferritin concentration, transferrin concentration and transferrin saturation were determined by the John Radcliffe clinical biochemistry laboratory.

Erythropoietin and IL-6 were measured from serum in duplicate by ELISA as per manufacturer's instructions (Quantikine® IVD human EPO and Quantikine® high sensitivity human IL-6, R&D Systems, Abingdon, United Kingdom). Duplicate plasma samples were used to measure sTfR (Quantikine® IVD human sTfR, R&D Systems, Abingdon, United Kingdom) and hepcidin (Bachem, Peninsula Laboratories, Torrance, USA) according to the manufacturer's instructions. See Chapter Two for detailed methods.

3.2.5 Hypoxic exposure and assessment of PASP

Two purpose-built chambers were used for all hypoxic exposures (Howard *et al.*, 1995). The target end-tidal PO_2 was 55 mmHg and the target end-tidal PCO_2 was the naturally occurring baseline end-tidal value for the participant. This was assessed with ten minutes of quiet air breathing, when the participant was connected to the monitoring system, at the beginning of each experimental day before hypoxic exposure.

A fine nasal catheter was used to continuously sample respired gas from each participant's nostrils and analysed for $P_{ET O_2}$ and $P_{ET CO_2}$. The chamber gas composition was automatically adjusted in responses to $P_{ET O_2}$ and $P_{ET CO_2}$ at five minute intervals or manually overridden, so as to minimise the difference in $P_{ET O_2}$ and $P_{ET CO_2}$ between the desired and measured end-tidal values.

Doppler echocardiography was used to measure PASP. The velocity of a small jet of physiological tricuspid regurgitation was used, and PASP was calculated from the modified Bernoulli equation, with a value of 5 mmHg used for the clinical estimate of right atrial pressure. PASP was measured at baseline and hourly during hypoxic exposures.

3.2.6 Statistical analysis

Statistical analysis was conducted using either IBM SPSS Statistics version 22.0 or using the nlme package in R (version R3.1.0 Vienna, Austria). Comparisons between groups were made using either an unpaired two-tailed Student's t-test, or linear mixed-effects modelling using participants as a random factor. For the blood results, where there was one value per participant per day, the fixed effects were group (saline or iron), day and the interactive term between the two. For the measurements of PASP, the fixed effects were group (saline or iron), day and 'state' – euoxia or sustained hypoxia (hours 4, 5, 6, where the measurements of PASP were relatively stable), together with their interactive terms. Statistical significance was assumed at $p < 0.05$.

3.3 Results

All participants were healthy and iron-replete. The groups were well matched at baseline. Participant demographics and day 0 venous blood analyses are given in Table 3.3. There were no significant adverse effects reported by participants and all infusions of IV iron were well tolerated.

Variable (normal range/units)	Saline Group (n = 11)	Iron Group (n = 11)	*p* value
Age (yr)	26.3 ± 8.6	33.5 ± 9.9	0.09
BMI (kg/m^2)	24.3 ± 1.6	24.9 ± 2.1	0.51
Sex	Female n = 4 Male n = 7	Female n = 4 Male n = 7	
Haemoglobin (12.0-17.0 g/dL)	13.9 ± 1.3	14.0 ± 0.9	0.81
Haematocrit (0.36-0.50%)	0.42 ± 0.04	0.42 ± 0.03	0.77
Mean Cell Volume (83-105 fL)	93.3 ± 3.8	90.8 ± 5.0	0.20
Erythropoietin (IU/L)	8.1 ± 3.4	9.3 ± 3.0	0.38
Serum Ferritin (10-300 µg/L)	66 ± 40	55 ± 37	0.52
Serum Transferrin (1.8-3.6 g/L)	2.6 ± 0.4	2.7 ± 0.3	0.44
Serum Transferrin Saturation (16-50%)	31.3 ± 11.3	29.3 ± 12.7	0.70
Hepcidin (µg/L)	51.5 ± 57.9	40.2 ± 34.2	0.58
Serum Transferrin Receptor (nmol/L)	20.6 ± 5.9	21.5 ± 6.1	0.73
Interleukin-6 (ng/L)	0.56 ± 0.41	0.56 ± 0.4	0.97
C-reactive protein (0-8 mg/L)	0.8 ± 0.6	2.5 ± 3.2	0.30

Table 3.3 Participant demographics and baseline venous blood analyses. Values are mean ± SD. An unpaired two-tailed Student's t-test was used to calculate the *p* value. BMI, body mass index.

3.3.1 Haematology variables

The two groups were well matched at day 0 for haemoglobin, haematocrit and mean cell volume (Table 3.3). There was no significant difference over time in these variables between the saline and iron groups (Figure 3.3). The groups were similarly well matched for EPO at day 0 (Table 3.3), but these values changed over time between the iron and saline group ($p < 0.02$ for change in EPO over time) (Figure 3.3). This change over time represents the difference between group, day and the interactive term between the two across all time points using linear mixed effects modelling. However, when the same data were analysed using unpaired Student's t-tests there was no significant difference between the iron and saline groups for each individual day. Values are means and error bars are ± SEM.

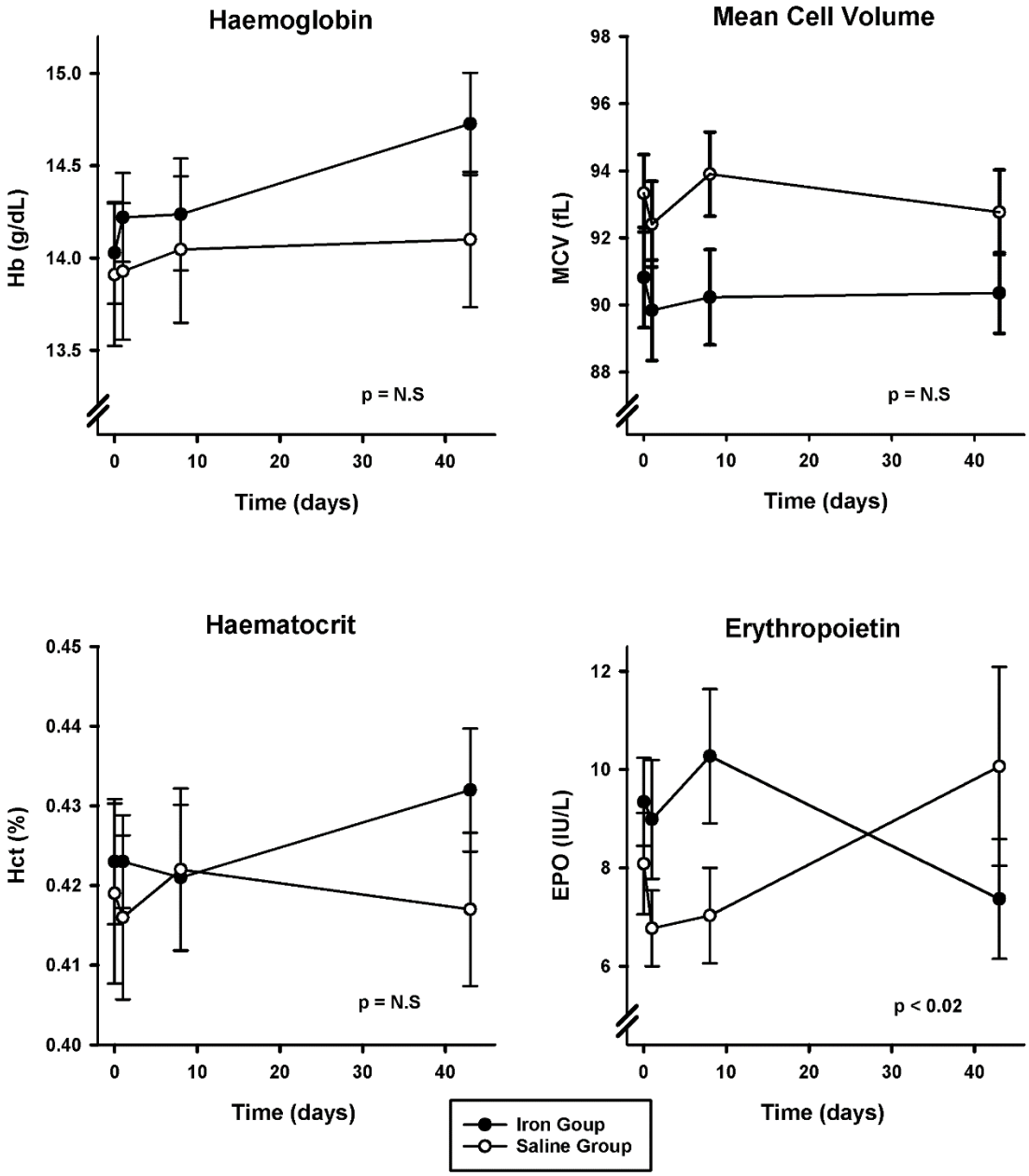

Figure 3.3 Haematology results. Following infusion of 1 g of iron, no significant changes were detected in haemoglobin concentration (Hb), haematocrit (Hct) or mean cell volume (MCV). There was a difference in erythropoietin concentration between the two groups ($p < 0.02$), which represents the difference between group, day and the interactive term between the two across all time points using linear mixed effects modelling. However, when the same data were analysed using unpaired Student's t-tests there was no significant difference between groups for each individual day. Values are means and error bars are ± SEM.

3.3.1 Assessment of iron status

Iron status values were well matched between the groups at day 0 (Table 3.3), including values for the concentrations of ferritin, transferrin, hepcidin, sTfR and transferrin saturation. Iron loading with 1g of iron led to an acute rise in ferritin concentration ($p < 0.001$), transferrin saturation ($p < 0.005$), and hepcidin concentration ($p < 0.001$) by day 8 (Figure 3.4). These changes remained significant at day 43. Transferrin concentrations fell post iron infusion and had not returned to pre-infusion levels by day 43 ($p < 0.001$) (Figure 3.4). Values for sTfR did not differ significantly over time between the saline and iron groups (Figure 3.4).

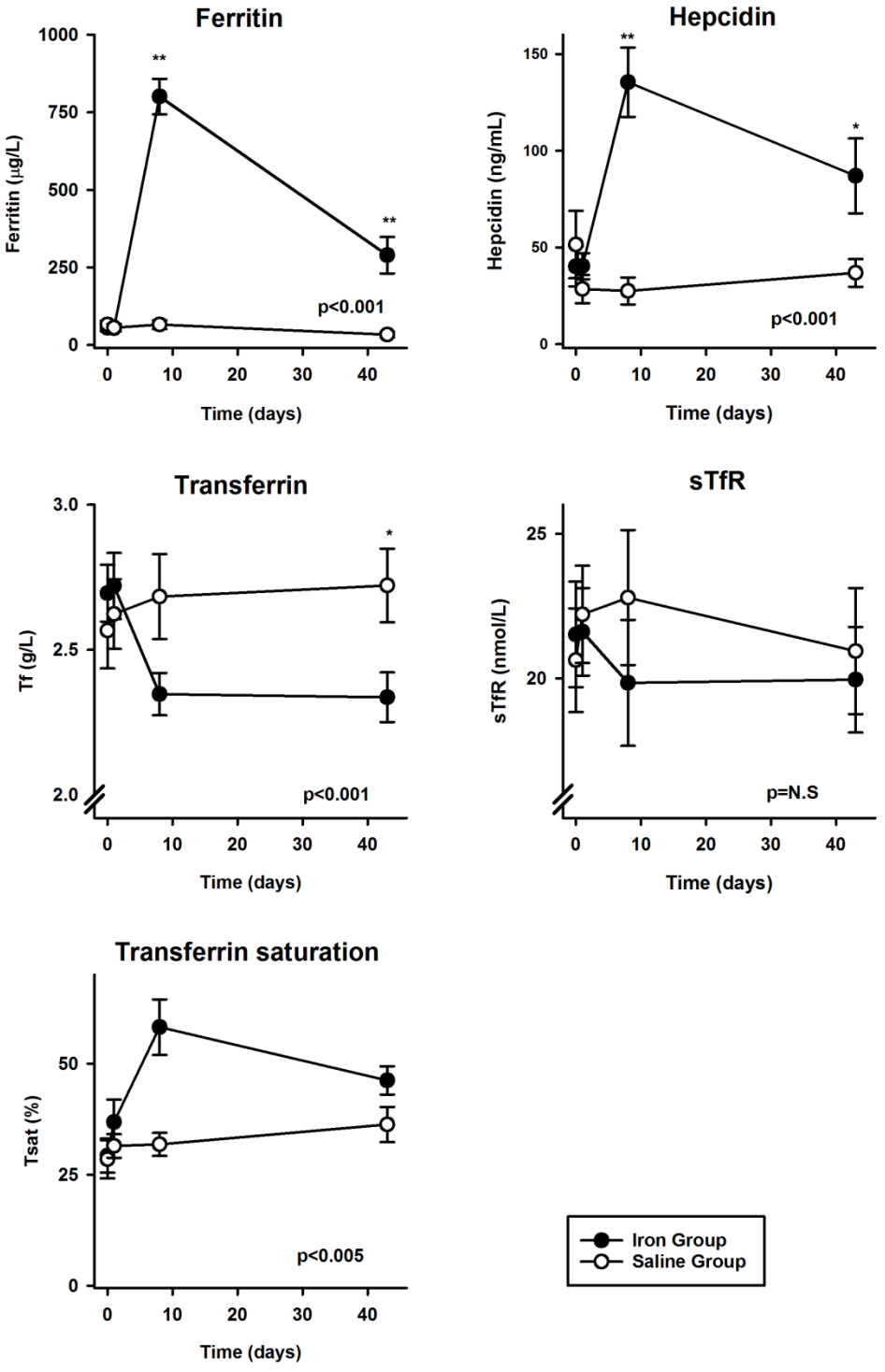

Figure 3.4 Iron indices. Following infusion of 1g of iron there were significant increases in the concentration of ferritin, transferrin saturation (Tsat) and hepcidin and a significant fall in transferrin concentration (Tf). No significant effects were observed in soluble transferrin receptor concentration (sTfR). Ferritin n = 6 for both groups at day 43. The ** represents a $p < 0.01$ and * represents $p < 0.05$. Values are means and error bars are ± SEM.

3.3.2 Inflammatory markers

Inflammatory markers CRP and IL-6 were well matched between groups at day 0 (Table 3.3). The normal value for CRP is 0.0-8.0 (mg/L), and no participant exceeded this value over the course of the study. Moreover, there were no acute rises in either inflammatory marker following a bolus of iron (Figure 3.5). There was no significant difference in CRP and IL-6 over time or between groups, suggesting that inflammation and infection did not confound the iron status results on any experimental day (Figure 3.5).

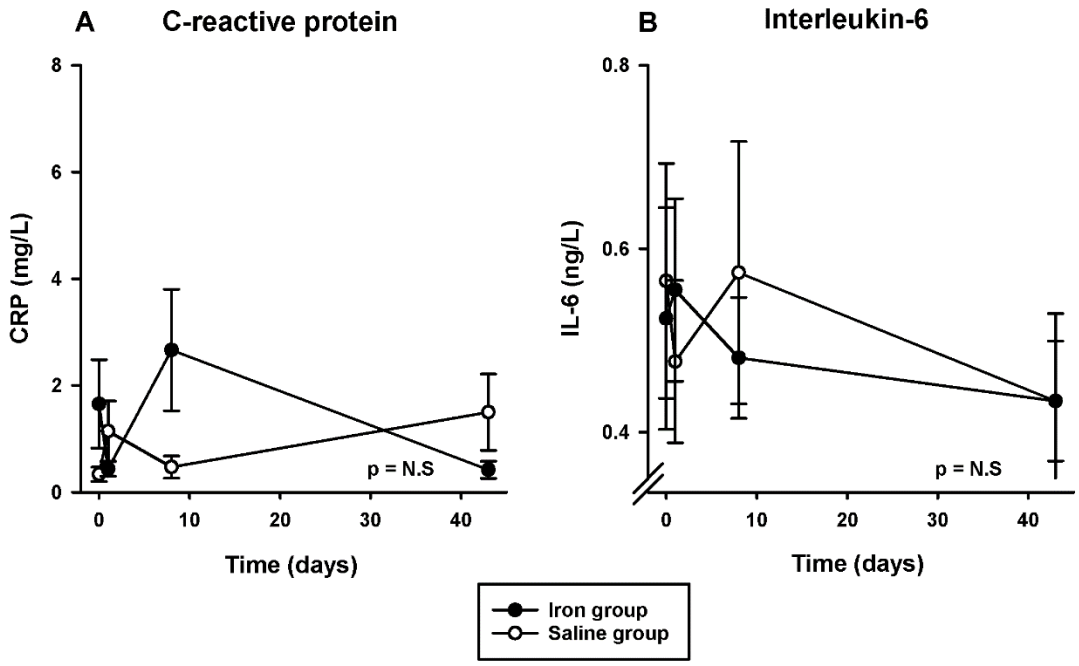

Figure 3.5 Inflammatory markers. **A** C-reactive protein (CRP). **B** Interleukin 6 (IL-6). There was no significant change in CRP and IL-6 over time. Neither CRP nor IL-6 went beyond the normal reference range during the course of the study. Values are means and error bars are ± SEM.

3.3.3 Gas control during sustained hypoxic exposure

Values for end-tidal P_{O_2} and P_{CO_2} were well matched between the two groups during the hypoxic exposures in the chamber on each experimental day (Figure 3.6 A/B and C/D respectively). Similarly, average oxyhaemoglobin saturation measured by pulse oximetry was well matched between the two groups across experimental days (Figure 3.6 E and F).

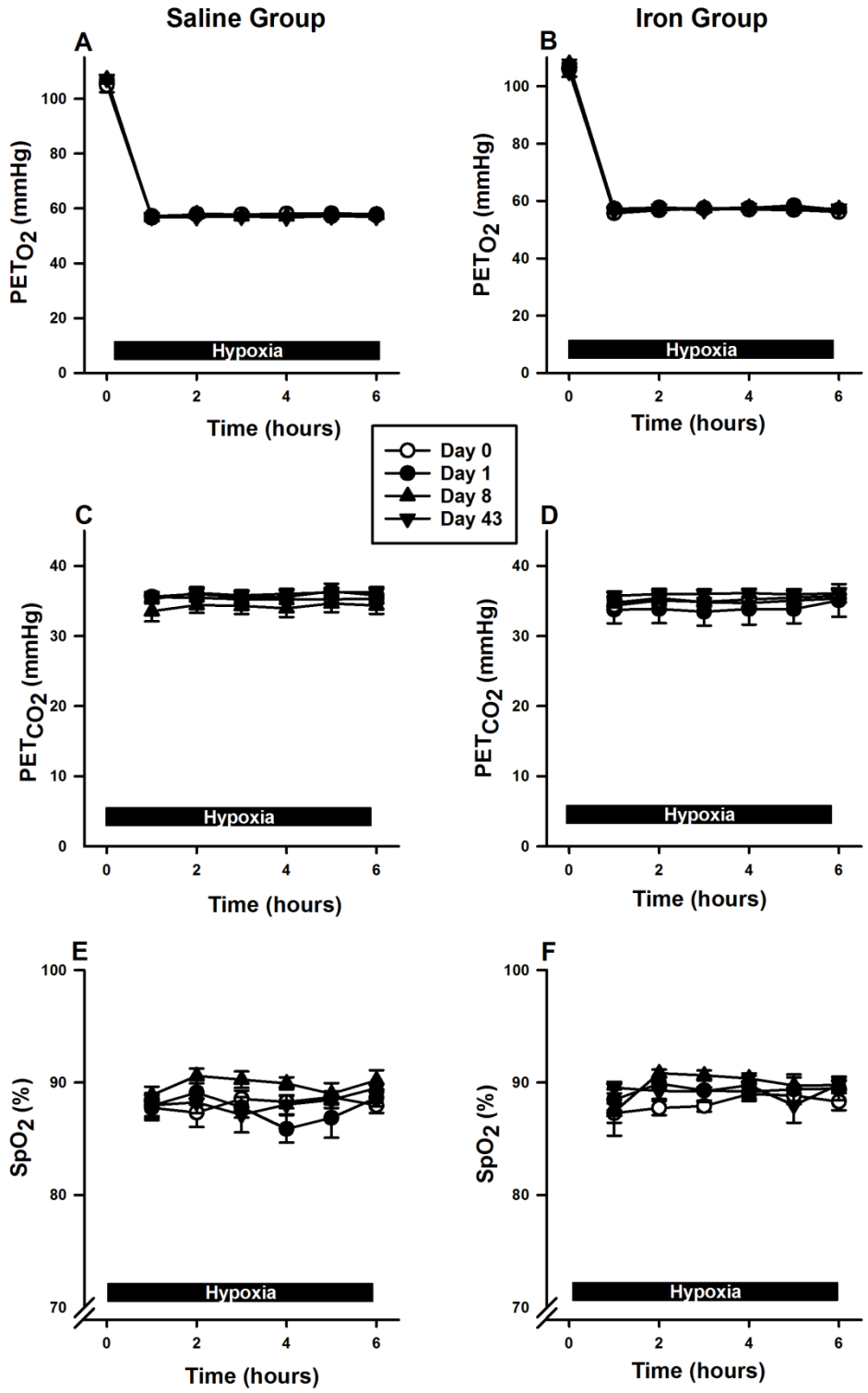

Figure 3.6 Gas control during exposure to hypoxia. **A, B** End-tidal oxygen (PET_{O2}) levels over six hours for saline and iron groups, respectively. **C, D** End-tidal carbon dioxide (PET_{CO2}) levels for saline and iron groups, respectively. **E, F** Arterial oxyhaemoglobin saturation (SpO_2) for saline and iron groups, respectively. Values are means at each hour of hypoxia and error bars are ± SEM.

Ventilation was not directly measured in this study. With sustained hypoxia there was a steady reduction in the inspired (chamber) P_{O_2} and increase in the inspired (chamber) P_{CO_2}, indirectly representing the increase in ventilation with sustained hypoxia. Figure 3.7 demonstrates the six hour chamber profile of one of the participants during hypoxia with $P_{ET_{O_2}}$ remaining constant at 55 mmHg and the $P_{ET_{CO_2}}$ remaining constant at the participant's own pre-determined end-tidal value. A steady fall in inspired (chamber) P_{O_2} (Figure 3.7 A) and corresponding rise in inspired (chamber) P_{CO_2} (Figure 3.7 B) can be seen.

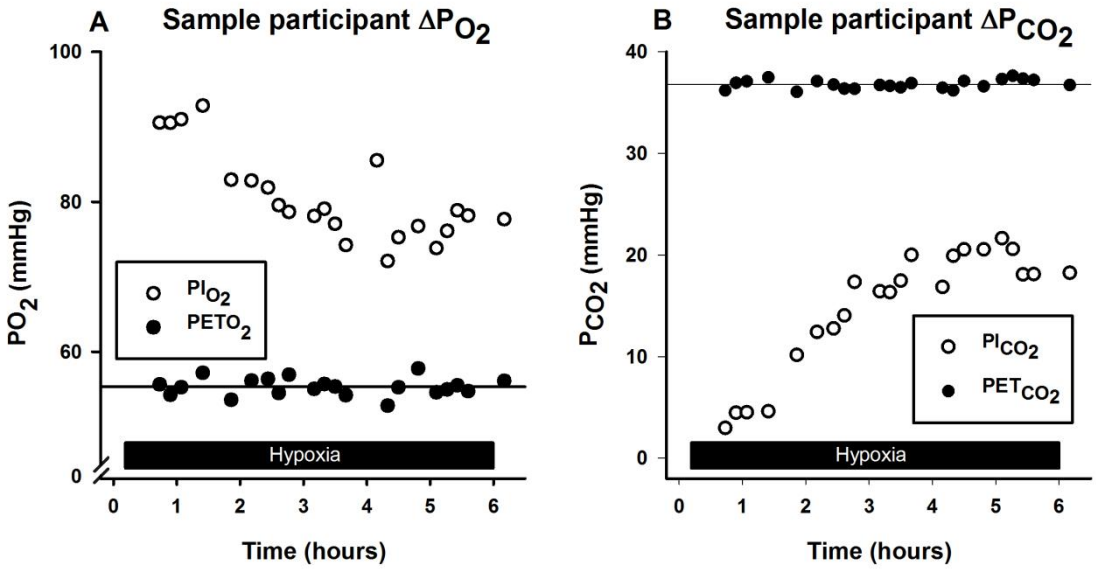

Figure 3.7 Sample participant partial pressure values A oxygen and **B** carbon dioxide during sustained isocapnic hypoxia. $P_{I_{O_2}}$ and $P_{I_{CO_2}}$ denote the inspired partial pressures of O_2 and CO_2 respectively. $P_{ET_{O_2}}$ and $P_{ET_{CO_2}}$ denote the end-tidal partial pressures of O_2 and CO_2 respectively. $P_{ET_{O_2}}$ was set at 55 mmHg; there was a fall in $P_{I_{O_2}}$ and a rise in $P_{I_{CO_2}}$ over time.

3.3.4 PASP response and EPO response to hypoxia

A progressive rise in PASP was observed in all participants over the six hour period of hypoxia. This rise in PASP over time is similar to what has been demonstrated in previous studies conducted both with echocardiography and invasively, which show a progressive rise in pulmonary vascular pressure with time (Dorrington *et al.*, 1997; Talbot *et al.*, 2014). There appeared to be a rapid rise in PASP over the first hour and then a slower rise, which plateaued over hours 4-6 (Talbot *et al.*, 2014).

Infusion of 1 g of IV iron attenuated the PASP response to hypoxia, and this remained significant at day 1, 8 and persisted at day 43 (Figure 3.8). Using linear mixed-effects modelling, the interactive terms between hypoxia and group on days 1, 8 and 43 were all significantly different from day 0 (each $p < 0.001$). The interactive term between hypoxia and group for day 0 (before iron was administered) was not significant. Thus, iron significantly attenuated the PASP response to hypoxia, by ~ 50% on day 1, and the response then remained suppressed over the six week study period.

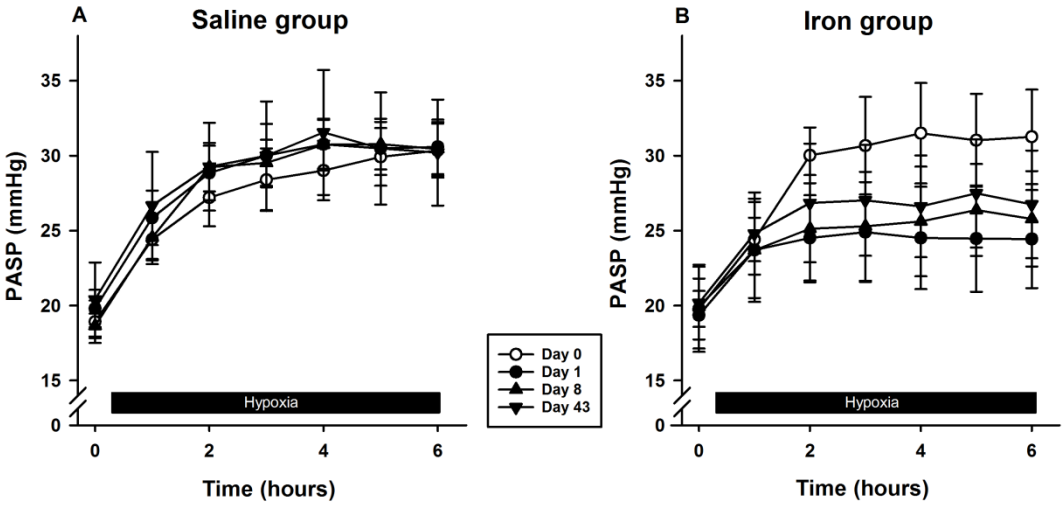

Figure 3.8 Pulmonary artery systolic pressure during sustained hypoxia. **A** saline group and **B** iron group. Responses to sustained hypoxia were blunted following iron infusion on day 1 and remained blunted at day 8 and 43 ($p < 0.001$). Values are means and error bars are ± SEM.

EPO concentration rose with exposure to hypoxia in both the iron and saline groups (Figure 3.9). There was no significant difference in the time-dependent rise in plasma EPO in response to six hours of sustained hypoxia between iron and saline groups (Figure 3.9).

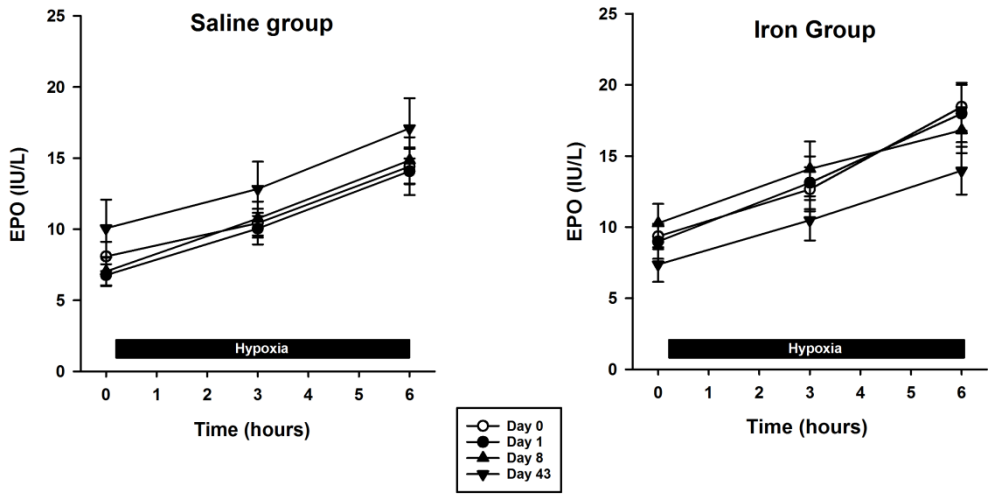

Figure 3.9 Time-dependent rise in erythropoietin with hypoxia. There was no significant difference in the rise in EPO between iron and saline groups. Values are means and error bars are ± SEM.

3.3.5 Change in hepcidin concentration with hypoxia

There was no significant difference in hepcidin concentration in response to six hours of sustained hypoxia between the iron and saline groups (Figure 3.10). There appeared to be no difference in hepcidin response with six hours of hypoxia, despite the 15-fold rise in the hepcidin concentration under euoxic conditions following iron infusion (Figure 3.10). In order to account for this, Δ hepcidin was used, which represents the difference between hour 6 and hour 0 for each individual participant on each chamber day. The groups were well matched for Δ hepcidin at baseline. There was no significant difference in the hepcidin response between iron and saline groups with exposure to hypoxia between days 0, 1, 8 and 43 using linear mixed-effects modelling. This is independent of the acute rise in hepcidin following iron infusion (Figure 3.10).

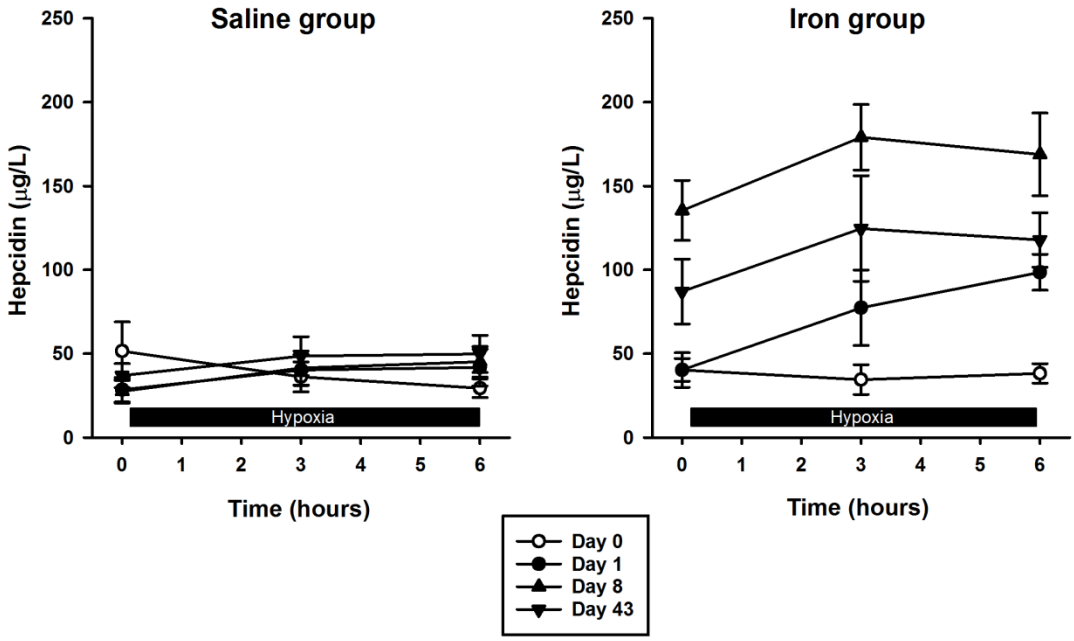

Figure 3.10 Change in hepcidin concentration with sustained hypoxia. There was an acute rise in hepcidin concentration following iron infusion. There was no significant difference in the hepcidin response between iron and saline groups with exposure to hypoxia. Values are means and error bars are ± SEM.

3.4 Discussion

3.4.1 Main finding: Intravenous iron abrogated the rise in PASP during sustained hypoxia

In this study, administration of a bolus dose of IV FCM resulted in a significant elevation in serum ferritin concentration, transferrin saturation and a decrease in transferrin concentration. This change in iron status resulted in an attenuation of the rise in PASP with sustained hypoxia. At 43 days post infusion, the change in iron markers remained significant, as did the effect of iron on PASP. Smith *et al.* have shown in humans that there is a close relationship between manipulation of iron status within the normal range and PASP (Smith *et al.*, 2008). These effects, however, were only measured in the short term (for up to four days) (Smith *et al.*, 2008; Smith *et al.*, 2009). Anker *et al.* have shown in heart failure patients that the effects of iron administration are long lasting, but the effects were measured qualitatively by NYHA class and symptom scores (Anker *et al.*, 2009). The results in this chapter demonstrate that iron administration affects blood iron parameters and pulmonary pressure measurements for up to six weeks post infusion.

3.4.2 Persistence of findings

The long lasting effects of IV iron on iron-replete individuals may be explained by the tight regulation of iron in the body. Administration of 1g of IV iron should elevate total iron body content by approximately 25% (Mole, 2010). The loss of iron from the body is approximately 1-2 mg/day (Finch & Huebers, 1982), suggesting that administration of 1g of iron, in the absence of any additional iron absorption or loss, will take 2-3 years for iron stores to return to levels seen prior to iron infusion.

We demonstrated that 1g of FCM resulted in a change in iron status with a significant rise in ferritin concentration and transferrin saturation and a decrease in transferrin concentration, which persisted for up to six weeks. As previously stated, FCM has a plasma half-life of 7-12 hours (Geisser & Banke-Bochita, 2010). By day 43, the foreign iron-sugar complex is eliminated from the body, suggesting that the iron has been incorporated into stores. Therefore, the change in iron markers is likely to be due to more than just the foreign iron-sugar complex itself.

What is not clear is the duration of the effect of these changes in ferritin concentration, transferrin saturation and transferrin concentration. This study shows that changes persist for 43 days. Anker *et al.* showed that iron parameters, including ferritin concentration and transferrin saturation, remained significantly elevated at week 24, with no change in haemoglobin in a non-anaemic population after iron infusion (Anker *et al.*, 2009). In that study, however, patients received 200 mg of FCM weekly until correction of their iron deficiency and then 200 mg monthly during the maintenance phase. In the current study, after a single bolus dose of iron, it took longer for iron markers to begin to fall back to baseline level when compared with a cohort of iron deficient patients in which iron levels decreased within 72 hours (Geisser & Banke-Bochita, 2010). This may reflect the different pharmacokinetics of IV iron depending on an individual's iron status. A question that still needs to be addressed is: when will these values return to normal in an iron-replete population whilst keeping other variables equal?

The present study shows an elevation in iron indices at days 8 and 43 following infusion of IV iron, by which time the iron-sugar complex has been eliminated from the body. This

suggests that the effects of iron administration on the pulmonary vasculature are due to something more than just the iron-sugar complex. This is in keeping with the rise in PASP with infusion of DFO with a time-course over eight hours that resembles that of exposure to hypoxia (Balanos *et al.*, 2002). In this study, the rise in PASP reached a steady state at three hours and remained elevated at eight hours.

Like the change in iron indices after the administration of IV iron, a similar uncertainty relates to the duration of the pulmonary vascular response to hypoxia. The persistent effect that iron has on hypoxia-induced PASP raises the question of whether this effect is related to circulating indices of iron status or more directly to the level of iron stores. It is of interest clinically that the effects of iron infusion on PASP are persistent, and are seen after the iron-sugar complex has been eliminated from the bloodstream, as iron stores could potentially be manipulated for therapeutic gain. An example of where this could be of benefit is in the management of pulmonary hypertension, which is associated with increased morbidity in hypoxic pulmonary disease (Barbera *et al.*, 2003). Moreover, in hypoxic lung disease, an intervention that could result in a persistent lowering of pulmonary vasoconstriction may be of benefit.

3.4.3 Mechanism behind the findings

This current study raises the question of the mechanism behind the effects of iron on the pulmonary vascular response to hypoxia. One hypothesis has been that iron availability affects the abundance of the transcription factor HIF, which itself is implicated in the pulmonary vascular response to hypoxia. It has been shown that iron modulates the HIF pathway (Wang & Semenza, 1993; Balanos *et al.*, 2002; Knowles *et al.*, 2006), and that

the HIF pathway is involved in the pulmonary vascular response to hypoxia (Yu *et al.*, 1999; Brusselmans *et al.*, 2003). Rare diseases of HIF upregulation demonstrate a phenotype which includes pulmonary hypertension and an exaggerated pulmonary vascular response. This is clearly seen in the case of Chuvash polycythaemia (Smith *et al.*, 2006b), as well as in other rare HIF-activating mutations linked to the development of pulmonary hypertension (Gale *et al.*, 2008).

If it were indeed the case that iron was acting via the HIF pathway, it could be expected that other HIF-regulated aspects of biology could be affected by manipulation of iron stores. For example, in humans it has been shown that iron chelation increases plasma EPO levels (Ren *et al.*, 2000). However, there have been no reports of iron manipulation affecting the ventilatory response to sustained hypoxia despite the fact that HIF has been implicated in this process (Kline *et al.*, 2002; Smith *et al.*, 2006b; Slingo *et al.*, 2014). This is similar to the PHD inhibitors, which have a marked effect on erythropoiesis, but to have minimal effects on the ventilatory response (Bishop *et al.*, 2013).

Biologically, there are many complex issues to consider. First, manipulation of iron stores may have much greater effects on some cell types than others. Therefore, the effects of manipulating the HIF pathway through iron stores may be specific to certain cell types. Secondly, HIF-1 and HIF-2 quite clearly have different actions (Loboda *et al.*, 2010; Prabhakar & Semenza, 2012a) and it may be the case that iron has differential effects in relation to these two isoforms (Hu *et al.*, 2003; Sowter *et al.*, 2003). Thirdly, although the classical effect of iron on the HIF pathway is through its involvement in the catalytic hydroxylation of HIF by the PHD enzymes, it has also been shown that cellular iron status

affects the translation of HIF-2α through the presence of an IRE in the 5' end of HIF-2α mRNA (Sanchez *et al.*, 2007; Wilkinson & Pantopoulos, 2013). Again, this is a potential mechanism that could underlie both cell-specific and isoform-specific actions of iron on the HIF pathway.

From this study and the literature to date, it cannot be concluded that the actions of iron availability on HIF are indeed the mechanism by which iron affects the pulmonary vascular response to hypoxia. The mechanisms of HIF pathway activation on the pulmonary vasculature are difficult to prove and as yet there is no method of quantifying HIF concentrations in the human lung. Moreover, HIF-gene targets such as EPO exert their effects more peripherally and may not reflect the effect of HIF within the lungs.

3.4.4 Alternative explanations for PASP response

Hypoxic pulmonary responses may be mediated by HIF-independent mechanisms. One theory is that free radicals generated by hypoxia cause a direct effect on potassium channels and consequently PASMC function (Weir & Archer, 1995). Iron may generate ROS, which may in turn affect voltage-gated potassium channels, which are known to play a role in the hypoxic pulmonary vascular response (Moudgil *et al.*, 2006). Of note, whether hypoxia causes an increase or decrease in ROS and the exact interaction with the pulmonary vasculature is unknown (Veit *et al.*, 2015). Iron seemed to have more of an effect on the second, slower phase of hypoxic pulmonary vasoconstriction in the current study, which has been seen previously (Talbot *et al.*, 2014). It could be expected that free radical generation may have a more immediate effect, as they could be limited by their

short half-life (Forkink *et al.*, 2010). The delay may, however, be attributed to the time taken for the free radicals to be taken up in the pulmonary arterial myocytes.

Another argument for HIF-independent mechanisms mediating hypoxic pulmonary vasoconstriction is seen in the conflicting results with ascorbic acid to date. The prolyl hydroxylases that hydroxylate HIF require oxygen as a substrate and iron and ascorbate as co-factors (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). Interestingly, variation in availability of ascorbate has been shown to have similar effects to iron in cultured cells (Knowles *et al.*, 2003). It can therefore be hypothesised that manipulation of ascorbate levels may have equally similar effects to iron *in vivo*, particularly in relation to pulmonary vasoconstriction. This theory is strengthened by case reports of patients with scurvy who present with severe pulmonary hypertension that is reversible after treatment with ascorbate (Kupari & Rapola, 2012; Duvall *et al.*, 2013). Against these findings, a study by Talbot *et al.* found no change in PASP in response to hypoxia after administration of 3 g of ascorbic acid (Talbot *et al.*, 2014). The normal dose to treat scurvy is 1 g, so a dose three times this was expected to have some effect. The lack of impact of ascorbic acid on PASP suggests that pulmonary vasoconstriction may be mediated by a yet undiscovered iron-dependent oxygen sensor. One likely source could be a member of the iron-dependent dioxygenase family. There are already some enzymes in this family that have been shown to sense changes in oxygen and iron availability (Elkins *et al.*, 2003; Elvidge *et al.*, 2006). An alternate explanation is that the pulmonary effects of iron are indeed HIF-dependent but via a mechanism that is independent of HIF hydroxylation.

3.4.5 Time course of pulmonary vascular response

In the current study, it was observed that the effects of iron on PASP appear to occur after a period of one hour of sustained hypoxia (Figure 3.8). Previous work has shown that the rise in PASP with exposure to hypoxia has two temporal components (Talbot *et al.*, 2005), and the second phase may be the result of the time for de novo vasoconstrictor substances to be synthesised after activation of gene expression. The effect of IV iron in the current study is consistent with the findings of Talbot *et al.* where the pulmonary vascular response to IV iron occurred only during the second phase (Talbot *et al.*, 2014), and the time lag was ~ 45 minutes. Taken together, these findings suggest that iron does not affect the initial hypoxic pulmonary vascular response, but rather the second phase of intensification of the hypoxic pulmonary vasoconstriction that begins after ~ 45 minutes and causes a further progressive rise in PASP (Talbot *et al.*, 2005). The fact that iron affects only the second phase, which is believed to be gene regulated, may implicate involvement of HIF-related hypoxia induced gene targets in this process.

3.4.6 Individual variation in hypoxia response

In our study we found that there was a range of individual PASP and EPO responses. Bärtsch and Swenson concluded in a review paper that 'there are currently no reliable tests to predict susceptibility to high altitude illnesses during an ascent' (Bartsch & Swenson, 2013). The importance of the role of HIF in coordinating the cellular response to hypoxia is well known, while the individual variation in response to hypoxia and its implications are still a source of investigation. For example, at a molecular level there may be inter-individual variation in the HIF system responsiveness (Brooks *et al.*, 2009). This is important clinically, because at present there are no reliable tests to determine an

individual's response to hypoxia. This form of testing might determine, for example, who will become unwell when ascending to altitude. In a hospital setting, such testing might predict the response of two different patients with similar co-morbidities to the same hypoxic insult. HIF variation at a systems level is demonstrated in a study of 80 participants that showed no correlation between measures of ventilatory and pulmonary vascular acclimatisation (Fatemian *et al.*, 2016). Similar findings were observed in a high altitude study, where authors found no correlation between the eucapnic hypoxic ventilatory responses (both poikilocapnic and isocapnic measurements) at sea level, and the increase in PASP at 5050 m elevation (Hoiland *et al.*, 2015).

In clinical disease states, there is great variation in the pathophysiological response to hypoxia. For example, in the spectrum of COPD patients, some develop type I respiratory failure (Pa_{O_2} < 60 mmHg and normal Pa_{CO_2}), whereas others develop type II respiratory failure (Pa_{O_2} < 60 mmHg and Pa_{CO_2} > 45 mmHg) despite the same degree of airflow obstruction and similar results on pulmonary function testing. Moreover, some patients with hypoxic lung disease may develop pulmonary hypertension that is disproportionate to their disease state (Nathan *et al.*, 2007; Harari, 2012), and subsequently clinically deteriorate (Oswald-Mammosser *et al.*, 1995; Nadrous *et al.*, 2005). There is certainly scope for future work with regards to phenotypic variation in hypoxic disease states and whether this relates to the HIF axis.

3.4.7 The effect of iron on erythropoiesis

The current study demonstrated the expected time-dependent rise in EPO with sustained hypoxia (Abbrecht & Littell, 1972; Richalet *et al.*, 1994; Smith *et al.*, 2008). IV iron

infusion did not alter this time-dependent rise, which was similar to previous studies in our laboratory (Smith *et al.*, 2008). If HIF was indeed the mechanism behind the attenuation in PASP observed in this study, it may be expected that the rise in EPO with hypoxia may be similarly attenuated. Infusion with DFO led to a rise in PASP, and demonstrated a similar time course to that of hypoxia (Balanos *et al.*, 2002), along with an increase in EPO levels (Ren *et al.*, 2000), which has been speculated to be via a HIF mediated mechanism. Interestingly, in a study conducted at an altitude of 4340 m there was a significant attenuation in PASP after administration of iron, but no difference between groups in the rise in EPO (Smith *et al.*, 2009). These results are similar to those in the present study. Of note, in a study investigating the differences in the hypoxic response between Tibetans and Han Chinese, Tibetans demonstrated hypo-responsiveness in pulmonary vasoconstriction, and the rise in EPO in this cohort correlated significantly with the EPAS1 and the EGLN1 loci (Petousi *et al.*, 2014). In addition, Tibetans had less activation of expression of HIF target genes such as VEGF A and aldolase C. One conclusion that could be drawn from this is that IV iron affects the pulmonary and erythropoietic cells via different unidentified mechanisms and that further gene studies are necessary to elucidate this.

3.4.8 Hepcidin response to iron and sustained hypoxia

In the current study, there was an acute rise in hepcidin concentration following administration of IV iron, an effect that persisted for six weeks. This is consistent with other recent studies following administration of similar doses of IV iron (Chand *et al.*, 2015; Gaillard *et al.*, 2016). There is a suggestion that the rate of hepcidin response may predict response to IV iron (Steensma *et al.*, 2015). Moreover, there is some evidence to suggest that this acute hepcidin rise after a large dose of IV iron may limit response to

further doses by decreasing the rate of iron supply to transferrin and thus limiting the utilisation of available iron. This may favour smaller incremental IV doses.

With chronic hypoxia there is a fall in hepcidin concentration in order to increase gastrointestinal absorption of iron to meet the body's increased erythropoietic drive (Nicolas *et al.*, 2002; Liu *et al.*, 2012; Talbot *et al.*, 2012). It is likely that hypoxemia itself, via an uncharacterised signalling mechanism, stimulates the fall in hepcidin rather than a drop in serum iron concentration. Erythroferrone, via a bone marrow dependent pathway, has been speculated to be one possible mechanism. The hormone, erythroferrone, is produced by erythroblasts and acts to suppress hepcidin transcription in the liver (Kautz *et al.*, 2014a). In further supporting evidence for this proposed mechanism, erythroferrone increases iron levels in periods of physiological stress, such as acute haemorrhage, which stimulates the recovery of erythrocytes (Kautz *et al.*, 2014a).

In this study there was a significant increase in hepcidin concentration after iron infusion, which has been shown previously after a bolus dose of iron (Lin *et al.*, 2007; Ganz *et al.*, 2008). In order to account for this, the delta hepcidin (h6-h0) for each day was analysed. On day 1 immediately post iron infusion, there was a significant difference in delta hepcidin between the iron group and the saline group with exposure to hypoxia ($p > 0.01$). This is likely to represent the acute rise in hepcidin post iron rather than any effect of interaction with hypoxia.

Talbot *et al.* found that at altitude there was a significant reduction of hepcidin concentration after 48 hours of hypoxic exposure (Talbot *et al.*, 2012). With prior iron

loading there was a delay in the hypoxia-driven decrease in hepcidin until after the transferrin saturation, rather than the ferritin concentration, normalised (Talbot *et al.*, 2012). In this study, the finding that there was no significant difference in delta hepcidin at days 8 and 43, despite the significant rise in hepcidin concentration, suggests that iron status does not affect the hepcidin response to hypoxia.

3.4.9 Methodological considerations

3.4.9.1 ΔPmax as a measurement of pulmonary artery systolic pressure.

The normal range for PASP in healthy individuals is 11-30 mmHg (Rudski *et al.*, 2010). PASP appears to rise with age, as does the sensitivity of pulmonary vasoconstriction to hypoxia (Pugh & Balanos, 2013; Balanos *et al.*, 2015). The mechanisms behind these changes may be related to a decline in peripheral arterial oxygen saturation (Turner *et al.*, 2015), increased left atrial pressures (Redfield *et al.*, 2003), as well as arterial remodelling with resultant vascular stiffening (Mackay *et al.*, 1978) that can all occur with aging. The normal values for the clinical estimate of right atrial pressure are -2 to 10 mmHg (Rudski *et al.*, 2010; Beigel *et al.*, 2013). Pathological increases beyond this range are attributed to derangements of fluid status and right heart strain or failure.

One limitation of this study is that cardiac output is not presented. However, previous studies have shown that PASP is relatively insensitive to changes in cardiac output, and is a reliable indicator of hypoxic pulmonary vasoconstriction (Balanos *et al.*, 2005). Balanos *et al.* investigated changes in these parameters with diurnal variation and hypoxia. In air breathing participants, cardiac output could vary by ~ 2.5 l/min with little variation in

ΔP_{max}. When eight of these participants were exposed to eight hours of hypoxia only ~ 5% of the changes in ΔP_{max} could be accounted for by concurrent changes in cardiac output (Balanos *et al.*, 2005). Previous studies in our laboratory have shown no effect of intravenous iron on cardiac output (Smith *et al.*, 2008; Talbot *et al.*, 2014).

The gold standard for assessment of pulmonary arterial pressure is a right heart catheterisation. This is an invasive test, and not ideal in a research setting. Therefore Doppler echocardiography was used as a surrogate measure. Yock and Popp found that Doppler echocardiography measurements and catheter values for PASP correlated well (r = 0.93) in a group of patients with measurable tricuspid regurgitation (Yock & Popp, 1984). A further study performed at altitude on 28 participants found that Doppler and invasive testing correlated well (Allemann *et al.*, 2000). The limitations of Doppler echocardiography include technician experience, the size of the tricuspid regurgitation jet and the angle of the continuous wave Doppler in relation to this. In the setting of hypoxia-driven tachypnoea, difficult apical windows can also constitute a limitation. Despite these limitations, Doppler echocardiography is considered a reliable and non-invasive technique for measurement of PASP (D'Alto *et al.*, 2013).

For all participants the right atrial pressure was assumed to be 5 mmHg as they were healthy and had no cardiopulmonary disease. One limitation of this study was that a normal RAP was assumed rather than confirmed by reviewing the size and collapsibility of the inferior vena cava as has been done in other studies (Rudski *et al.*, 2010; Beigel *et al.*, 2013).

3.5 Conclusions

This study demonstrates that in healthy, iron-replete and non-anaemic participants, infusion of 1g of IV iron leads to a persistent rise in ferritin concentration and transferrin saturation with a decrease in transferrin concentration. The persistent elevation in iron indices continues long after the foreign iron-sugar complex has been eliminated from the blood stream. The change in iron indices corresponded to a persistent attenuation in the rise in PASP during exposure to sustained hypoxia. This has implications for hypoxic lung diseases, where pulmonary hypertension is associated with increased morbidity and mortality.

There is much scope for future work, which would include studies that attempt to understand the underlying mechanisms behind the attenuation in PASP with iron infusion, focusing particularly on the role of HIF and HIF-related gene targets in the slower, latent stage of pulmonary vasoconstriction.

CHAPTER FOUR:

STUDY B

4 Study B: Effects on iron indices of modest iron loading in healthy individuals

4.1 Introduction

Iron deficiency is clinically important even in the absence of anaemia. However, the threshold values of various iron indices, on which a diagnosis of iron deficiency can be made, are not clearly defined. Central to the diagnosis of iron deficiency is serum ferritin for concentrations < 12 µg/L are very likely to be associated with absolute iron deficiency (Cook & Skikne, 1982; Cavill, 1999). However, as ferritin is also an acute phase reactant, its value may be falsely high in the presence of inflammation. Transferrin saturation may also be helpful, with a value > 20% being associated with normal red cell indices and a value of < 16% indicating an insufficient supply of iron to the bone marrow, such that anaemia will eventually ensue (Bainton & Finch, 1964).

Many studies in this area use different criteria for defining iron deficiency. In two major randomised control trials investigating the use of IV iron in treating patients with iron deficiency and chronic heart failure, a diagnostic threshold for iron deficiency was ferritin of < 100 µg/L, or alternatively a ferritin of < 300 µg/L in combination with a transferrin saturation of < 20% (Anker *et al.*, 2009; Ponikowski *et al.*, 2015). These are much less stringent definitions of iron deficiency than ferritin < 12 µg/L (see above). In comparison, a recent cross-sectional study of iron deficiency in a population of patients with chronic obstructive pulmonary disease (COPD) showed a median value for ferritin of 28 µg/L in the iron deficient group (defined as those with either a transferrin saturation < 16%, or an elevated soluble transferrin receptor > 28.1 nmol/L), and a median ferritin value of 80

µg/L in the iron-replete group (Nickol *et al.*, 2015). This study reported a prevalence of iron deficiency of 18% and 5% for the COPD and control groups, respectively. Moreover, if the diagnostic criteria for iron deficiency of a ferritin < 100 µg/L was applied to the same cohort, 60% of the participants would be diagnosed with iron deficiency, which would include both those with COPD and those who were regarded as healthy individuals.

Iron supplementation is possible via both oral and IV routes. While the oral route may seem more practicable, the tight regulatory control of iron balance may lead to the potential for non-absorption in iron-replete individuals. Some disease states result in an elevated level of hepcidin, meaning that oral iron may not be sufficiently absorbed in the gastrointestinal tract due to the regulatory action of this hormone. In addition, oral iron supplementation carries the risk of gastrointestinal side effects; IV iron administration circumvents these issues.

Intravenous iron is a more appropriate route of administration in some groups, such as those with chronic renal failure where inflammation and an inappropriate hepcidin response play a role (Charytan *et al.*, 2005; Qunibi *et al.*, 2011). Administration of IV iron in this population is also associated with a significant improvement in haemoglobin compared to oral iron, with fewer side effects. Iron supplementation may have a therapeutic benefit in the setting of a systemic hypoxic insult. For example, perioperative adjustment in iron stores reduces peri-procedure transfusion requirements and associated morbidity (Bisbe *et al.*, 2005; Karkouti *et al.*, 2006; Bisbe *et al.*, 2011).

Intravenous iron has effects on the pulmonary vasculature, with iron infusion protecting individuals against the progressive rise in PASP that occurs with sustained hypoxia (Smith *et al.*, 2008; Smith *et al.*, 2009). This observation appears to be related to the recent finding of rapid evolutionary selection in Tibetans for a functionally hypomorphic allele of the transcription factor known as HIF-2α *(EPAS1*) (Beall *et al.*, 2010), a gene that encodes for significant parts of the HIF axis. Decreasing iron availability promotes the translation of HIF-2α mRNA and also limits the degradation of the HIF-2α protein (Ghosh *et al.*, 2013; Wilkinson & Pantopoulos, 2013). Studies in Tibetan populations have shown that they have a blunted hypoxic pulmonary vascular response and higher pulmonary ventilation relative to metabolism (Petousi *et al.*, 2014).

In humans exposed to hypoxia, it may be beneficial to elevate iron to mildly supra-normal levels. Despite the laxity of the inclusion criteria for iron deficiency in the major randomised control trials of IV iron in chronic heart failure (Anker *et al.*, 2009; Ponikowski *et al.*, 2015), it is clear that patients benefitted from iron infusion. This benefit was not restricted to particular subgroups and was independent of haemoglobin. As such, an alternative interpretation of these trial results is that it may be beneficial to modestly elevate patients' iron stores above their normal physiological values in chronic heart failure, and this may extend to other hypoxic cardiorespiratory conditions. If so, it then becomes important to understand the dose-response relationship between additional iron load and serum values for various iron indices.

Aims of Study B:

To determine a dose for IV administration of iron that will cause a mild elevation of iron indices and is therefore practical in a health care setting.

1) To determine the effects of supra-therapeutic iron levels on haematological parameters, iron haematinics, and a downstream marker of hypoxia inducible factor i.e. EPO.
2) To determine whether smaller, incremental iron doses influence the pulmonary vascular response after a period of sustained hypoxia.

4.2 Methods

4.2.1 Sample size calculation

The purpose of this study was to define a response between iron loading and ferritin level. The SD for the within-subject variation in ferritin concentration, as measured at the John Radcliffe Hospital laboratory, Oxford, is < 2 μg/L, and at 15 days post infusion of 1 g of iron, a change in ferritin of ~ 250 μg/L is expected. The study was powered at 80%, with a two-tailed significance of $p < 0.05$. A sample size of 18 was determined, with 12 participants randomised to receive iron infusions, and the anticipated outcome was to be able to define the response of each subject in terms of iron status. In the design of the study, minimal variation in iron status was expected in the placebo group. The 12 individual responses from participants receiving iron were expected to provide a good measure of the range of variation in response observed between healthy subjects after receiving IV iron infusions.

4.2.2 Protocol

The study received ethical approval by the South Central National Research Ethics Service (11/SC/0530) and was performed in accordance with the *Declaration of Helsinki*. The study protocol is detailed in Figure 4.1. This was a double-blind, placebo controlled study. Eighteen healthy men were recruited via advertisement. Participants were required to be non-smokers and iron-replete. Individuals with ferritin > 300 μg/L and a normal CRP or with a transferrin saturation of > 45% were excluded from the study. Individuals with no detectable tricuspid regurgitation, as assessed by Doppler echocardiography, were excluded from the study as this was required to assess pulmonary arterial pressure. All

participants provided written, informed consent. Participants were randomised 2:1 to iron (n = 12) or saline (n = 6) infusions. A similar method of randomisation using permuted blocks is shown in Chapter Three.

There were two full study days at the beginning and end of six months (month 0 and month 6) in each arm of the study. At month 0, baseline blood measurements for haematology variables, red cell mass and iron indices were taken. In addition, physiological baseline variables, including PASP and Δ PASP, were measured. Each month over a six month period, participants underwent a blood test and based on the result received an infusion of either saline or iron. An unblinded investigator who was not involved in any of the analysis administered the infusions, and participants wore a blindfold so that they were blinded to the infusions. At the beginning and end of six months, participants spent a period of eight hours in a specialised chamber and were exposed to sustained hypoxia and hourly echocardiographic measurements. The protocol for each individual chamber day is seen in Figure 4.2. At months 0, 3 and 6, participants underwent a closed circuit re-breathing technique with carbon monoxide to obtain red cell mass measurements.

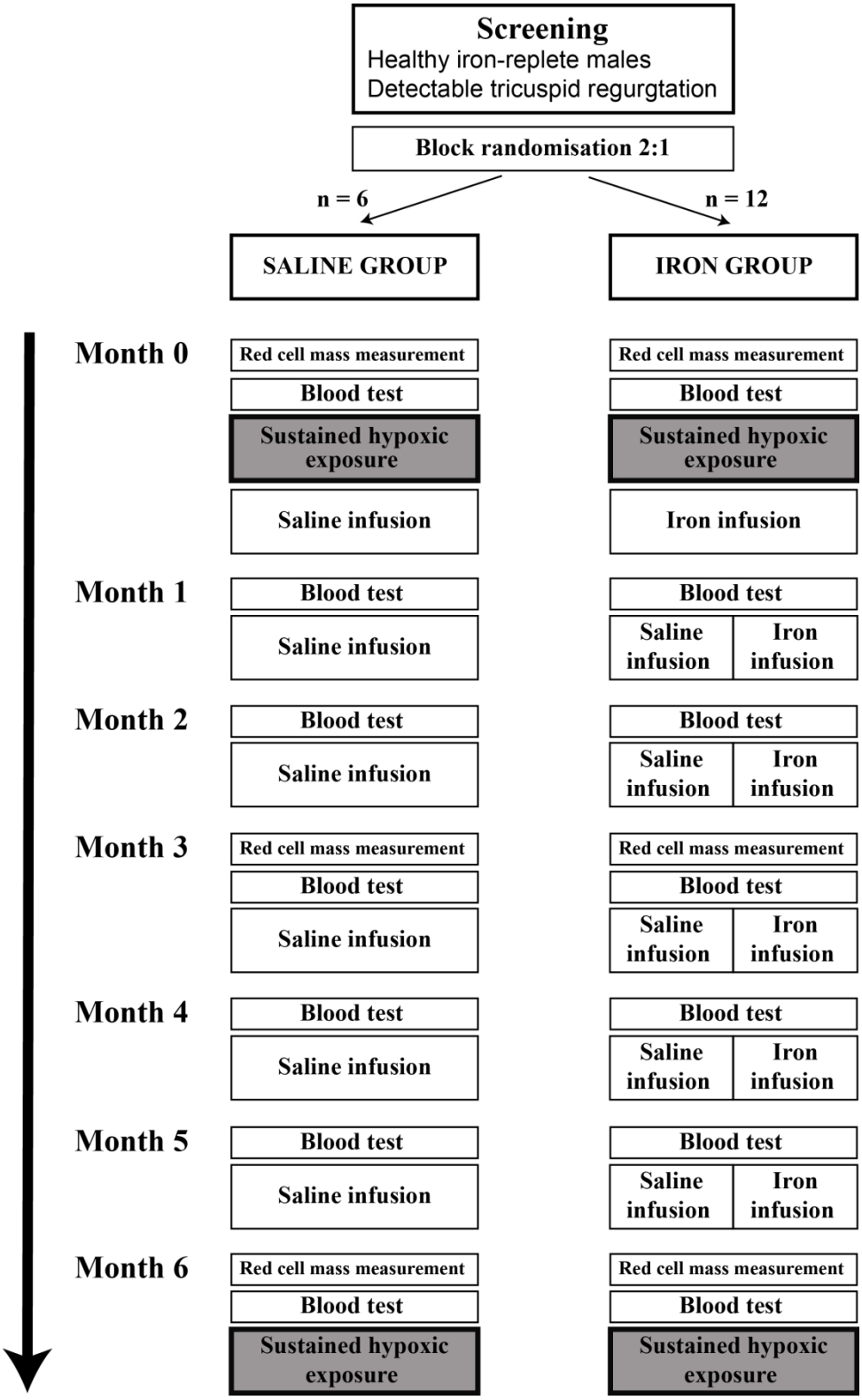

Figure 4.1 Six month protocol for Study B. Eighteen participants were recruited and block randomised to saline (n = 6) or iron (n = 12). Participants had a blood test and subsequent infusion of either saline or IV iron every month for the six month period. Participants were exposed to sustained isocapnic hypoxia at the beginning and end of the six month period.

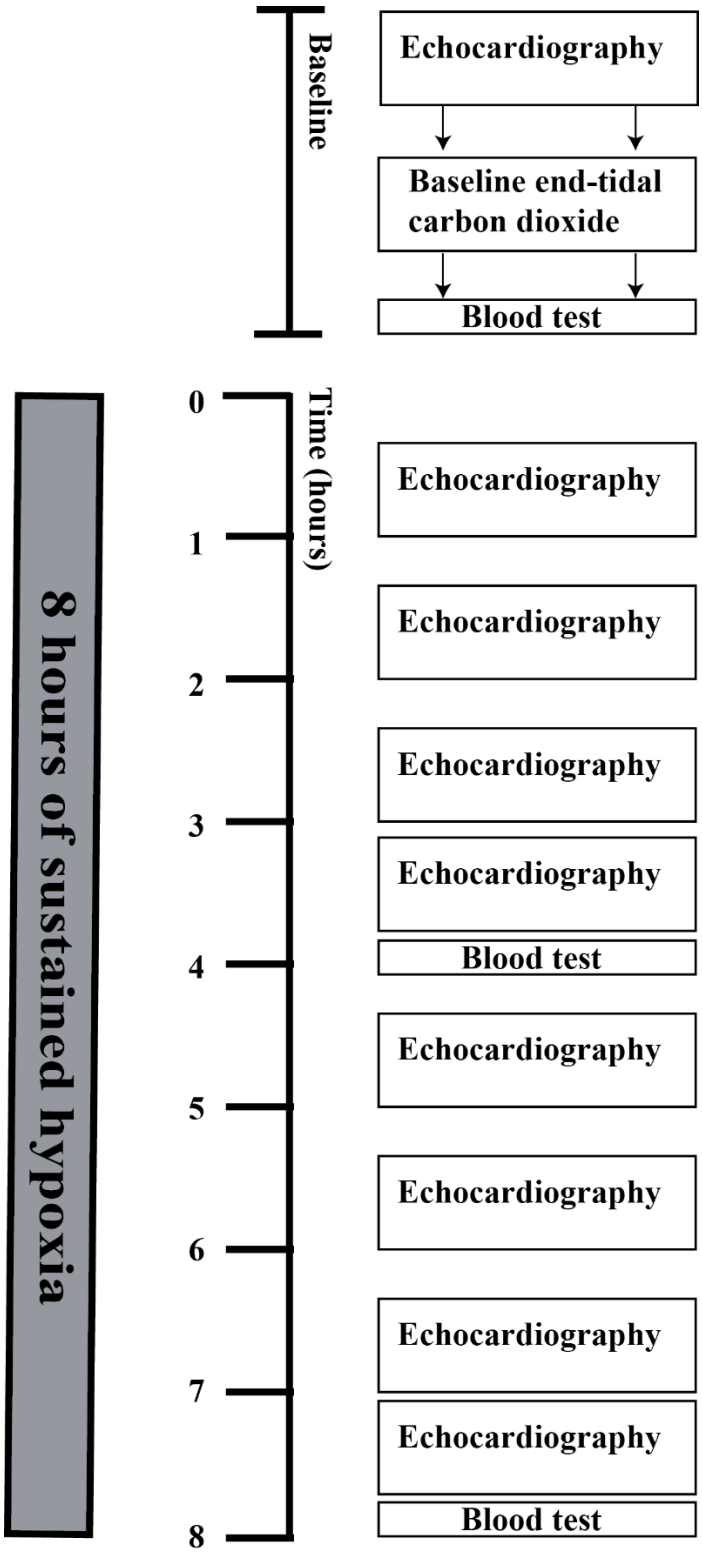

Figure 4.2 Protocol for each experimental day. There were two full study days (month 0 and month 6) in each arm of the study. Baseline measurements including blood tests, echocardiography and end-tidal gases were conducted at the start of the experimental day. Participants were then exposed to eight hours of sustained isocapnic hypoxia, during which they had hourly echocardiograms and blood tests at hours 4 and 8.

4.2.3 Iron infusion and venous blood sampling

Participants received an IV infusion of either placebo (0.9% m/V NaCl) or FCM (Ferinject®,Vifor Pharma Ltd) at a dose of 3.75 mg/kg (maximum dose of 250 mg which is 25% of the maximal single dose that can be administered) diluted in 50 mL sterile 0.9% m/V NaCl (iron group). Infusions were administered over 15 minutes via a syringe infusion pump (Graseby™ 3100 Syringe Pump, Smith Medical International Ltd, UK). Participants received one infusion of either placebo or iron every month for six months. Blood tests were taken each month before the infusion. If the participant's ferritin was < 300 μg/L with a normal CRP and their transferrin saturation was < 45%, then they received a further increment in FCM at 3.75 mg/kg. The maximum total dose of iron received by any individual during this protocol was limited to 1.5 g.

Each month over the six month period, venous blood samples were taken from each participant. Haematology including a full blood count, haematocrit and mean cell volume, was performed by the John Radcliffe Hospital NHS Foundation Trust haematology laboratory. Iron markers, including ferritin concentration, transferrin concentration and transferrin saturation, were determined by the clinical biochemistry laboratory at the same hospital.

During the six month period, unblinded medical practitioners had access to blood test results. An unblinded medical practitioner reviewed the results of the blood test and decided whether to administer iron or saline according to the set protocol. Any blood test

abnormalities were managed appropriately in conjunction with the participant's nominated general practitioner.

Enzyme-linked immunosorbent assays were performed in duplicate on serum to measure EPO and interleukin 6 (IL-6) (Quantikine® IVD human EPO and Quantikine® high sensitivity human IL-6, R&D Systems, Abingdon, United Kingdom). Plasma samples were used to measure sTfR (Quantikine® IVD human sTfR, R&D Systems, Abingdon, United Kingdom) and hepcidin (Bachem, Peninsula Laboratories, Torrance, USA) according to the manufacturers' instructions.

4.2.4 Red cell mass measurements

A closed circuit rebreathing technique was used to assess changes in red cell mass. This technique, based on a modified Prommer protocol (Prommer & Schmidt, 2007), was shown to be highly reliable in a meta-analysis, comparing rebreathing to other techniques, such as radioisotope labelling, showing a typical error of 1.1% in test-retest experiments (Gore *et al.*, 2005).

The technique involved participants breathing 1 mL/kg of carbon monoxide in 4 L of 100% oxygen for 3.5 minutes. Venous blood samples were taken at 0, 10, 12 and 15 minutes during the experiment to measure carboxyhaemoglobin. The temperature, barometric pressure and change in carboxyhaemoglobin between baseline and the 10 minute time point was used to calculate red cell mass. The full protocol for red cell mass measurements can be found in Chapter Two.

4.2.5 Hypoxic exposure and assessment of PASP

At the beginning and end of the six month study period, participants underwent a full day of sustained isocapnic hypoxia in a purpose-built chamber. The protocol for each experimental day is shown in Figure 4.2. The first chamber day was conducted prior to any infusion. Participants arrived in the laboratory one hour prior to the start of the sustained hypoxia protocol for baseline measurements and were observed for a minimum of 30 minutes after the end of the experiment. Eight hours of sustained hypoxia with the end-tidal oxygen concentration set at 55 mmHg was chosen as it has been shown to reliably induce pulmonary vascular responses and ventilatory acclimatisation to hypoxia (Smith *et al.*, 2008). A full description of the chamber methodology is available in Chapter Two.

At baseline, and every hour for eight hours, PASP was measured by recording the maximum systolic pressure gradient across the tricuspid valve and using the modified Bernoulli equation with an estimated right atrial pressure of 5 mmHg (Yock & Popp, 1984; Allemann *et al.*, 2000), as described in Chapter Two.

4.2.6 Statistical analysis

An unpaired Student's t-test was used to carry out comparisons on the baseline values between the two groups. The remainder of the statistical analysis was conducted using linear mixed-effects modelling (IBM SPSS V.20). For the analysis of blood results, participants were considered a random factor; group and month were fixed factors and dose of iron was a covariate in the model. For the analysis of PASP measurements, the response to sustained hypoxia was taken as the average value for PASP over 3 to 8 hours

(hypoxia), where the response appeared to have plateaued, minus the starting value at hour 0 (euoxia). Participants were taken as a random factor, group as a fixed factor, and either the dose of iron or the change in serum ferritin concentration over the six months as a covariate.

4.3 Results

4.3.1 Participant characteristics

Physical characteristics and baseline blood values for haematology indices and iron values are shown in Table 4.1. All participants were healthy and iron-replete. Participants were well matched for age and baseline blood parameters.

Variable (normal range/units)	Saline group	Iron group	*p* value
Age (yr)	32.5 ± 14.5	29.1 ± 11.0	0.61
BMI (kg/m^2)	24.8 ± 1.7	25.4 ± 4.4	0.67
Haemoglobin (12.0-17.0 g/dL)	15.1 ± 1.1	14.6 ± 0.8	0.24
Haematocrit (0.36-0.50%)	0.45 ± 0.03	0.44 ± 0.02	0.52
Mean Cell Volume (83-105 fL)	89.0 ± 4.0	91.0 ± 5.2	0.46
Erythropoietin (IU/L)	7.7 ± 3.9	7.6 ± 2.7	0.94
Serum Ferritin (10-300 µg/L)	98.4 ± 65.9	117.3 ± 67.1	0.58
Serum Transferrin (1.8-3.6 g/L)	2.7 ± 0.3	2.6 ± 0.3	0.75
Serum Transferrin Saturation (16-50%)	37.1 ± 10.3	33.7 ± 6.3	0.39
Soluble transferrin receptor (nmol/L)	23.7 ± 4.5	20.6 ± 3.5	0.12
Hepcidin (µg/L)	38.3 ± 21.0	81.0 ± 68.1	0.15
Interleukin- 6 (ng/L)	0.68 ± 0.26	0.54 ± 0.19	0.22
C-reactive protein (0-8 mg/L)	0.52 ± 0.04	0.68 ± 0.44	0.37

Table 4.1 Participant demographics and baseline venous blood analyses. Values are mean ± SD. BMI, body mass index; IL-6, interleukin 6; CRP, C-reactive protein. *p* values were calculated with a two-tailed Student's t-test.

4.3.2 Safety

Infusions were well tolerated and there were no major adverse events during the experiment. There were two notable minor events during the experimental period. On the day of the chamber experiment at the end of six months, one participant experienced a vasovagal episode at hour four of sustained hypoxia immediately following venesection. The experiment on that participant was abandoned after this. The participant was observed for one hour in euoxia and was then referred to his general practitioner for review. A second participant suffered gastrointestinal discomfort characterised by nausea and diarrhoea three weeks following iron infusion. These symptoms may be attributed to iron infusion as this is a known side effect of iron although uncommon with IV preparation**.** Due to the three week lag time from iron infusion, the time course suggested these symptoms were more likely attributable to a viral gastrointestinal illness. The participant's infusion for that month was delayed. The participant had repeat blood tests one week after resolution of symptoms and the infusion administered was based on these results due to concerns that infection/inflammation could falsely elevate the ferritin concentration. The participant had no further symptoms throughout the remainder of the study.

4.3.3 Dosing

The maximum total dose possible for a participant randomised to the iron group was 1.5 g. It was the intention to administer a dose of 250 mg of IV iron every month if the participant met the pre-specified criteria of ferritin concentration < 300 µg/L and transferrin saturation < 45%.The general pattern of administration of iron is seen in Figure 4.3. No participant received the maximal possible study dose of 1.5 g. Only five

participants received the next highest dose of 1.25 g. The lowest dose received in the study was 500 mg and the highest 1.25 g.

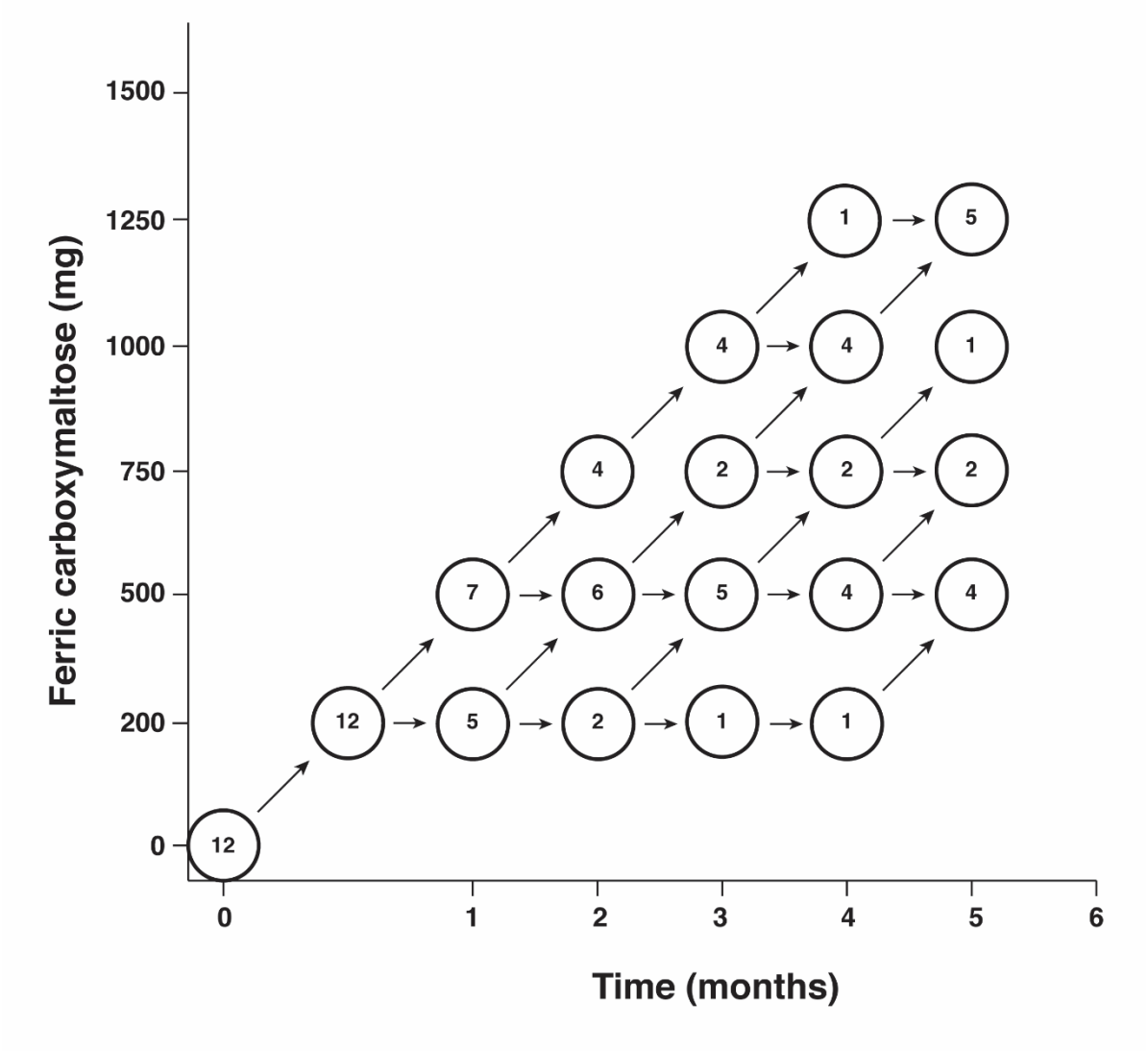

Figure 4.3 Patterns of administration of IV iron. Iron group participants received FCM or saline based on their iron indices. Over the six month period, the minimum dose administered in the study was 500 mg and the maximum dose was 1.25 g. The number in the circle represents number of participants at a given dose for that time point.

The parameters for excluding a participant from receiving an iron infusion are seen in Figure 4.4. In the first half of the study more infusions were excluded due to transferrin saturation than ferritin concentration, and as the ferritin levels increased in the second half of the study this limited more participants from receiving an iron infusion. Throughout the study both ferritin concentration and transferrin saturation levels, being above the pre-determined safety range, limited participants from receiving iron.

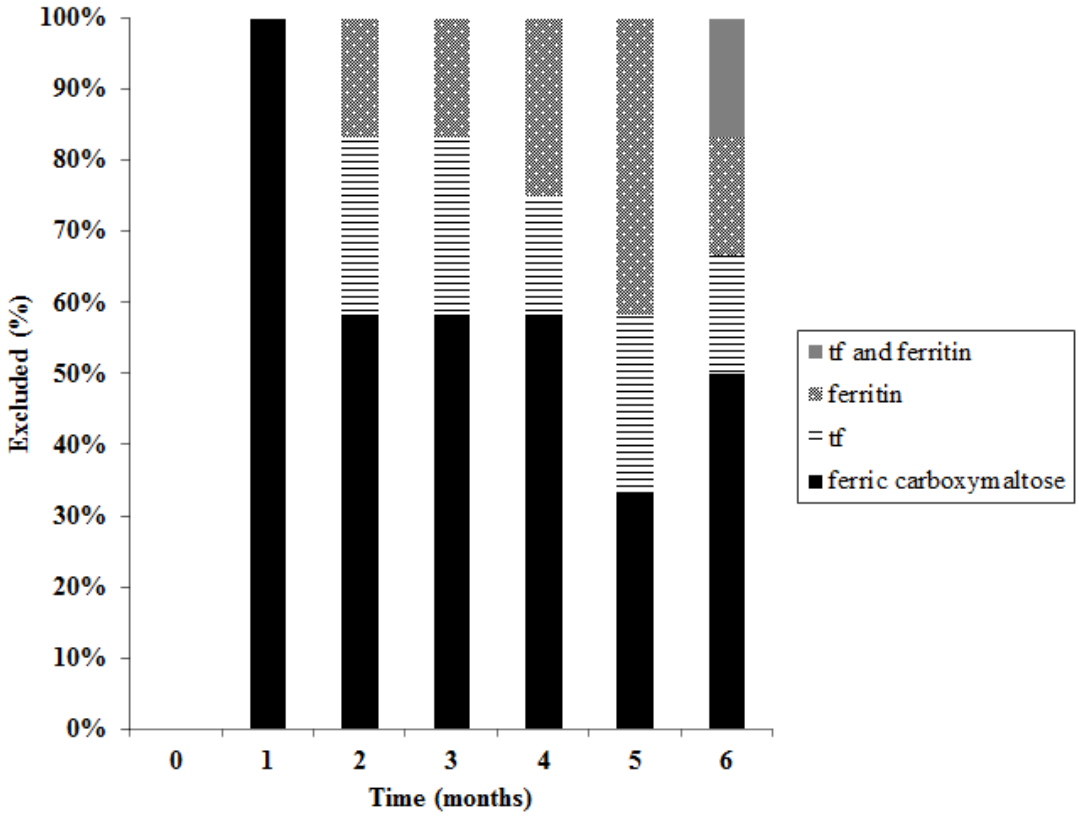

Figure 4.4 Exclusions met for IV iron infusion. Participants randomised to iron received saline if their ferritin > 300 μg/L or transferrin saturation > 45%. Initially it was transferrin saturation that was the main exclusion, however, by months 4-6 it was ferritin concentration which was the main excluding factor.

4.3.4 Haematological indices and red cell mass

The two groups were well matched at month 0 for haemoglobin, haematocrit and mean cell volume (Table 4.1). There was no significant difference over time in haemoglobin, haematocrit and mean cell volume between the iron and saline groups as seen in Figure 4.5. The two groups were well matched at baseline for red cell mass. Figure 4.6 shows the results for total haemoglobin concentration at 0, 3 and 6 months. Iron administration had no measurable effect on this index of total red cell mass.

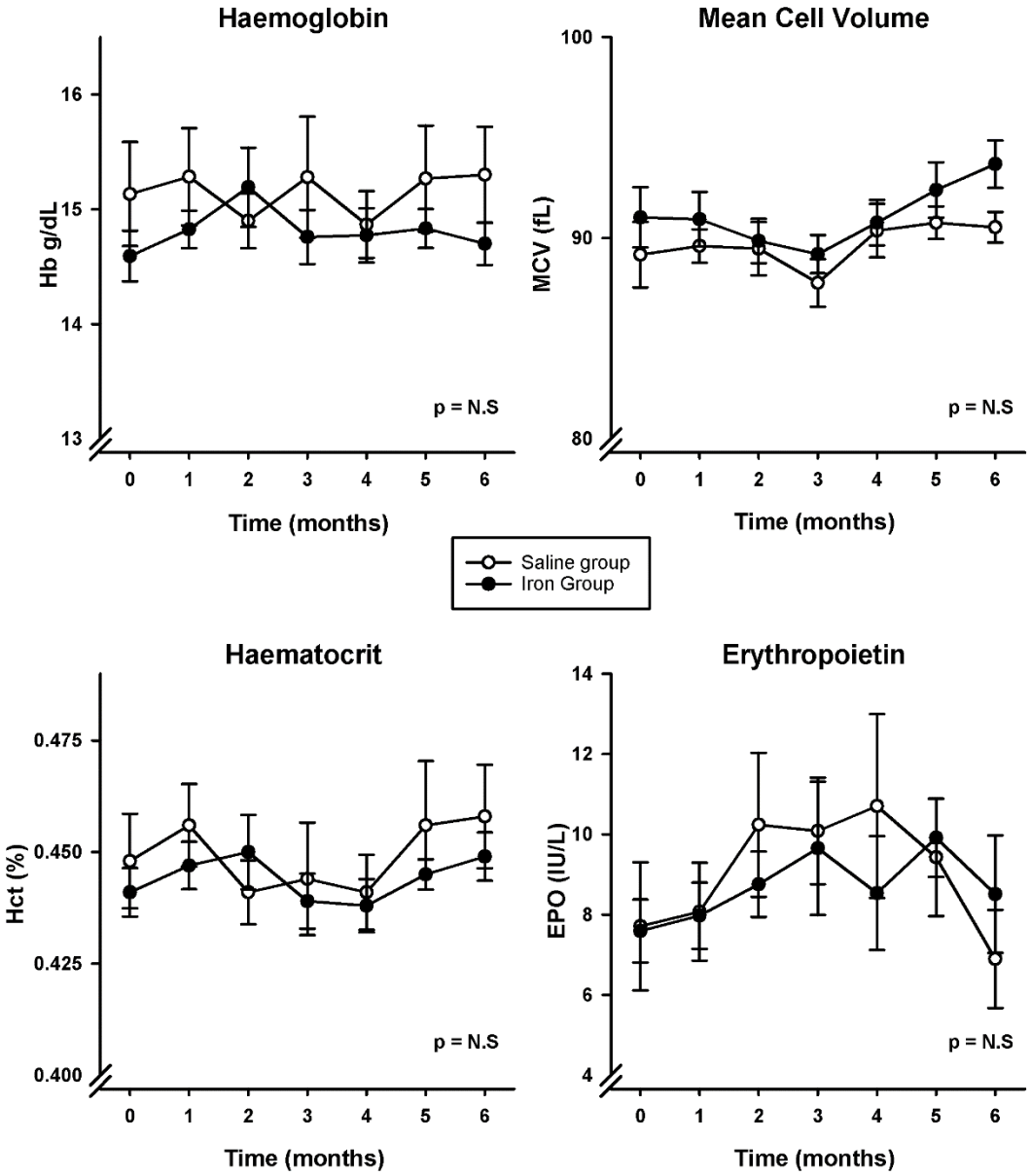

Figure 4.5 Haematology results. The groups were well matched at baseline for haematology values. There was no significant difference in haemoglobin concentration, haematocrit, mean cell volume (MCV) or erythropoietin (EPO) between saline and iron groups. Values are means and error bars are ± SEM.

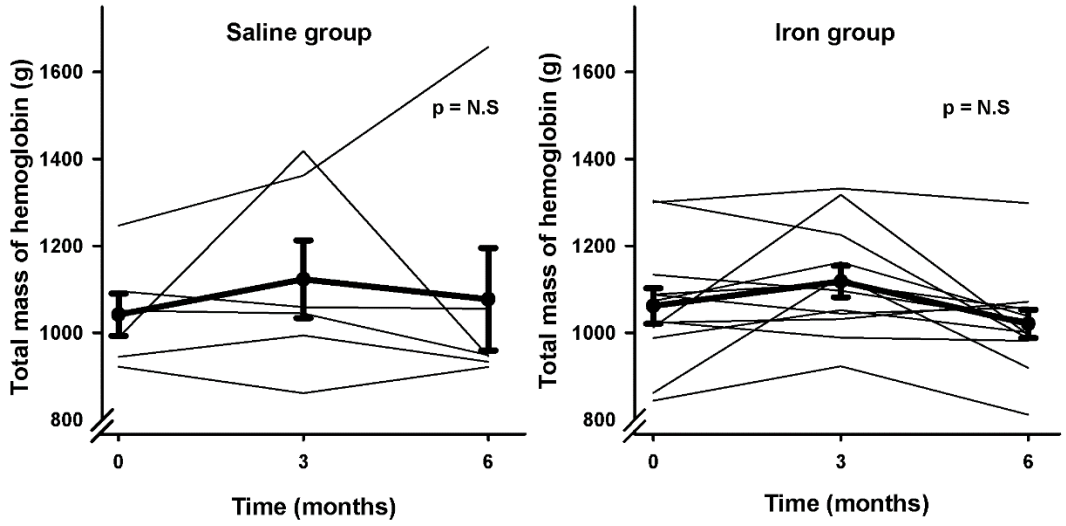

Figure 4.6 Haemoglobin mass over time. The two groups were well matched for haemoglobin mass at baseline. There was no significant change in haemoglobin mass over time for either the saline or iron groups. Each individual line represents a single volunteer and the thicker line represents the mean ± SEM.

4.3.5 Assessment of iron status and inflammation

The two groups were well matched overall in iron status at baseline. There was a linear and highly significant increase in ferritin concentration with iron administration ($p < 0.001$) (Figure 4.7B). Using linear mixed-effects modelling, the slope of the relationship between ferritin concentration (µg/L) and iron dose (mg) was 0.21 (95% CI 0.15-0.26) µg/L/mg.

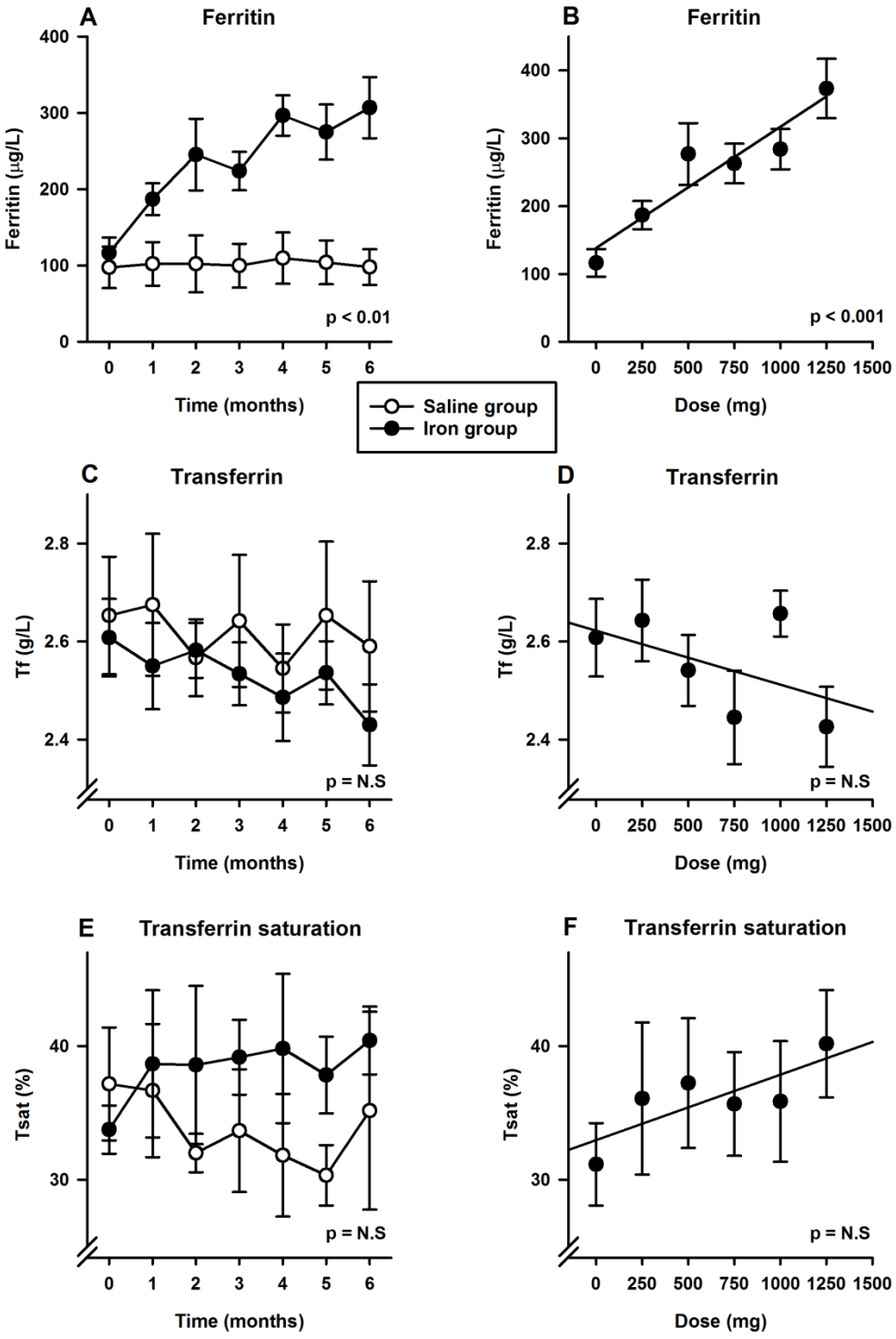

Figure 4.7 Iron indices. A, **C** and **E** illustrate ferritin concentration, transferrin concentration and transferrin saturation (mean ± SEM) respectively, for the saline group (open circles) and iron group (closed circles) across the six month period. **B**, **D**, and **F** illustrate ferritin concentration, transferrin concentration and transferrin saturation (mean ± SEM) respectively, for iron participants only. **B** There was a significant positive linear

relationship between ferritin concentration and iron dose. **D** The relationship between transferrin concentration and iron dose was not statistically significant. **F** The relationship between transferrin saturation and iron dose was not statistically significant.

The inverse linear relationship between transferrin concentration and iron dose did not reach statistical significance (Figure 4.7D), nor did the positive linear relationship between transferrin saturation and increasing iron dose (Figure 4.7F). Surprisingly, hepcidin concentration appeared to be unaffected by this level of iron loading and sTfR was similarly unaffected (Figure 4.8 C, D).

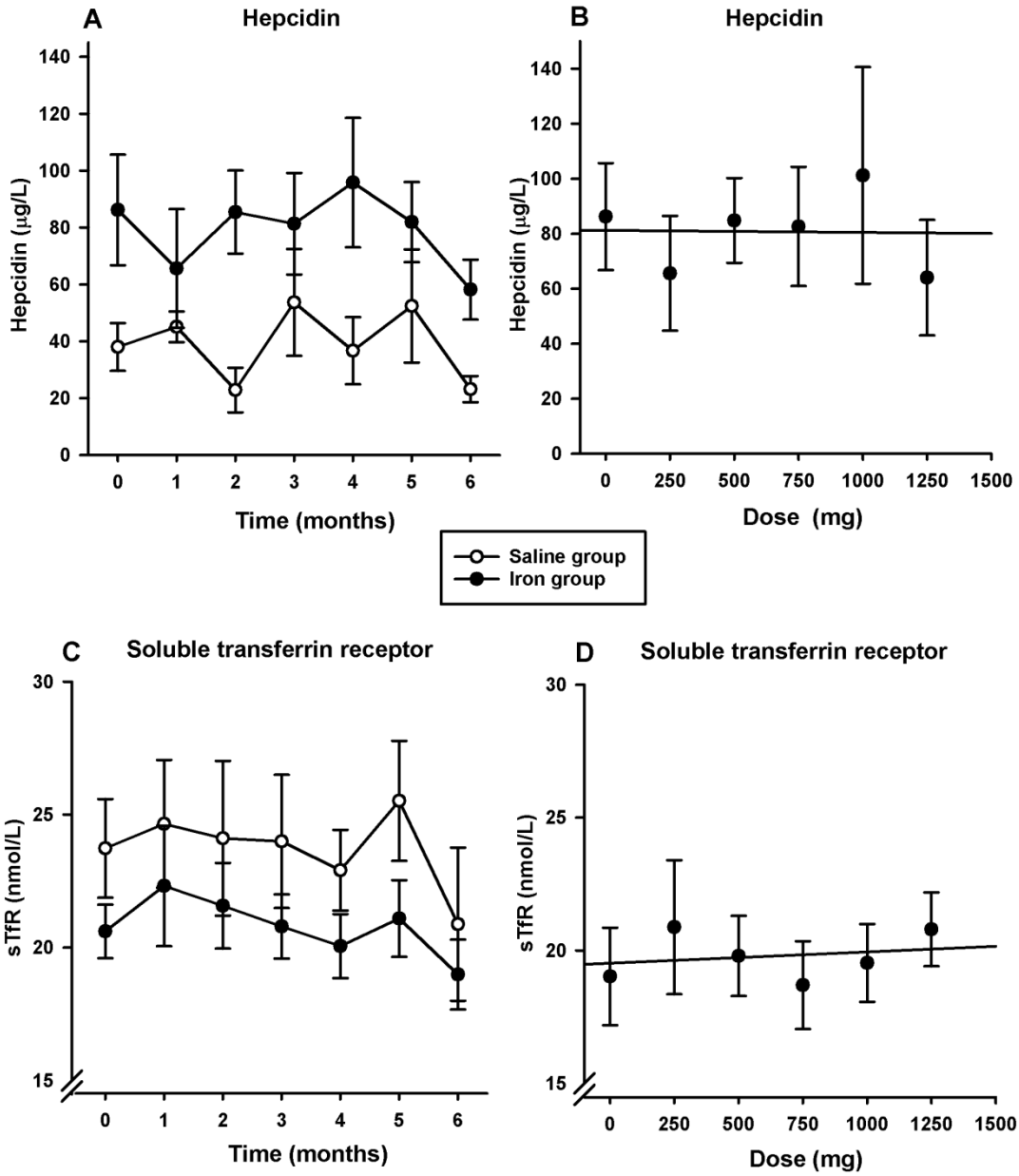

Figure 4.8 Hepcidin and soluble transferrin receptor concentration. A, **C** show mean ± SEM for saline groups (open circles) and iron group (closed circles) across the six month period for hepcidin concentration and soluble transferrin receptor concentration (sTfR), respectively. Graphs **B**, **D** represent hepcidin concentration and sTfR plotted against cumulative dose for the iron group only. There were no statistically significant effects with dose.

Values for CRP and IL-6 were well matched at baseline. The values for CRP and IL-6 at baseline and each month over the six month period are shown in Figure 4.9 A and B respectively. There was no significant change in either of the inflammatory markers over time suggesting that neither inflammation nor infection confounded the ferritin results. Note that blood tests were conducted one month following infusion and so it is not possible to determine whether there was an acute rise in inflammatory markers immediately following iron infusion.

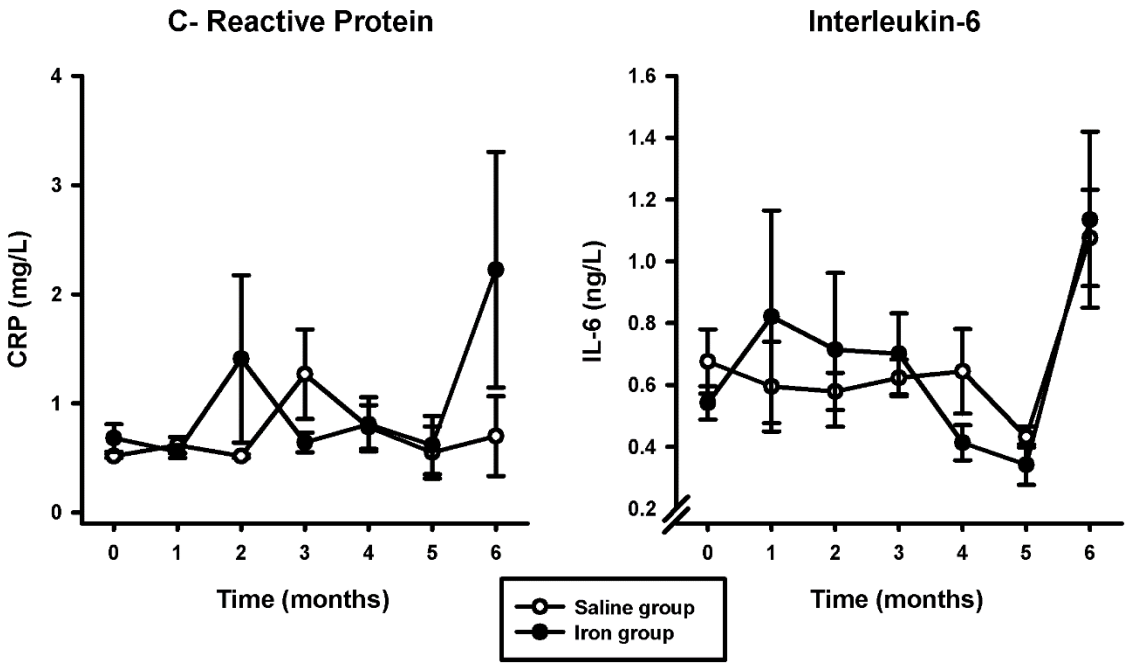

Figure 4.9 Inflammatory markers. A C-Reactive protein (CRP). **B** Interleukin 6 (IL-6). There was no significant change in CRP and IL-6 over time. Neither CRP nor IL-6 went beyond the normal reference range during the course of the study. Values are means and error bars are ± SEM

4.3.6 PASP response and gas control during sustained hypoxic exposures

Values for end-tidal P_{O_2} and P_{CO_2} together with values for arterial oxyhaemoglobin saturation were well matched between the two groups at day 1 and month 6 (Figure 4.10).

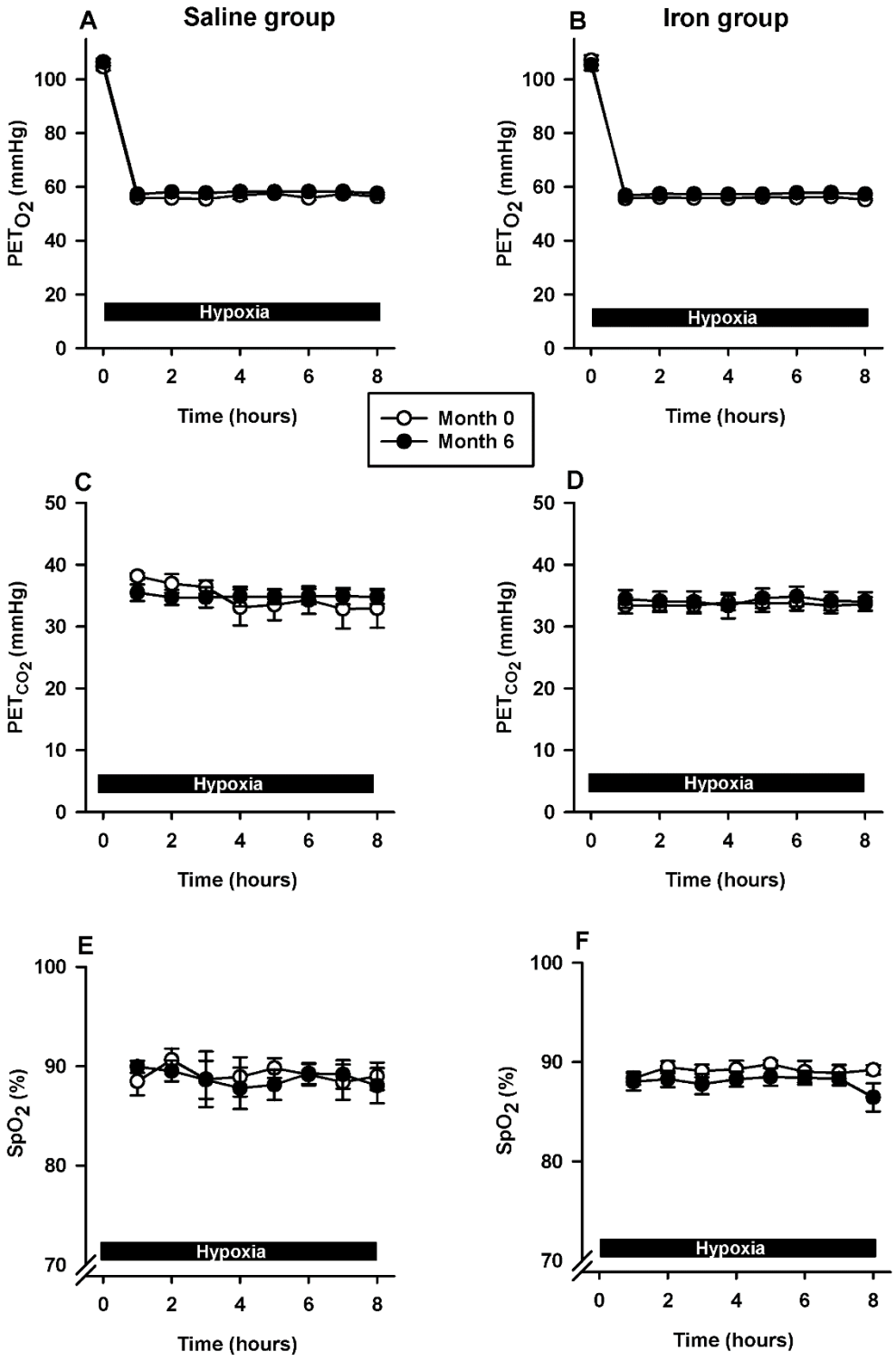

Figure 4.10 Gas control during exposure to hypoxia. **A, B** End-tidal oxygen (PET_{O2}) levels over eight hours for saline and iron groups, respectively. **C, D** End-tidal carbon dioxide (PET_{CO2}) levels for saline and iron groups, respectively. **E, F** Arterial oxyhaemoglobin saturation (SpO_2) for saline and iron groups, respectively. Values are means at each hour of hypoxia and error bars are ± SEM.

The responses of PASP to hypoxia are shown in Figure 4.11. There was a progressive rise in PASP with hypoxia observed in all participants over the eight hour hypoxic exposure. Similar to the results shown in Chapter Three, there appeared to be a more rapid initial rise in PASP in the first hour, with a slower, more progressive rise over the next few hours.

A comparison of the pulmonary vascular responses to hypoxia between the iron and saline groups at baseline (before the six month period) suggests that the groups were not as well matched as they could have been, with the responses for the saline group being larger than for the iron group, however this did not reach statistical significance. The attenuation of the rise in PASP in the iron group with hypoxia was not statistically significant (Figure 4.11A, B). There was however, a significant dose-dependent fall in Δ PASP with hypoxic exposure in the iron group. The magnitude of the decrease in the hypoxic pulmonary vascular response were found to become larger with increasing doses of iron ($p < 0.05$), as shown in (Figure 4.11C). The magnitude of fall in PASP was also related to increasing ferritin concentration ($p < 0.01$) (Figure 4.11D).

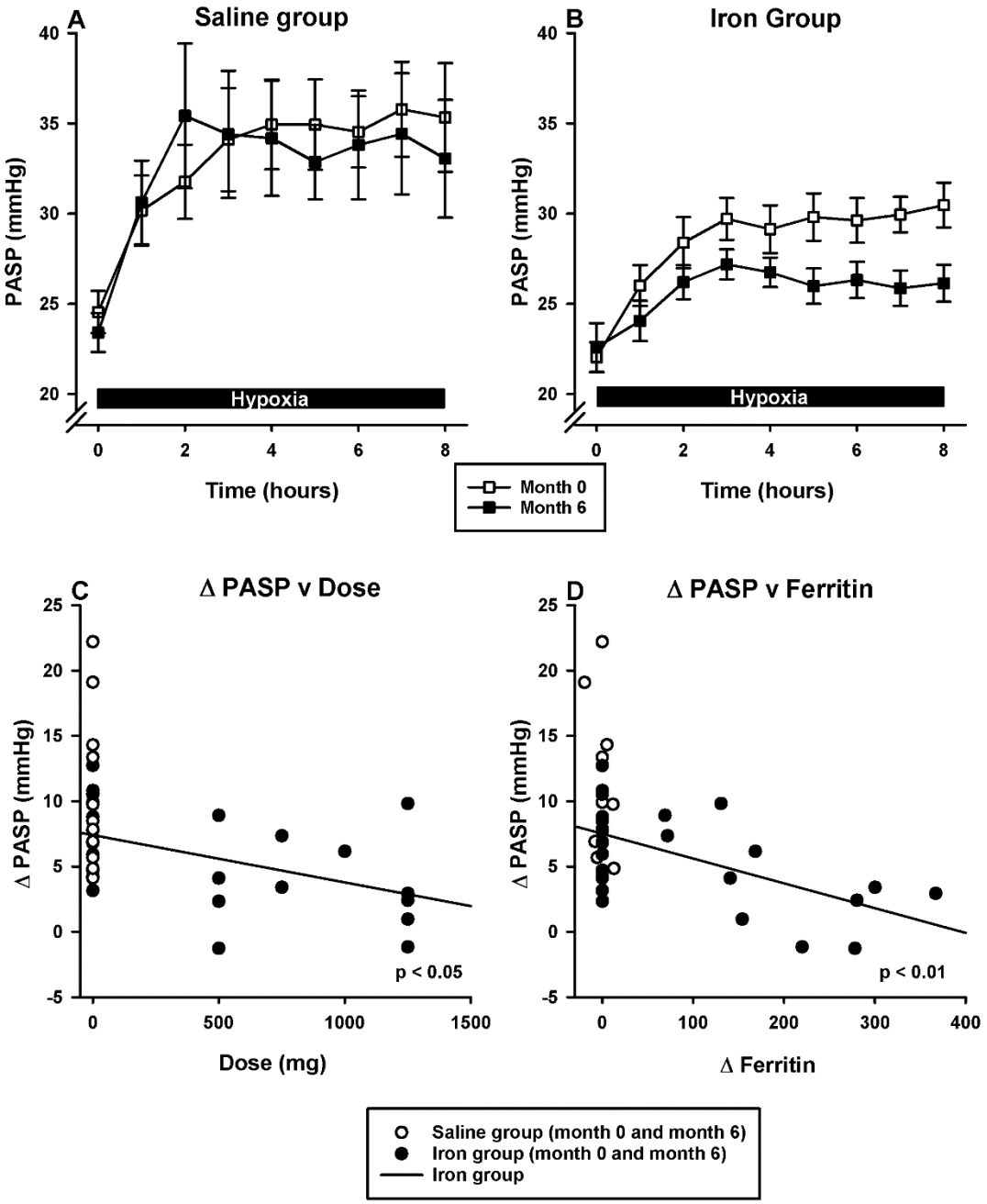

Figure 4.11 Pulmonary artery systolic pressure during sustained hypoxic exposure. **A** saline group and **B** iron group at the beginning (open squares) and end (closed squares) of the six month protocol. There was no significant difference in the PASP response to hypoxia between the two groups. **C** Increment in PASP (ΔPASP) during the eight hour exposure to hypoxia plotted against cumulative dose of iron for the saline group (open circles) and iron group (closed circles). **D** Increment in PASP (ΔPASP) during the eight hour exposure to hypoxia plotted against the change in ferritin (Δ ferritin) from baseline to the end of six months. There was a statistically significant effect of both dose ($p < 0.05$) and Δ ferritin ($p < 0.01$) on Δ PASP.

4.3.7 EPO and hepcidin response to hypoxia

Exposure to sustained hypoxia resulted in a rise in EPO concentration in both the iron and saline groups (Figure 4.12). There was no significant difference in the time-dependent rise in plasma EPO in response to eight hours of sustained hypoxia between iron and saline groups (Figure 4.12).

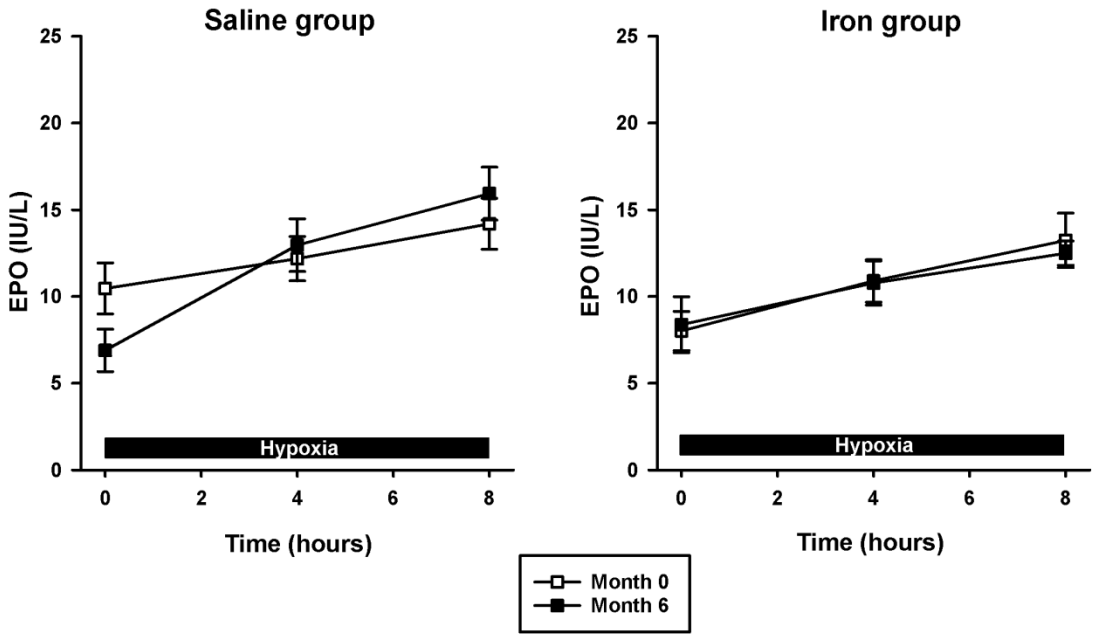

Figure 4.12 Time-dependent rise in erythropoietin with hypoxia. EPO rose in both groups. There was no significant difference in the rise in EPO between iron and saline groups. Values are means and error bars are ± SEM.

There was no statistically significant difference in the baseline values for hepcidin concentration, nor over the six month period for hepcidin concentration between the iron and saline groups. There was no significant difference in the hepcidin response to sustained hypoxia between the two groups (Figure 4.13).

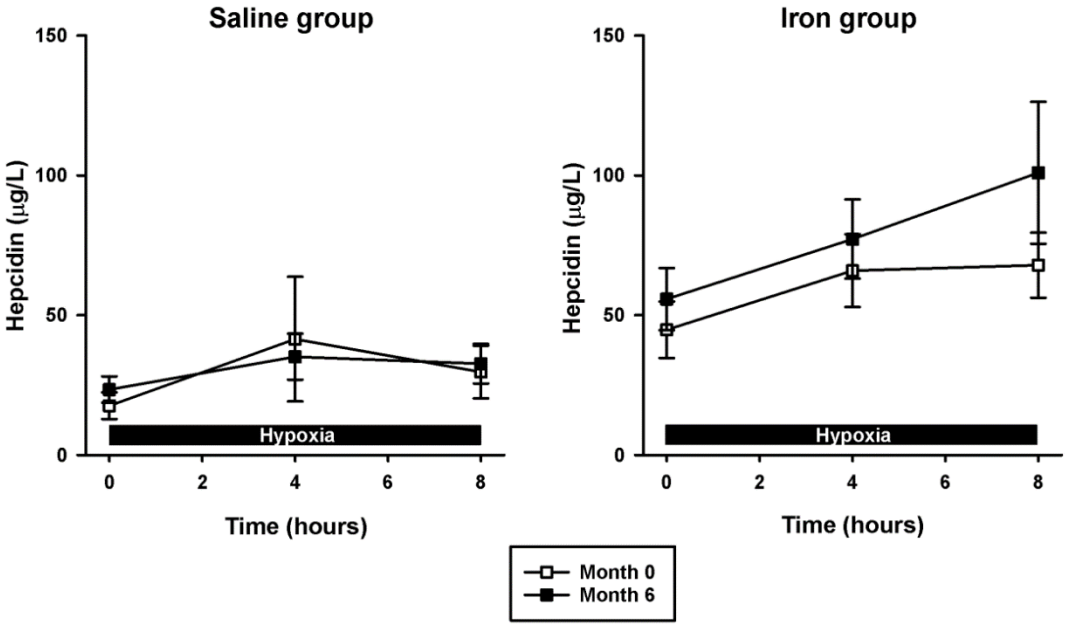

Figure 4.13 Change in hepcidin with sustained hypoxia. There was no significant difference in the rise in hepcidin between iron and saline groups. Values are means and error bars are ± SEM.

4.4 Discussion

This study is the first to define a dose-response relationship for the effects of IV iron administration on indices of iron stores in individuals who are already iron-replete. All other studies have been concerned with the dose of iron required to replenish stores in individuals with established iron deficiency (Geisser & Banke-Bochita, 2010; Krayenbuehl *et al.*, 2011). In our iron-replete participants, serum ferritin concentration rose by ~ 20 µg/L for every 100 mg of IV iron. Ferritin appeared to be the most robust marker of iron loading of all the iron indices measured. Transferrin did not decrease and transferrin saturation did not increase. This demonstrates that ferritin represents a better marker for iron status than other variables measured.

Another biomarker of iron status that can be measured in serum is hepcidin. Hepcidin limits the availability of iron by blocking absorption of iron from the gut and inhibiting release of iron from the reticuloendothelial system into the circulation (Nemeth *et al.*, 2004b). Interestingly, hepcidin is considered the central regulator of iron availability (Nemeth *et al.*, 2004b), but despite the six months of iron loading carried out in this study, no significant change in hepcidin concentration was observed over this period. This is a relatively surprising result, given that doses of iron of up to 1.25 g were given over the six month period. In Study A, there was a significant increase in hepcidin concentration with acute administration of iron. In this current study, it was thought that the cumulative dose would have increased iron stores by up to 25%, assuming that an average healthy human male has a total body iron content of 4 g. Moreover, hepcidin concentration could be expected to change in response to the repeated dosing of iron, alongside 'supra-normal' ferritin levels. The lack of hepcidin response seen in this current study may be due to the

timing of measurement of hepcidin. In a study of oral iron replacement, hepcidin concentration appeared to peak at 24 hours and then fall (Moretti *et al.*, 2015). The change in hepcidin concentration may also reflect the baseline iron status and final ferritin concentration in the current study. In a large study looking at a population of patients with chronic kidney disease who were administered repeated doses of FCM, hepcidin concentration appeared to correlate with dose of FCM and ferritin levels (Gaillard *et al.*, 2016). Of interest, at the higher ferritin levels the correlation between ferritin and hepcidin concentrations appeared to plateau, which may correlate with findings upon administration of iron to iron-replete individuals. We found no significant correlation between ferritin and hepcidin concentrations, which may reflect the baseline normal iron-replete status as well as the manipulation of iron stores within the physiological range.

The hepcidin response to iron status may be evolutionarily conserved, with some studies showing that limiting iron availability may be useful in hindering invading pathogens. This is the case in malaria, where high hepcidin levels limit iron supply to pathogens (de Mast *et al.*, 2009a; de Mast *et al.*, 2009b). There is some debate that iron supplementation may predispose certain patients with chronic disease to an increased risk of infection (Pieracci & Barie, 2005; Maynor & Brophy, 2007). Thus, iron infusion should be limited to stable patients with chronic disease without active infection, determined clinically by measuring inflammatory markers such as CRP and IL-6, as in this study.

Hepcidin levels increase in infection and inflammation, and this is a factor in the mechanism of anaemia of chronic disease (Weinstein *et al.*, 2002). One study looking at a cohort of COPD patients showed those with iron deficiency had inappropriately high

hepcidin and ferritin concentrations compared with the iron deficient healthy control group. Moreover, CRP was a determining factor in both hepcidin and ferritin concentrations (Nickol *et al.*, 2015). In chronic disease states, overall iron levels may be low despite a slightly higher ferritin, itself an acute phase reactant, and therefore it may be of benefit to supplement these patients with iron. In this current study, by ensuring that CRP and IL-6 levels were negligible at each time point, it could be confirmed that inflammation or infection did not factor into the change in ferritin concentration observed.

Genetic factors have been shown to play a significant role in determining the concentrations of markers of iron status. In one genome-wide association study of monozygotic twins, three variants in the transferrin gene and the C282Y variant of the HFE gene accounted for 40% of the variation in transferrin concentration (Benyamin *et al.*, 2009b). In a meta-analysis of an Italian and American Caucasian population, Tanaka *et al.* showed that the single-nucleotide polymorphism, most strongly associated with lower serum iron concentration, is located on the *TMPRSS6* gene which is involved in hepcidin regulation (Tanaka *et al.*, 2010). Interestingly, Tanaka *et al.* did not show the same significant genetic association with the transferrin and HFE genes that Benyamin *et al.* had shown, although the latter looked exclusively at twins.

The *TMPRSS6* gene is thought to regulate iron levels through suppression of hepcidin production (Du *et al.*, 2008; Silvestri *et al.*, 2008). *TMPRSS6* deficiency in humans has been linked with anaemia refractory to oral iron and a poor response to IV iron (Finberg et al., 2008). This gene has also been implicated in the ability to sense iron deficiency (Du *et*

al., 2008). Furthermore, variants in the *TMPRSS6* gene are associated with variation in haemoglobin concentration (Benyamin *et al.*, 2009a; Chambers *et al.*, 2009).

Tibetan highlanders have been shown to have genetic selection for two alleles *EPAS1*and *ENGLN1*, resulting in haemoglobin levels similar to low land residents (Beall *et al.*, 2010). For sea level residents, increased red cell mass is a known complication of high altitude exposure, with an ability to increase haemoglobin by up to 50% at 4,500 m (Reynafarje *et al.*, 1959), which is coupled with increased intestinal absorption of iron to meet erythropoietic demand. Further investigation needs to address whether iron supply limits, at least to some degree, red cell mass (Friedmann *et al.*, 2001; Garvican *et al.*, 2011; Wachsmuth *et al.*, 2015). In the present study, no evidence was found that iron infusion increases red cell mass in iron-replete individuals. Since iron deficiency is the single most common medical condition in the world (McLean *et al.*, 2009), it is possible that the observed genetic association arises from the presence of a significant number of individuals who are iron deficient within the association studies (Finberg *et al.*, 2008; Chambers *et al.*, 2009; Soranzo *et al.*, 2009).

4.4.1 Administration of ferric carboxymaltose in a health care setting

This study has established a safe and effective dosing regimen for FCM in a healthy, non-anaemic population. This is an important result if the manipulation of iron stores is to be used for therapeutic benefit clinically, as currently there are no clear criteria or dosage regimens in the absence of anaemia. Moreover, the Ganzoni formula, which has traditionally been used to dose patients with IV iron is inconsistently used (Kulnigg *et al.*, 2008), and may not achieve iron repletion in some patient cohorts (Koch *et al.*, 2015). In

patients with iron deficiency anaemia and chronic renal failure, two 750 mg infusions of FCM are safe and effective, in comparison to multiple lower dose infusions of IV iron sucrose (200 mg administered in up to five infusions in 14 days) (Onken *et al.*, 2014). A recent meta-analysis compared a cumulative high dose (1500 mg) of FCM versus a cumulative lower dose (1000 mg) in an iron deficient population and showed that 1500 mg was closer to the actual iron requirement for these patients (Koch *et al.*, 2015). A dose of 1500 mg also resulted in a more pronounced haemoglobin response in these patients, which is in contrast to the results in this study. The current study of iron-replete individuals, in which the criteria of ferritin < 300 μg/L and transferrin saturation < 45% were used, iron stores were safely manipulated with doses between 500 mg and 1.25 g with no participant needing a cumulative dose higher than 1.25 g. This may reflect altered pharmacokinetics of FCM depending on baseline iron status (Geisser & Banke-Bochita, 2010) and suggests the need for individual patient dosing protocols.

The administration of multiple small doses of IV iron in an iron-replete cohort was shown to be safe in this study. This is consistent with larger scale studies that showed that FCM has an improved safety profile compared to other iron preparations, although the long term effects (over 20 years) of FCM are not currently known (Lyseng-Williamson & Keating, 2009). Iron dextran, which is no longer used, is responsible for the reputation of IV iron causing anaphylaxis (Wang *et al.*, 2015). There are currently no documented case reports of anaphylaxis with FCM. Moreover, a protocol for desensitisation in the setting of FCM hypersensitivity reactions has now been published (Montandon *et al.*, 2016). Iron sucrose has been shown to cause transient proteinuria and oxidative stress after IV administration (100 mg iron-sucrose) in a cohort of patients with chronic renal failure (Agarwal *et al.*,

2004). Another study demonstrated that IV iron sucrose administration caused accelerated atherogenesis in a chronic kidney disease population (Kuo *et al.*, 2014). This is in contrast with a study that showed that IV iron sucrose reduced inflammatory markers in patients with heart failure and chronic kidney disease (Toblli *et al.*, 2015b).

Unlike iron sucrose, FCM is a complex similar to ferritin and is robust and stable within its carbohydrate shell, meaning less labile iron is released and that higher single doses can be administered (Toblli *et al.*, 2012). The ferrous component generates toxic free radicals and ROS. A recent study found that the ferrous component of FCM is markedly smaller than that of iron sucrose (1.4% and 12.2% respectively) (Gupta *et al.*, 2016). Despite this, given that it is a newer iron preparation, the exact nature of oxidative stress caused by FCM is still unclear. One animal model has shown that FCM, compared to iron sucrose, results in increased oxidative/nitrosative stress, pro-inflammatory and apoptosis markers in end organs such as the heart and liver (Toblli *et al.*, 2015a). In contrast, a rat study found that FCM attenuated the effects of iron deficiency on cardio-renal oxidative stress in rats that were concomitantly administered chemotherapy in the form of doxorubicin (Toblli *et al.*, 2014). In human studies, Agarwal *et al.* have shown oxidative stress with iron sucrose, but to date there have not been similar results found with FCM (Agarwal *et al.*, 2004). In the current study we found no change in CRP or IL-6, which could be used as indirect markers of oxidative stress, after IV FCM administration.

Iron overload and potential oxidative stress was prevented in this study by applying clear parameters based on which IV FCM could be administered. If the participant's ferritin was < 300 μg/L with a normal CRP and their transferrin saturation was < 45% then they were

given a further dose of IV FCM. Iron overload can be seen biochemically with an increase in ferritin concentration, transferrin saturation and decrease in transferrin concentration, but more importantly can result in end organ damage such as skin pigmentation, heart failure and diabetes (Bothwell & MacPhail, 1998; Townsend & Drakesmith, 2002). There were no significant adverse effects in this current study. In numerous large cohort studies on the use of FCM since its approval, it has been deemed to be a safe and effective treatment for iron deficiency (Kulnigg *et al.,* 2008; Geisser & Banke-Bochita, 2010; Bach *et al.*, 2015).

4.4.2 Iron stores and pulmonary vascular response

Iron stores themselves may have a close relationship with the rise in PASP with exposure to hypoxia. In this current study we found a significant attenuation in PASP that was FCM dose dependent. Moreover, the attenuation in PASP was also significantly affected by the change in ferritin concentration over time, suggesting that it may be beneficial to further supplement iron stores in a population of participants previously considered 'iron-replete'. Iron deficiency has been found to result in an exaggerated rise in PASP with exposure to hypoxia in otherwise healthy individuals. The PASP response in patients with iron deficiency is attenuated with a greater magnitude to the response seen in individuals who are iron-replete with IV iron infusion (Frise 2016). Thus, iron deficiency, even in the absence of anaemia, can have a deleterious effect in cardiorespiratory disease states. Iron deficiency has been reported with a high prevalence in pulmonary arterial hypertension (Rhodes *et al.*, 2011a) and COPD (Nickol *et al.*, 2015). More recently, iron deficiency itself has been shown to play a role in pulmonary vascular remodelling in a rat model of pulmonary hypertension (Cotroneo *et al.*, 2015).

Models of genetic up or down regulation provide evidence implicating HIF in the PASP response. For example, the PASP response after iron administration resembles that seen in the Tibetans, and the blunting in this sub-group is likely to be related to genetic adaptation (Petousi *et al.*, 2014). In the case of CP, where there is stabilisation of HIF in euoxia, patients have a higher baseline PASP and an exaggerated PASP response to hypoxia (Bushuev *et al.*, 2006; Smith *et al.*, 2006b). Furthermore, CP patients with lower ferritin concentrations have a correspondingly higher baseline PASP (Sable *et al.*, 2012). More direct evidence of the role of HIF in the pulmonary vascular response is seen in iron deficient rats which exhibit increased lung expression of HIF-1α and HIF-2α (Cotroneo *et al.*, 2015).

Results presented in this chapter provide further evidence for the mechanism behind the blunting of PASP with iron infusion being related to HIF. The current study demonstrated that hypoxic pulmonary vasoconstriction was modulated with chronic small doses of iron, and that after the administration of iron, the time course of the PASP response is modified. This is particularly in the second delayed phase of HPV, which is likely to be related to changes in downstream gene targets of HIF. These alterations take time and similar effects of iron on the second stage of HPV have been observed in other studies (Smith *et al.*, 2008; Talbot *et al.*, 2014).

There may be other mechanisms that can explain the effect of IV iron on pulmonary vasculature. One theory is that of the effect of ROS on PASP (Waypa *et al.*, 2001; Waypa

et al., 2002). FCM has been shown to comprise of only 1.2% Fe^{2+} species, and to have extremely low reactivity when compared to other iron preparations (Gupta *et al.*, 2016), therefore this goes against the ROS theory in the context of this study. One study investigating the effect of hyperoxia, and thus generation of ROS, found no changes in PASP (Cheng, personal communication). If it was the preparation of IV iron itself, or its generation of ROS causing changes in the pulmonary vasculature, attenuation in PASP might be expected across all patient groups. There was a difference, however, in the observed changes in PASP with iron administration in a population of patients with chronic mountain sickness, when compared with healthy participants (Smith *et al.*, 2009). In this study, a population of patients with chronic mountain sickness underwent staged venesection, which resulted in progressive iron deficiency, and at altitude, an exaggerated pulmonary vascular response (Smith *et al.*, 2009). Interestingly, after administration of IV iron to this population there was no significant effect on PASP, indicating the increase in PASP was not acutely attenuated by IV iron infusion. In contrast, administration of iron to an iron-replete population resulted in a 40% reduction in the PASP response (Smith *et al.*, 2009).

An alternate explanation to HIF activation and/or ROS generation, by which iron status affects PASP, could be via TfR1. TfR1 is essential for cellular iron uptake and its expression is induced in iron deficiency (Cook *et al.*, 1990; Cook *et al.*, 1993). In humans, levels of TfR1 are higher in patients with pulmonary arterial hypertension (Rhodes *et al.*, 2011a). In rats, deletion of TfR1 decreases both the degree of pulmonary vascular remodelling and right ventricular failure (Naito *et al.*, 2016). Moreover, i*n vitro,* TfR1 deletion also limited PASMC proliferation (Naito *et al.*, 2016). An important, but yet unanswered question is whether genetic variants that cause alterations in iron status may

be important markers for variation in the pulmonary vascular response, and consequently, the development of pulmonary hypertension.

4.4.3 Methodological considerations

There are some methodological considerations to note in this study. There was a trend towards the iron group being more 'iron-replete' at baseline, suggesting some mismatch in baseline randomisation despite blinding. This was not statistically significant, however physiological variation in iron status may be important. There was variation present in baseline hepcidin levels, which has been shown to be a factor that contributes to baseline ferritin concentration as well as other iron indices (Roe *et al.*, 2009). The iron group and saline group had some differences in their baseline pulmonary vascular response to hypoxia, albeit none significant, which may be a result of small differences in baseline iron status. Iron deficiency itself can affect the pulmonary vascular response, and this may also be true for individual variation in iron status (Frise *et al.*, 2016). The variation in baseline values measured between the two groups is most likely a reflection of the relatively small sample size in this study, especially the control group of six participants.

The study method used a closed circuit re-breathing technique for the measurement of red cell mass rather than radio-dilution technique. One concern with these measurements of total mass of haemoglobin is that the absolute values obtained appear over large (Gore *et al.*, 2005). There is no clear explanation for why this is the case, although it is recognised that some overestimate of total haemoglobin will arise because of carbon monoxide binding to haem groups other than to haemoglobin in the body (Gore *et al.*, 2005).

Cardiac ultrasound is operator-dependent, and over the six month study period there was a change in operator experience between the start and end of the study. As discussed in Chapter Three, there is a wide range in inter-individual physiological baseline tricuspid regurgitation, as well as variability in human pulmonary vascular response both during exercise (Reeves *et al.*, 2005; Kiencke *et al.*, 2008), and with hypoxia (Groves *et al.*, 1987; Bartsch *et al.*, 2001) making this a point of consideration. Alongside this, there are limitations in two-dimensional echocardiography in the assessment of the right ventricle. This may have implications in the assessment of the degree of tricuspid regurgitation as flow is dependent on preload, afterload and changes in right ventricular contractility. The limitations are anatomical, based on the retrosternal location of the right ventricle (Haddad *et al.*, 2009) and the complex pyramidal structure of the right ventricle, as well as physiological limitations, with the right ventricle having many functional indices that demonstrate load dependence (Dell'Italia, 1991; Ho & Nihoyannopoulos, 2006). There are studies which suggest that acute hypoxia does not affect the right ventricle, both in healthy participants (Oliver *et al.*, 1991), and in a group of COPD patients (Akgul *et al.*, 2007). In contrast, other studies have demonstrated that acute hypoxia adversely affects both right and left diastolic function (Huez *et al.*, 2005).

In clinical practice, particularly in the diagnosis of pulmonary hypertension, additional echocardiographic measurements have now been incorporated into the assessment of right ventricular function, including tricuspid annular plane systolic excursion, right ventricular index of myocardial performance, indexed right atrial volumes and right ventricular strain pattern (Rudski *et al.*, 2010; Kossaify, 2015). These were not conducted in the present study. Moreover, additional imaging methods such as cardiac magnetic resonance imaging (MRI) have now surpassed echocardiography for the measurement of right ventricular

structure and function (Aebischer *et al.*, 1998; Puchalski *et al.*, 2007). In an ongoing study looking at a pulmonary arterial hypertension, both MRI and right heart catheterisation are being used in conjunction for the assessment of changes in pulmonary vasoconstriction in response to iron infusion rather than echocardiography alone (Howard *et al.*, 2013).

The present study did not measure changes in left ventricular diastolic dysfunction or ejection fraction, as a marker of systolic function. It therefore did not take into account the impact of left ventricular function on right ventricular haemodynamics and thus pulmonary pressures; this is something to be considered for future studies. Anker *et al.* showed that iron infusion benefited patients with left heart failure (Anker *et al.*, 2009), although the underlying mechanism is yet to be understood. Toblli *et al.*, have expanded on that work to demonstrate that IV iron results in a reduction in left ventricular systolic diameter and left ventricular diastolic diameter (Toblli *et al.*, 2015b). Right ventricular function is interdependent with left ventricular function, predominately by way of the shared inter-ventricular septum (Santamore & Dell'Italia, 1998). All stages of the cardiac cycle are energy dependent, and it has been shown that, in healthy individuals, impairment of cardiomyocyte contractility can occur after less than 18 hours of hypoxic exposure. In one study, left ventricular energetics, when measured in terms of phosphocreatine: ATP ratio and diastolic function (measured by the E/ E') were affected by 20 hours of sustained isocapnic hypoxia in a similar chamber experiment to that used in the present study (Holloway *et al.*, 2011).

4.5 Conclusions

In conclusion, this study establishes a dose-response relationship between ferritin concentration and cumulative iron dose in individuals who are not iron deficient. The slope of this relationship between serum ferritin and added iron is ~ 0.2 µg/L/mg of iron administered. By manipulating iron stores, there was a dose-dependent and ferritin concentration dependent decrease in the rise in PASP with hypoxia. This suggests that there may be a benefit of IV iron infusion in populations that may not traditionally be considered 'iron deficient', or scope for changing the definition of 'iron deficiency' in chronic disease states. Furthermore, these results may help guide potential future therapies using IV iron supplementation to alter physiological iron status in patients who are not formally iron deficient.

There is great potential for future work to build on this study. In particular, investigating manipulation of iron stores in chronic hypoxic cardiopulmonary disease states.

CHAPTER FIVE:

DISCUSSION AND CONCLUSIONS

5 Discussion and conclusions

5.1 General Discussion

The work presented in this thesis demonstrates the clear links between iron, oxygen and the control of pulmonary blood flow. Iron plays an important role in oxygen sensing and in the delivery of oxygen to tissues. Manipulation of iron stores in the human body with IV iron may influence the cellular and systemic response to hypoxia. There is a multi-system response to low oxygen in human physiology, through the increase in minute ventilation, cardiac output, pulmonary vascular resistance and erythropoiesis.

This work adds to the body of knowledge in the area of hypoxia and iron physiology. It provides information as to the attenuation of pulmonary vasoconstriction with iron, and the duration of these effects. Importantly, it demonstrates a previously unclear link between iron stores and PASP, as well as the dose-response relationship between iron and ferritin concentration. This will provide the basis for future work in this area, some of which is already underway.

5.1.1 Mechanism behind pulmonary vascular findings

The role of the HIF family of transcription factors may provide the most likely explanation of how iron status can affect the human hypoxic response. HIF hydroxylation requires oxygen as a substrate and iron as a co-factor; it is the crucial step in HIF stabilisation and thereby upregulation of downstream targets. These targets have a myriad of physiological

functions including angiogenesis, metabolism, hormonal regulation, and a role in iron homeostasis itself.

To date, there are no studies that clearly describe the mechanism by which iron may modulate the pulmonary vascular tone. One of the limitations of the two studies presented here is that they do not provide a direct mechanism to explain the observations made, primarily because HIF activity is difficult to measure in human subjects. In animal models, targeted disruption of HIF manifests in a reproducible phenotype characterised by pulmonary hypertension and altered oxygen sensing (Ghosh *et al.*, 2013; Tan *et al.*, 2013). The rare genetic condition of Chuvash polycythaemia demonstrates the consequence of HIF stabilisation in euoxia, characterised by polycythaemia, resting pulmonary hypertension and increased ventilation (Ang *et al.*, 1999; Ang *et al.*, 2002). When exposed to hypoxia, these patients have an exaggerated increase in PASP (Smith *et al.*, 2006b) further emphasising the link between HIF activation and HPV.

An accurate and safe way of measuring HIF in humans needs to be developed in order to confirm that the effect of iron on the pulmonary vasculature is mediated by HIF, rather than relying on the measurement of downstream HIF target genes. Without the capacity to measure HIF concentrations, either in the pulmonary vasculature or even systemically, there is currently no way of attributing the results of this work, or other studies, to the HIF pathway. The results may be alternatively explained by other vasoactive mediators such as nitric oxide, prostaglandins or endothelin, which are all implicated in hypoxic pulmonary vasoconstriction. Even so, these mediators may function as HIF related targets for which the HRE has not yet been identified (Semenza, 2012). Other than the aforementioned

vasoactive mediators, iron could modulate HPV via ROS generation or alterations in other gene targets i.e.TfR1 and *TMPRSS6.*

It is expected that the results of direct HIF measurement will be confirmatory of this study. It is already known that iron deficient rats have increased expression of HIF-1 and HIF-2 in the lung in response to hypoxia (Cotroneo *et al.,* 2015). Moreover, in these animals, the adverse effects of iron deficiency, such as pulmonary hypertension, improve with iron infusion. At present, however, there is no workable method of translating these findings to humans.

If the striking effect that iron has on the pulmonary vasculature was HIF mediated, the exact mechanism by which this occurs is not immediately apparent, as HIF-1 and HIF-2 exert quite different actions, and iron may affect each isoform differently (Raval *et al.*, 2005). Furthermore, the presence of an IRE in the 5'-untranslated region of HIF-2 mRNA, but not in HIF-1 mRNA (Sanchez *et al.,* 2007), may play an important and as yet uncharacterised role in the pulmonary vascular response to hypoxia. IRP1 has an inhibitory effect on HIF-2, and pulmonary hypertension is a prominent phenotypic feature in mice with deletion of IRP1 (Ghosh *et al.,* 2013).

5.1.2 Influence of iron bioavailability on the hypoxic pulmonary response

Taken together, the findings of both studies presented in this thesis suggest that it is overall iron stores that affect the pulmonary vasculature rather than the acute influence of IV iron itself. This builds on work that considers the manipulation of body iron stores previously

conducted in the same laboratory (Smith *et al.*, 2008; Smith *et al.*, 2009), and sits alongside parallel work on the profound effects of iron deficiency on the pulmonary vasculature (Frise *et al.*, 2016).

If indeed these findings are applicable to clinical medicine, the definition of 'iron deficiency' must be clarified. In the absence of inflammation, a serum ferritin of < 12 µg/L is very likely to be associated with absolute iron deficiency (Cook & Skikne, 1982; Cavill, 1999). In the trials concerning patients with heart failure, a diagnostic threshold for ferritin of < 100 µg/L (or even a ferritin of < 300 µg/L if the transferrin saturation was < 20%) was used, and even with these relatively lax criteria, patients still benefited from iron replacement.

The concept of 'functional iron deficiency' is more nuanced. This is where there is insufficient iron to fulfil physiological demands, such as oxygen sensing and response. The criteria for functional iron deficiency may need to be reviewed given recent findings. In Study B (see Chapter Four), participants had a starting ferritin of ~100 µg/L, traditionally a 'normal value'. The significant inverse relationship between Δ ferritin and Δ PASP in this study suggests that whether occupational, recreational or in a health setting, patients with potential hypoxic insults should maintain a higher ferritin concentration. In addition, there is evidence to suggest that screening for iron deficiency should be conducted routinely in patients with cardiorespiratory conditions who are at risk of hypoxia. There is also a suggestion that guidelines need to be reviewed in terms of the optimal threshold for ferritin concentration in both the presence and absence of inflammation.

One question that should be raised is whether oral iron supplementation would be sufficient to achieve the same results. From a public health and cost perspective this would be more practicable. There are potential obstacles to this. The tight regulation of iron absorption in the gastrointestinal system limits the efficacy of orally administered iron in the setting of 'replete' bone marrow iron stores (Ganz, 2003; Roe *et al.*, 2009). Indeed, iron administered orally has been shown to be ineffective in the setting of some acute and chronic medical conditions, particularly in patients with high hepcidin levels (Nemeth *et al.*, 2003; Goodnough, 2012; Bregman *et al.*, 2013). Despite this, there certainly remains scope to study the effect of oral iron supplementation on the human hypoxic response.

5.1.3 Is the effect of iron on the pulmonary vasculature independent of haemoglobin?

Recent clinical studies have shown that iron deficiency itself is detrimental to human health in the absence of anaemia (Anker *et al.*, 2009; Viethen *et al.*, 2014). The inverse may also be true with iron replacement, without augmentation of haemoglobin, proving beneficial. Given the well-recognised role of haemoglobin in oxygen transport, this is an interesting concept. In both studies there was no effect of IV iron on any haematological index (haemoglobin concentration, haematocrit, mean cell volume and red cell mass). It is well known that, in the setting of iron deficiency, IV iron significantly increases haemoglobin levels (Kulnigg *et al.*, 2007; Litton *et al.*, 2013), yet we did not find this to be the case in an iron-replete population. Moreover, iron supply is thought to limit red cell mass (Friedmann *et al.*, 2001; Garvican *et al.*, 2011; Wachsmuth *et al.*, 2015), and genetic studies have linked the *TMPRSS6* gene with haemoglobin concentration (Chambers *et al.*, 2009; Soranzo *et al.*, 2009). The *TMPRSS6* gene has also been linked with iron deficiency

anaemia that is refractory to iron replacement (Melis *et al.*, 2008; Kodama *et al.*, 2014). In this present study, there was no evidence to show that iron infusion increased red cell mass.

Genetic adaptation studies for certain high altitude populations have shown that there is natural selection for HIF related genes that result in lower haemoglobin levels. In Tibetan populations *EPAS1*, which encodes for HIF-2, and *EGLN 1*, which encodes for PHD2, have been associated with lower haemoglobin and haematocrit levels at altitude in comparison to other high altitude populations (Beall *et al.*, 2010; Simonson *et al.*, 2010). Tibetans appear to have a blunted hypoxic pulmonary vascular response to both acute and sustained hypoxia (Petousi *et al.*, 2014). It is interesting that the results of this thesis show changes in the pulmonary vascular response to hypoxia with iron loading, with no demonstrable change in haematological indices.

This again raises an additionally complex issue as to the pleiotropic effects of HIF, as well as the divergent effects of activating or silencing one aspect of the HIF pathway. For example, DFO has been shown to increase EPO, but have no effect on ventilation (Ren *et al.*, 2000). Equally, specific PHD inhibitors affect erythropoiesis, but not ventilation (Bishop *et al.*, 2013). We speculated that the changes seen in the pulmonary vasculature may be mediated by HIF-2α rather than HIF-1α, although HIF-2α is also known to play a role in determining haemoglobin concentration. It is of note, therefore, that Tibetans with HIF-2α genetic mutations have both lower haemoglobin concentrations and an attenuation of the hypoxic pulmonary vascular response (Petousi *et al.*, 2014). These possible

phenotypic manifestations of HIF-2α genetic mutations prompt further consideration of the exact mechanism that relates the action of iron to hypoxic pulmonary vasoconstriction.

Haemoglobin concentration itself has been shown to have an effect on pulmonary pressure. In dogs, lower haemoglobin concentrations lead to a reduction in the rise in pulmonary pressure with hypoxia (Kerbaul *et al.*, 2004). In patients with chronic lung disease, repeated small volume venesection, with a consequent reduction in haemoglobin concentration, resulted in a fall in mean pulmonary artery pressure (Weisse *et al.*, 1975). In both studies presented in this thesis, there was no change in haemoglobin between groups and so this was not considered to be a confounding factor. Indeed, in human patients with secondary polycythaemia, there is an initial decrease in the degree of pulmonary hypertension with venesection, but this is not sustained after repeated venesections due to the development of iron deficiency (Lewis *et al.*, 1952). This demonstrates the role of iron, independent of haemoglobin concentration, in the pulmonary vascular response.

5.1.4 Iron loading and hepcidin

Hepcidin is recognised as the master regulator of iron homeostasis (Ganz, 2003). Study A (Chapter Three) showed that the administration of a single bolus dose of iron led to an acute rise in hepcidin, likely to be related to hyperferraemia. In contrast, with incremental doses of iron over six months there was no significant change in hepcidin levels as shown in Study B (Chapter Four). This is surprising given the central role of hepcidin in iron regulation. In addition, in Study B there was a marked increase in ferritin concentration, which is considered a strong predictor of hepcidin concentration (Weinstein *et al.*, 2002; Weiss *et al.*, 2009; Kuragano *et al.*, 2010).

A dysfunctional hepcidin response is a hallmark feature of both iron overload and iron deficiency syndromes. For example in anaemia of chronic disease (Weinstein *et al.*, 2002), and IRIDA (De Falco *et al.*, 2013), patients have inappropriately high hepcidin levels, whereas the converse is true is some forms of haemochromatosis (Ahmad *et al.*, 2002; Muckenthaler *et al.*, 2003; Nicolas *et al.*, 2003). Hepcidin may be a valuable biomarker in predicting response to oral iron (Bregman *et al.*, 2013) and IV iron. Furthermore, it is purported to be a potential biomarker in the diagnosis and management of iron deficiency disorders. For these reasons, one interesting result is that with a large bolus dose of IV iron (Study A) there was an acute rise in hepcidin concentration, while with smaller incremental doses there was no change in hepcidin concentration (Study B). This may be related to the type of iron loading. Despite the differences in hepcidin response between the two groups, both studies showed that with iron loading there was a change in the pulmonary vascular response to hypoxia; this may be related to the ferritin response. In Study A, with administration of a large bolus of iron there was an acute rise in ferritin that did not return to baseline by six weeks. In Study B there was an incremental and significant increase in ferritin concentration. Moreover, we found that ferritin concentration and dose of iron appeared to correlate with the degree of PASP attenuation.

Hepcidin levels are modulated both by iron and hypoxia (Nicolas *et al.*, 2002), but the interaction is complex. The initial belief was that hepcidin suppression was mediated by hypoxia itself, potentially via HIF (Peyssonnaux *et al.*, 2007), and this is supported by low hepcidin levels in patients with congenital upregulation of HIF (Gordeuk *et al.*, 2011). With exposure to hypoxia, iron supply needs to meet the increasing demands of erythropoiesis to support oxygen transport (Richalet *et al.*, 1994). Recent work in human participants exposed to hypobaric hypoxia has suggested that it is not serum iron

concentration itself that suppresses hepcidin (Talbot *et al.*, 2012), but signalling from the bone marrow instead. A potential mediator of this mechanism is erythroferrone (Kautz *et al.*, 2014b). Further evidence for an erythroid regulator is seen in chronic mountain sickness, characterised by polycythaemia and pulmonary hypertension, where progressive venesection results in a fall in hepcidin concentration (Talbot *et al.*, 2016). By maintaining a steady-state hypoxia, this study suggests that it is erythropoietic drive rather than hypoxia itself which influences hepcidin concentration (Talbot *et al.*, 2016).

Individuals susceptible to HAPE have an exaggerated hypoxic pulmonary vascular response (Grunig *et al.*, 2000). During the acute erythropoietic response that occurs on ascent to altitude, humans can become iron deficient (Richalet *et al.*, 1994). A recent study has shown that a rise in hepcidin concentration is likely to be mediated by inflammation in individuals with HAPE, but did not find any association with serum iron levels (Altamura *et al.*, 2015). This is interesting given the attenuation of PASP observed here with IV iron. In addition, the symptoms present in acute mountain sickness appear to be attenuated by IV iron (Talbot *et al.*, 2011), adding further weight to the need for additional studies to examine the complex relationship between iron, hepcidin and hypoxia.

5.1.5 Variation of human response to hypoxia

There is significant heterogeneity in the pulmonary vascular response to hypoxia (Weitzenblum *et al.*, 1988) which changes with age (Balanos *et al.*, 2015) and degree of hypoxic exposure. Pulmonary hypertension is an umbrella term for a group of distinct disease states, each with varying aetiology and treatment responses (Simonneau *et al.*, 2013). In long standing pulmonary hypertension, vascular remodelling is a prominent

feature. The two studies in this thesis only included participants with no concomitant cardiorespiratory disease. Though these studies are important in identifying the effects of iron on normal individuals, it is unclear whether it will have similar effects in patients with pulmonary hypertension. It is for this reason that larger clinical trials are now being undertaken to further investigate some of these questions (Howard *et al.*, 2013).

5.1.6 The possible clinical implications of tricuspid regurgitation?

In these studies tricuspid regurgitation was used to calculate pulmonary artery systolic pressures, and participants without detectable tricuspid regurgitation were excluded. One limitation in the methodology is the uncertainty surrounding the significance of physiological tricuspid regurgitation. The degree of severity of tricuspid regurgitation correlates strongly with a poorer prognosis in heart failure (Behm *et al.*, 2004; Nath *et al.*, 2004), while physiological tricuspid regurgitation appears to have no effect on mortality (Lam *et al.*, 2009). In patients who receive heart transplants, the degree of tricuspid regurgitation correlates with five year mortality (Wartig *et al.*, 2014). One limitation of these studies is that the biological or clinical relevance of detectable tricuspid regurgitation in the response to hypoxia in 'normal' participants is unknown.

5.2 Overall Conclusions:

The pulmonary vasculature is a good model for investigating the impact of iron availability on the physiological response to hypoxia. It has already been shown that a bolus of IV iron reduces the rise in PASP by ~50% on the day of the infusion (Smith *et al.*, 2008). Iron supplementation lowers the increase in pulmonary vascular pressure in response to hypoxia, while iron depletion exacerbates the rise in PASP (Smith *et al.*, 2008; Smith *et al.*, 2009).

Independent of the presence of anaemia, iron deficiency is a negative prognostic indicator in both acute (Jankowska *et al.*, 2014) and chronic cardiorespiratory conditions (Comin-Colet *et al.*, 2013; Klip *et al.*, 2013), including acute cardiac failure, chronic systolic heart failure, pulmonary hypertension and COPD. There is increasing evidence that IV iron supplementation is beneficial in these disease states, and this may influence clinical practice and decision making.

The experiments in this thesis were designed to address: *(i)* the physiological effect of bolus and incremental doses of iron *(ii)* the duration of the effects of IV iron on the responses to isocapnic hypoxia, and *(iii)* the dose of iron required to increase iron status.

5.2.1 Physiological effects of bolus and incremental doses of iron

One of the main findings of this work is that IV iron attenuates hypoxic pulmonary vasoconstriction and the effect persists for at least six weeks. The degree of suppression is

in the magnitude of ~50% on the day of the infusion, which is consistent with previous studies (Smith *et al.*, 2008). PASP remains suppressed, although to a lesser degree over time. Similar to the effect of a single bolus dose of iron, with careful manipulation of iron stores over six months, the magnitude of pulmonary hypertension induced by hypoxia is also abrogated. Taken together, these findings suggest that iron stores play a central role in the human physiological response to hypoxia.

Both studies in this thesis demonstrate that by taking a cohort traditionally considered 'iron-replete' and supplementing their iron stores, there is a reduction in the degree of pulmonary vasoconstriction. This effect lasts beyond the half-life of the iron preparation (Geisser & Banke-Bochita, 2010), suggesting that it is manipulation of iron status rather than the artificial iron-sugar complex itself that affects the hypoxic response. The degree to which iron stores affect the pulmonary vascular response is quantitative, with the change in ΔPASP being dose-dependent. This work has shown that the effect of iron on ΔPASP was significant, whether the iron loading was expressed in terms of cumulative dose ($p < 0.05$) or its effect on serum ferritin concentration ($p < 0.01$). This suggests that more careful consideration of iron status needs to be made in clinical practice, particularly in diseases where hypoxia is central to the pathogenesis.

The quantitative effect of iron stores on ΔPASP has great clinical relevance because iron deficiency is the most common medical condition world-wide (Camaschella, 2015) and is an independent risk factor for mortality in chronic hypoxic conditions (Barbera *et al.*, 2003; Rhodes *et al.*, 2011a; Jankowska *et al.*, 2014). Up to one-third of patients with chronic heart failure or pulmonary arterial hypertension are thought to be iron deficient.

The importance of iron stores in oxygen sensing shown in this work is linked to parallel work in the same laboratory. Iron deficient patients (mean ferritin 6.4 μg/L, mean transferrin saturation 8.4%), demonstrated an excess of 50% rise in PASP compared with iron-replete controls with exposure to six hours of sustained hypoxia (P_{ETO_2} 55 mmHg) (Frise *et al.*, 2016). Balanos *et al.* showed that infusion of DFO, an iron chelator, leads to a progressive rise in PASP that mimics the time course of hypoxia (Balanos *et al.*, 2002). This result is confounded by the fact that DFO has a role in oxidation and reduction reactions and can result in free radical formation. Free radicals are believed to alter the HIF pathway and thus oxygen sensing, which could therefore constitute an alternative mechanism to explain the effects of DFO on the pulmonary vasculature (Salceda & Caro, 1997; Zhu *et al.*, 1998). Unlike studies using DFO, that of Frise *et al.* was able to demonstrate that it is iron status itself which is implicated in hypoxic vasoconstriction (Frise *et al.*, 2016).

In addition to exacerbating the acute rise in PASP with hypoxia (Frise *et al.*, 2016), iron deficiency may also contribute to longer term pulmonary vascular remodelling (Barbera *et al.*, 2003). In an animal model, rats fed an iron deficient diet demonstrated medial hypertrophy, inflammatory changes, raised pulmonary artery pressure and right ventricular hypertrophy (Cotroneo *et al.*, 2015). The pulmonary vascular remodelling in these rats was reversed with administration of FCM. Translated to clinical studies, this work may explain why iron status has an impact on both acute and chronic outcomes in hypoxic cardiorespiratory disease.

5.2.2 Duration of effects of intravenous iron

While previous work has demonstrated that iron infusion affects the pulmonary vascular response on the day of iron infusion (Smith *et al.*, 2008), and for up to three days post infusion (Smith *et al.*, 2009), the duration beyond this was unknown. Study A confirms that after administration of 1 g of IV iron the effect on both iron indices and pulmonary vasculature persisted at days 1, 8 and 43.

The concentrations of ferritin and transferrin, as well as transferrin saturation, remained elevated at day 43. A question which remains outstanding is whether the effect on PASP persists because of the persistence of circulating iron markers, or whether it is related to overall iron stores. Ferritin is considered a good marker of iron stores, and Study B shows manipulation of these iron stores by incrementally infusing IV iron with a resultant increase in ferritin concentration. In this study, there was no significant decrease in transferrin concentration or rise in transferrin saturation with iron loading. In the absence of a significant change in transferrin concentration and transferrin saturation, there was a reduction in the rise in PASP with sustained hypoxia. Frise *et al.* showed that iron deficiency itself exacerbated the rise in PASP (Frise *et al.*, 2016). Taken together, these findings suggest that it is iron stores themselves that influence the pulmonary vasculature.

Yet to be answered is what the effect of IV iron on both iron indices and the systemic hypoxic response is beyond 43 days. Assuming losses of iron of 1-2 mg/day without other confounding factors, it would take 2-3 years for iron stores to return to their pre-infusion values. Evidence that the effect of IV iron persists beyond this time is demonstrated in a study of patients with heart failure who were administered six weeks of IV iron therapy for

'iron deficiency', and an additional dose of 500 mg at 12, 24 and 32 weeks if they still met iron deficiency criteria (Ponikowski *et al.*, 2015). Patients randomised to FCM had a significantly greater six minute walk distance and improved self-reported global assessment and fatigue score at week 52. Moreover, a post-hoc analysis of this study showed reduced hospitalisations in patients who had been randomised to the FCM arm of this trial. In a cohort of patients with pulmonary arterial hypertension, a single dose of 1 g of FCM led to improvements in quality of life and six minute walk distance at eight weeks (Viethen *et al.*, 2014). These studies contribute evidence to the persistence of the effect of FCM, albeit with qualitative end points, but do not provide a link to an underlying mechanism.

Study A demonstrated that the effect of iron on the pulmonary vasculature persisted until at least day 43. This is well beyond the half-life of FCM and is likely to be due to the effects of iron being incorporated into iron stores. One limitation of both studies is that there is no evidence that the iron-sugar complex FCM has been eliminated, although this is likely (Geisser & Banke-Bochita, 2010).

5.2.3 Dose of iron needed for modest iron loading

There is a growing body of evidence demonstrating the importance of iron replacement in chronic cardiorespiratory conditions and modern medical therapeutic goals are increasingly tailored to each individual patient. This work meaningfully contributes to this by providing a safe dosing schedule for IV iron in a population traditionally considered to be 'iron-replete'.

IV iron has a significant effect on iron stores, and ferritin concentration is the most reliable marker of this. Both a single bolus dose and incremental smaller doses of IV iron increase ferritin concentration in a significant manner. The increase in ferritin is robust and persists for at least six weeks. We showed a clear linear relationship of ~20 μg/ L increase in ferritin concentration for every 100 mg of iron administered. With an increasing body of evidence showing that iron deficiency is a poor prognostic indictor, even in the absence of anaemia, this could provide an important incentive for the administration of IV iron to patients who are not considered 'iron deficient' by conventional definitions.

There are still a number of questions that need to be addressed that will form the basis of future work in this field. These questions include investigating the mechanisms underlying the relationship between iron and the pulmonary vasculature, the dose-response relationship of iron and PASP, the role of iron independent of haemoglobin, and the link between hepcidin and hypoxia. Finally, there are methodological questions of whether these studies in normal participants are relevant to patients with systemic pathology, as well as the clinical significance of tricuspid regurgitation as a surrogate measure for pulmonary vasoconstriction.

5.3 Future work

5.3.1 Mechanistic link between HIF, iron and the pulmonary vasculature

Future work into the mechanism behind the findings in this work is needed as there are no practical ways of measuring HIF levels in humans at present. Currently, a study is being designed in our laboratory using the same hypoxic chamber methodology to investigate the effects of the administration of digoxin on the pulmonary vasculature. Digoxin is a member of a family of cardiac glycosides known to inhibit HIF-1α translocation and block its activity (Zhang *et al.*, 2008a; Yoshida *et al.*, 2010). Digoxin administration reduces right ventricular hypertrophy, pulmonary vascular remodelling and increases in PASMC calcium concentration in mice exposed to chronic hypoxia (Abud *et al.*, 2012). In humans, digoxin improves right ventricular dysfunction in the short term. Digoxin administration may be a good way of assessing HIF blockade with sustained hypoxia. As yet, however, there would be no way of definitively attributing any of these results to the HIF pathway.

5.3.2 Large cohort clinical studies in hypoxic cardiorespiratory disease

Currently a larger study is underway based on this work and previous studies in the same laboratory, in which the proof of concept is currently being extended to patients with pulmonary hypertension. Howard *et al.* utilise right heart catheterisation to evaluate the effect of FCM on this patient cohort, with the primary outcome being measurement of change in resting pulmonary vascular resistance from baseline at 12 weeks (Howard *et al.*, 2013). Though there is a well described, close correlation with echocardiography and pulmonary artery catheterisation, the latter remains the gold standard. The twelve week time point will also answer whether the effect of IV iron on PASP persists beyond day 43, further improving our understanding of the time course of the effect of FCM on the

pulmonary vasculature. Furthermore, this study is the first to use cardiac MRI to look at the effects of IV FCM on this patient cohort. This will provide insight into right ventricular remodelling and changes in cardiac anatomy after iron infusion. Comparisons between this work and the results of the upcoming study may help to delineate whether there are similar or diverging pulmonary vascular responses between a 'normal' healthy cohort and individuals with pulmonary arterial hypertension who have established pulmonary vascular remodelling.

There is great scope for future work in this area with regards to treatment of heart failure with IV iron. Larger clinical studies with qualitative end-points are already underway to further evaluate the effect of IV iron in chronic hypoxic cardiopulmonary disease. FAIR-HF2, which builds on the work of Anker *et al.* (Anker *et al.*, 2009) has been designed to look at the effect of IV iron on morbidity and mortality in patients with heart failure with reduced ejection fraction. The FAIR-HFpEF study is being designed to look at similar outcomes in those patients with heart failure with a preserved ejection fraction.

No study of heart failure to date has demonstrated the mechanism behind the poor outcomes associated with iron deficiency. Iron has a multitude of biochemical and physiological effects. Based on the results here one could postulate that disorders in oxygen sensing via the HIF pathway may be one explanation for poorer outcomes in these patients. Incorporating right heart catheter assessments and measurements of mean PASP and pulmonary vascular resistance into these larger studies on FCM will provide useful data. Unfortunately, it would be difficult to ethically justify including an acute hypoxic challenge in these already unwell patients, so an appropriate methodology would need to

be carefully considered. Like the study designed by Howard *et al.* (Howard *et al.*, 2013), the incorporation of the technique of cardiac MRI, particularly to examine right ventricular remodelling and hypertrophy, could produce valuable data.

5.3.3 Dosing

From a clinical perspective the practical finding of this study, namely the robust linear relationship with IV iron and ferritin concentration, needs to be expanded into a health setting. Certainly clear guidelines are required relating to screening for iron deficiency in chronic hypoxic disease states and for setting out criteria for iron replacement.

The question of dose and potential application of orally administered iron is being studied in a trial of high altitude miners in Peru. This will follow directly on from the work in this thesis. In this study, oral iron will be administered and the effects on the human pulmonary and haematological response will be studied in a high altitude setting. The primary goal of this study is an understanding of the relationship between the duration of oral iron administration at altitude and measurements of iron status (ferritin concentration, transferrin saturation and soluble transferrin receptor).

REFERENCES

Abboud S & Haile DJ. (2000). A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *The Journal of Biological Chemistry* **275,** 19906-19912.

Abbrecht PH & Littell JK. (1972). Plasma erythropoietin in men and mice during acclimatization to different altitudes. *Journal of Applied Physiology* **32,** 54-58.

Abnova. (2010). ELISA Pairs Kits. Available at: http://www.abnova.com/support/resources.asp?switchfunctionid=%7B70196CA1-59B1-40D0-8394-19F533EB108F%7D URL [Accessed December 28, 2016].

Abud EM, Maylor J, Undem C, Punjabi A, Zaiman AL, Myers AC, Sylvester JT, Semenza GL & Shimoda LA. (2012). Digoxin inhibits development of hypoxic pulmonary hypertension in mice. *Proceedings of the National Academy of Sciences of the United States of America* **109,** 1239-1244.

Aebischer N, Meuli R, Jeanrenaud X, Koerfer J & Kappenberger L. (1998). An echocardiographic and magnetic resonance imaging comparative study of right ventricular volume determination. *International Journal of Cardiac Imaging* **14,** 271-278.

Agarwal R, Vasavada N, Sachs NG & Chase S. (2004). Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. *Kidney International* **65,** 2279-2289.

Ahmad KA, Ahmann JR, Migas MC, Waheed A, Britton RS, Bacon BR, Sly WS & Fleming RE. (2002). Decreased liver hepcidin expression in the *HFE* knockout mouse. *Blood Cells, Molecules, and Diseases* **29,** 361-366.

Akgul F, Batyraliev T, Karben Z & Pershukov I. (2007). Effects of acute hypoxia on left and right ventricular contractility in chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease* **2,** 77-80.

Aldenderfer M. (2011). Peopling the Tibetan plateau: insights from archaeology. *High Altitude Medicine & Biology* **12,** 141-147.

Alfrey CP, Udden MM, LeachHuntoon C, Driscoll T & Pickett MH. (1996). Control of red blood cell mass in spaceflight. *Journal of Applied Physiology* **81,** 98-104.

Allemann Y, Sartori C, Lepori M, Pierre S, Melot C, Naeije R, Scherrer U & Maggiorini M. (2000). Echocardiographic and invasive measurements of pulmonary artery pressure correlate closely at high altitude. *American Journal of Physiology Heart and Circulatory Physiology* **279,** H2013-2016.

Allen J, Backstrom KR, Cooper JA, Cooper MC, Detwiler TC, Essex DW, Fritz RP, Means RT, Meier PB, Pearlman SR, Roitman-Johnson B & Seligman PA. (1998). Measurement of soluble transferrin receptor in serum of healthy adults. *Clinical Chemistry* **44,** 35-39.

Altamura S, Bartsch P, Dehnert C, Maggiorini M, Weiss G, Theurl I, Muckenthaler MU & Mairbaurl H. (2015). Increased hepcidin levels in high-altitude pulmonary edema. *Journal of Applied Physiology* **118,** 292-298.

Anderson ER, Xue X & Shah YM. (2011). Intestinal hypoxia-inducible factor-2alpha (HIF-2alpha) is critical for efficient erythropoiesis. *The Journal of Biological Chemistry* **286,** 19533-19540.

Andrews NC. (2008). Forging a field: the golden age of iron biology. *Blood* **112,** 219-230.

Andriopoulos B, Corradini E, Xia Y, Faasse SA, Chen SZ, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY & Babitt JL. (2009). BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nature Genetics* **41,** 482-487.

Ang SO, Buchanan GR, Gordeuk VR & Prchal JT. (1999). Putative dysregulation in the HIF-1 oxygen sensing pathway in congenital Chuvash polycythemia. *Blood* **94,** 412a-412a.

Ang SO, Chen H, Hirota K, Gordeuk VR, Jelinek J, Guan YL, Liu EL, Sergueeva AI, Miasnikova GY, Mole D, Maxwell PH, Stockton DW, Semenza GL & Prchal JT. (2002). Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nature Genetics* **32,** 614-621.

Anker SD, Comin Colet J, Filippatos G, Willenheimer R, Dickstein K, Drexler H, Luscher TF, Bart B, Banasiak W, Niegowska J, Kirwan BA, Mori C, von Eisenhart Rothe B, Pocock SJ, Poole-Wilson PA & Ponikowski P. (2009). Ferric carboxymaltose in patients with heart failure and iron deficiency. *The New England Journal of Medicine* **361,** 2436-2448.

Ashenden MJ, Gore CJ, Martin DT, Dobson GP & Hahn AG. (1999). Effects of a 12-day "live high, train low" camp on reticulocyte production and haemoglobin mass in elite female road cyclists. *European Journal of Applied Physiology and Occupational Physiology* **80,** 472-478.

Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC & Morrell NW. (2002). Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* **105,** 1672-1678.

Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC & Lin HY. (2006). Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nature Genetics* **38,** 531-539.

Bach M, Geisel T, Martin J, Schulze B, Schaefer R, Virgin G & Stein J. (2015). Efficacy and safety of intravenous ferric carboxymaltose in geriatric inpatients at a German tertiary university teaching hospital: A retrospective observational cohort study of clinical practice. *Anemia* **2015,** 647930.

Bainton DF & Finch CA. (1964). The diagnosis of iron deficiency anemia. *The American Journal of Medicine* **37,** 62-70.

Balanos GM, Dorrington KL & Robbins PA. (2002). Desferrioxamine elevates pulmonary vascular resistance in humans: potential for involvement of HIF-1. *Journal of Applied Physiology* **92,** 2501-2507.

Balanos GM, Pugh K, Frise MC & Dorrington KL. (2015). Exaggerated pulmonary vascular response to acute hypoxia in older men. *Experimental Physiology* **100,** 1187-1198.

Balanos GM, Talbot NP, Dorrington KL & Robbins PA. (2003). Human pulmonary vascular response to 4 h of hypercapnia and hypocapnia measured using Doppler echocardiography. *Journal of Applied Physiology* **94,** 1543-1551.

Balanos GM, Talbot NP, Robbins PA & Dorrington KL. (2005). Separating the direct effect of hypoxia from the indirect effect of changes in cardiac output on the maximum pressure difference across the tricuspid valve in healthy humans. *Pflugers Arch European Journal of Physiology* **450,** 372-380.

Balga I, Solenthaler M & Furlan M. (2000). Should whole-body red cell mass be measured or calculated? *Blood Cells Molecules and Diseases* **26,** 25-31.

Barbera JA, Peinado VI & Santos S. (2003). Pulmonary hypertension in chronic obstructive pulmonary disease. *The European Respiratory Journal* **21,** 892-905.

Bartsch P & Swenson ER. (2013). Clinical practice: Acute high-altitude illnesses. *The New England Journal of Medicine* **368,** 2294-2302.

Bartsch P, Swenson ER & Maggiorini M. (2001). Update: High altitude pulmonary edema. *Advances in Experimental Medicine and Biology* **502,** 89-106.

Beall CM, Cavalleri GL, Deng L, Elston RC, Gao Y, Knight J, Li C, Li JC, Liang Y, McCormack M, Montgomery HE, Pan H, Robbins PA, Shianna KV, Tam SC, Tsering N, Veeramah KR, Wang W, Wangdui P, Weale ME, Xu Y, Xu Z, Yang L, Zaman MJ, Zeng C, Zhang L, Zhang X, Zhaxi P & Zheng YT. (2010). Natural selection on *EPAS1 (HIF2alpha)* associated with low hemoglobin concentration in Tibetan highlanders. *Proceedings of the National Academy of Sciences of the United States of America* **107,** 11459-11464.

Beguin Y. (1992). The Soluble Transferrin Receptor - Biological Aspects and Clinical Usefulness as Quantitative Measure of Erythropoiesis. *Haematologica* **77,** 1-10.

Behm CZ, Nath J & Foster E. (2004). Clinical correlates and mortality of hemodynamically significant tricuspid regurgitation. *The Journal of Heart Valve Disease* **13,** 784-789.

Beigel R, Cercek B, Luo H & Siegel RJ. (2013). Noninvasive evaluation of right atrial pressure. *Journal of the American Society of Echocardiography* **26,** 1033-1042.

Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP, Hottenga JJ, Henders AK, Campbell MJ, Wallace L, Frazer IH, Heath AC, de Geus EJ, Nyholt DR, Visscher PM, Penninx BW, Boomsma DI, Martin NG, Montgomery GW & Whitfield JB. (2009a). Common variants in *TMPRSS6* are associated with iron status and erythrocyte volume. *Nature Genetics* **41,** 1173-1175.

Benyamin B, McRae AF, Zhu G, Gordon S, Henders AK, Palotie A, Peltonen L, Martin NG, Montgomery GW, Whitfield JB & Visscher PM. (2009b). Variants in *TF* and *HFE* explain approximately 40% of genetic variation in serum-transferrin levels. *The American Journal of Human Genetics* **84,** 60-65.

Berger M, Hecht SR, Van Tosh A & Lingam U. (1989). Pulsed and continuous wave Doppler echocardiographic assessment of valvular regurgitation in normal subjects. *Journal of the American College of Cardiology* **13,** 1540-1545.

Bisbe E, Garcia-Erce JA, Diez-Lobo AI, Munoz M & Anaemia Working Group E. (2011). A multicentre comparative study on the efficacy of intravenous ferric carboxymaltose and iron sucrose for correcting preoperative anaemia in patients undergoing major elective surgery. *British Journal of Anaesthesia* **107,** 477-478.

Bisbe E, Rodriguez C, Ruiz A, Saez M, Castillo J & Santiveri X. (2005). Preoperative use of intravenous iron: a new transfusional therapy. *Revista Espanola de Anestesiologia y Reanimacion* **52,** 536-540.

Bishop T, Talbot NP, Turner PJ, Nicholls LG, Pascual A, Hodson EJ, Douglas G, Fielding JW, Smith TG, Demetriades M, Schofield CJ, Robbins PA, Pugh CW, Buckler KJ & Ratcliffe PJ. (2013). Carotid body hyperplasia and enhanced ventilatory responses to hypoxia in mice with heterozygous deficiency of PHD2. *The Journal of Physiology* **591,** 3565-3577.

Borman MA, MacDonald JA, Muranyi A, Hartshorne DJ & Haystead TA. (2002). Smooth muscle myosin phosphatase-associated kinase induces Ca^{2+} sensitization via myosin phosphatase inhibition. *The Journal of Biological Chemistry* **277,** 23441-23446.

Bothwell TH & MacPhail AP. (1998). Hereditary hemochromatosis: etiologic, pathologic, and clinical aspects. *Seminars in Hematology* **35,** 55-71.

Bradford JR & Dean HP. (1894). The Pulmonary Circulation. *The Journal of Physiology* **16,** 34-158 125.

Bregman DB, Morris D, Koch TA, He A & Goodnough LT. (2013). Hepcidin levels predict nonresponsiveness to oral iron therapy in patients with iron deficiency anemia. *American Journal of Hematology* **88,** 97-101.

Brooks JT, Elvidge GP, Glenny L, Gleadle JM, Liu C, Ragoussis J, Smith TG, Talbot NP, Winchester L, Maxwell PH & Robbins PA. (2009). Variations within oxygen-regulated gene expression in humans. *Journal of Applied Physiology* **106,** 212-220.

Bruner AB, Joffe A, Duggan AK, Casella JF & Brandt J. (1996). Randomised study of cognitive effects of iron supplementation in non-anaemic iron-deficient adolescent girls. *Lancet* **348,** 992-996.

Brusselmans K, Compernolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D & Carmeliet P. (2003). Heterozygous deficiency of hypoxia-inducible factor–2α protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *The Journal of Clinical Investigation* **111,** 1519-1527.

Burge CM & Skinner SL. (1995). Determination of hemoglobin mass and blood volume with CO: evaluation and application of a method. *Journal of Applied Physiology* **79,** 623-631.

Bushuev VI, Miasnikova GY, Sergueeva AI, Polyakova LA, Okhotin D, Gaskin PR, Debebe Z, Nekhai S, Castro OL, Prchal JT & Gordeuk VR. (2006). Endothelin-1, vascular endothelial growth factor and systolic pulmonary artery pressure in patients with Chuvash polycythemia. *Haematologica* **91,** 744-749.

Camaschella C. (2015). Iron-deficiency anemia. *The New England Journal of Medicine* **372,** 1832-1843.

Carlsson AJ, Bindslev L, Santesson J, Gottlieb I & Hedenstierna G. (1985). Hypoxic Pulmonary Vasoconstriction in the human lung - the effect of prolonged unilateral hypoxic challenge during anesthesia. *Acta Anaesthesiologica Scandinavica* **29,** 346-351.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D & Keshet E. (1998). Role of HIF-1 alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394,** 485-490.

Cavill I. (1999). Iron status as measured by serum ferritin: The marker and its limitations. *American Journal of Kidney Diseases* **34,** S12-S17.

Chambers JC, Zhang W, Li Y, Sehmi J, Wass MN, Zabaneh D, Hoggart C, Bayele H, McCarthy MI, Peltonen L, Freimer NB, Srai SK, Maxwell PH, Sternberg MJ, Ruokonen A, Abecasis G, Jarvelin MR, Scott J, Elliott P & Kooner JS. (2009). Genome-wide association study identifies variants in *TMPRSS6* associated with hemoglobin levels. *Nature Genetics* **41,** 1170-1172.

Chand S, Ward DG, Ng ZY, Hodson J, Kirby H, Steele P, Rooplal I, Bantugon F, Iqbal T, Tselepis C, Drayson MT, Whitelegg A, Chowrimootoo M & Borrows R. (2015). Serum hepcidin-25 and response to intravenous iron in patients with non-dialysis chronic kidney disease. *Journal of Nephrology* **28,** 81-88.

Chaouat A, Naeije R & Weitzenblum E. (2008). Pulmonary hypertension in COPD. *The European Respiratory Journal* **32,** 1371-1385.

Charytan C, Qunibi W, Bailie GR & Venofer Clinical Studies G. (2005). Comparison of intravenous iron sucrose to oral iron in the treatment of anemic patients with chronic kidney disease not on dialysis. *Nephron Clinical Practice* **100,** c55-62.

Christman BW, McPherson CD, Newman JH, King GA, Bernard GR, Groves BM & Loyd JE. (1992). An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *The New England Journal of Medicine* **327,** 70-75.

Comin-Colet J, Enjuanes C, Gonzalez G, Torrens A, Cladellas M, Merono O, Ribas N, Ruiz S, Gomez M, Verdu JM & Bruguera J. (2013). Iron deficiency is a key determinant of health-related quality of life in patients with chronic heart failure regardless of anaemia status. *European Journal of Heart Failure* **15,** 1164-1172.

Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D & Carmeliet P. (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nature Medicine* **8,** 702-710.

Connor JR, Ponnuru P, Wang XS, Patton SM, Allen RP & Earley CJ. (2011). Profile of altered brain iron acquisition in restless legs syndrome. *Brain* **134,** 959-968.

Cook JD, Dassenko S & Skikne BS. (1990). Serum transferrin receptor as an index of iron absorption. *British Journal of Haematology* **75,** 603-609.

Cook JD & Skikne BS. (1982). Serum ferritin: a possible model for the assessment of nutrient stores. *The American Journal of Clinical Nutrition* **35,** 1180-1185.

Cook JD, Skikne BS & Baynes RD. (1993). Serum transferrin receptor. *Annual Review of Medicine* **44,** 63-74.

Cooperman SS, Meyron-Holtz EG, Olivierre-Wilson H, Ghosh MC, McConnell JP & Rouault TA. (2005). Microcytic anemia, erythropoietic protoporphyria, and neurodegeneration in mice with targeted deletion of iron-regulatory protein 2. *Blood* **106,** 1084-1091.

Cotroneo E, Ashek A, Wang L, Wharton J, Dubois O, Bozorgi S, Busbridge M, Alavian KN, Wilkins MR & Zhao L. (2015). Iron homeostasis and pulmonary hypertension: iron deficiency leads to pulmonary vascular remodeling in the rat. *Circulation Research* **116,** 1680-1690.

Covic A & Mircescu G. (2010). The safety and efficacy of intravenous ferric carboxymaltose in anaemic patients undergoing haemodialysis: a multi-centre, open-label, clinical study. *Nephrology Dialysis Transplantation* **25,** 2722-2730.

Curran-Everett D. (2005). A classic learning opportunity from Fenn, Rahn, and Otis (1946): the alveolar gas equation. *Advances in Physiology Education* **30,** 58-62

D'Alto M, Romeo E, Argiento P, D'Andrea A, Vanderpool R, Correra A, Bossone E, Sarubbi B, Calabro R, Russo MG & Naeije R. (2013). Accuracy and precision of echocardiography versus right heart catheterization for the assessment of pulmonary hypertension. *International Journal of Cardiology* **168,** 4058-4062.

Davies KJ, Maguire JJ, Brooks GA, Dallman PR & Packer L. (1982). Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion. *The American Journal of Physiology* **242,** E418-427.

De Falco L, Sanchez M, Silvestri L, Kannengiesser C, Muckenthaler MU, Iolascon A, Gouya L, Camaschella C & Beaumont C. (2013). Iron refractory iron deficiency anemia. *Haematologica* **98,** 845-853.

de Mast Q, Nadjm B, Reyburn H, Kemna EH, Amos B, Laarakkers CM, Silalye S, Verhoef H, Sauerwein RW, Swinkels DW & van der Ven AJ. (2009a). Assessment of urinary concentrations of hepcidin provides novel insight into disturbances in iron homeostasis during malarial infection. *The Journal of Infectious Diseases* **199,** 253-262.

de Mast Q, van Dongen-Lases EC, Swinkels DW, Nieman AE, Roestenberg M, Druilhe P, Arens TA, Luty AJ, Hermsen CC, Sauerwein RW & van der Ven AJ. (2009b). Mild increases in serum hepcidin and interleukin-6 concentrations impair iron incorporation in haemoglobin during an experimental human malaria infection. *British Journal of Haematology* **145,** 657-664.

Dehnert C, Risse F, Ley S, Kuder TA, Buhmann R, Puderbach M, Menold E, Mereles D, Kauczor HU, Bartsch P & Fink C. (2006). Magnetic resonance imaging of uneven pulmonary perfusion in hypoxia in humans. *American Journal of Respiratory and Critical Care Medicine* **174,** 1132-1138.

Dell'Italia LJ. (1991). The right ventricle: anatomy, physiology, and clinical importance. *Current Problems in Cardiology* **16,** 653-720.

Dipp M, Nye PC & Evans AM. (2001). Hypoxic release of calcium from the sarcoplasmic reticulum of pulmonary artery smooth muscle. *American Journal of Physiology Lung Cellular and Molecular Physiology* **281,** L318-325.

Domkowski PW, Cockerham JT, Kot PA, Myers JL, Wallace RB & Hopkins RA. (1995). The role of N omega-nitro-L-arginine in modulation of pulmonary vascular tone in the maturing newborn pig. *The Journal of Thoracic and Cardiovascular Surgery* **110,** 1486-1492.

Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC & Zon LI. (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403,** 776-781.

Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S & Andrews NC. (2005). The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metabolism* **1,** 191-200.

Dorrington KL, Clar C, Young JD, Jonas M, Tansley JG & Robbins PA. (1997). Time course of the human pulmonary vascular response to 8 hours of isocapnic hypoxia. *The American Journal of Physiology* **273,** H1126-1134.

Douglas C, Haldane J, Henderson Y & Schneider E. (1913). Physiological observations made on Pike's Peak, Colorado, with special reference to adaptation to low barometric pressure. *Philosophical Transactions of the Royal Society B* **203,** 185–318.

Douglas PS, Garcia MJ, Haines DE, Lai WW, Manning WJ, Patel AR, Picard MH, Polk DM, Ragosta M, Ward RP & Weiner RB. (2011). ACCF/ASE/AHA/ASNC/HFSA/HRS/SCAI/SCCM/SCCT/SCMR 2011 Appropriate Use Criteria for Echocardiography. *Journal of the American College of Cardiology* **57,** 1126-1166.

Du X, She E, Gelbart T, Truksa J, Lee P, Xia Y, Khovananth K, Mudd S, Mann N, Moresco EM, Beutler E & Beutler B. (2008). The serine protease *TMPRSS6* is required to sense iron deficiency. *Science* **320,** 1088-1092.

Dunwoodie SL. (2009). The role of hypoxia in development of the mammalian embryo. *Developmental Cell* **17,** 755-773.

Duvall MG, Pikman Y, Kantor DB, Ariagno K, Summers L, Sectish TC & Mullen MP. (2013). Pulmonary hypertension associated with scurvy and vitamin deficiencies in an autistic child. *Pediatrics* **132,** e1699-1703.

Eckardt KU, Boutellier U, Kurtz A, Schopen M, Koller EA & Bauer C. (1989). Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *Journal of Applied Physiology* **66,** 1785-1788.

Ekblom B. (1996). Blood doping and erythropoietin - The effects of variation in hemoglobin concentration and other related factors on physical performance. *American Journal of Sports Medicine* **24,** S40-S42.

Elkins JM, Hewitson KS, McNeill LA, Seibel JF, Schlemminger I, Pugh CW, Ratcliffe PJ & Schofield CJ. (2003). Structure of factor-inhibiting hypoxia-inducible factor (HIF) reveals mechanism of oxidative modification of HIF-1 alpha. *The Journal of Biological Chemistry* **278,** 1802-1806.

Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J & Gleadle JM. (2006). Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. *The Journal of Biological Chemistry* **281,** 15215-15226.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ & Ratcliffe PJ. (2001). C. elegans EGL-9 and

mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107,** 43-54.

Epstein FH, Agmon Y & Brezis M. (1994). Physiology of renal hypoxia. *Annals of the New York Academy of Sciences* **718,** 72-81; .

Evans SS, Repasky EA & Fisher DT. (2015). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nature Reviews Immunology* **15,** 335-349.

Evstatiev R, Marteau P, Iqbal T, Khalif IL, Stein J, Bokemeyer B, Chopey IV, Gutzwiller FS, Riopel L, Gasche C & Group FS. (2011). FERGIcor, a randomized controlled trial on ferric carboxymaltose for iron deficiency anemia in inflammatory bowel disease. *Gastroenterology* **141,** 846-853 e841-842.

Fairbanks VF. (2000). Commentary: Should whole-body red cell mass be measured or calculated. *Blood Cells Molecules and Diseases* **26,** 32-36.

Fatemian M, Herigstad M, Croft QP, Formenti F, Cardenas R, Wheeler C, Smith TG, Friedmannova M, Dorrington KL & Robbins PA. (2016). Determinants of ventilation and pulmonary artery pressure during early acclimatization to hypoxia in humans. *The Journal of Physiology* **594,** 1197-1213.

Favier J, Buffet A & Gimenez-Roqueplo AP. (2012). *HIF2A* mutations in paraganglioma with polycythemia. *The New England Journal of Medicine* **367,** 2161; author reply 2161-2162.

Feder JN. (1999). The hereditary hemochromatosis gene (*HFE*): a MHC class I-like gene that functions in the regulation of iron homeostasis. *Immunologic Research* **20,** 175-185.

Feldman HI, Joffe M, Robinson B, Knauss J, Cizman B, Guo W, Franklin-Becker E & Faich G. (2004). Administration of parenteral iron and mortality among hemodialysis patients. *Journal of the American Society of Nephrology : JASN* **15,** 1623-1632.

Fenn W, Rahn H & Otis A. (1946). A theoretical study of the composition of the alveolar air at altitude. *The American Journal of Physiology* **146,** 637–653.

Fielitz J, Philipp S, Herda LR, Schuch E, Pilz B, Schubert C, Gunzler V, Willenbrock R & Regitz-Zagrosek V. (2007). Inhibition of prolyl 4-hydroxylase prevents left ventricular remodelling in rats with thoracic aortic banding. *European Journal of Heart Failure* **9,** 336-342.

Finberg KE, Heeney MM, Campagna DR, Aydinok Y, Pearson HA, Hartman KR, Mayo MM, Samuel SM, Strouse JJ, Markianos K, Andrews NC & Fleming MD. (2008). Mutations in *TMPRSS6* cause iron-refractory iron deficiency anemia (IRIDA). *Nature Genetics* **40,** 569-571.

Finch C. (1994). Regulators of iron balance in humans. *Blood* **84,** 1697-1702.

Finch CA & Huebers H. (1982). Perspectives in iron metabolism. *The New England Journal of Medicine* **306,** 1520-1528.

Fleming MD, Trenor CC, Su MA, Foernzler D, Beier DR, Dietrich WF & Andrews NC. (1997). Microcytic anaemia mice have a mutation in *Nramp2*, a candidate iron transporter gene. *Nature Genetics* **16,** 383-386.

Forkink M, Smeitink JA, Brock R, Willems PH & Koopman WJ. (2010). Detection and manipulation of mitochondrial reactive oxygen species in mammalian cells. *Biochimica et Biophysica Acta* **1797,** 1034-1044.

Formenti F, Beer PA, Croft QP, Dorrington KL, Gale DP, Lappin TR, Lucas GS, Maher ER, Maxwell PH, McMullin MF, O'Connor DF, Percy MJ, Pugh CW, Ratcliffe PJ, Smith TG, Talbot NP & Robbins PA. (2011). Cardiopulmonary function in two human disorders of the hypoxia-inducible factor (HIF) pathway: von Hippel-Lindau disease and HIF-2alpha gain-of-function mutation. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **25,** 2001-2011.

Friedmann B, Weller E, Mairbaurl H & Bartsch P. (2001). Effects of iron repletion on blood volume and performance capacity in young athletes. *Medicine and Science in Sports and Exercise* **33,** 741-746.

Frise MC, Cheng HY, Nickol AH, Curtis MK, Pollard KA, Roberts DJ, Ratcliffe PJ, Dorrington KL & Robbins PA. (2016). Clinical iron deficiency disturbs normal human responses to hypoxia. *The Journal of Clinical Investigation* **126,** 2139-2150.

Gaillard CA, Bock AH, Carrera F, Eckardt KU, Van Wyck DB, Bansal SS, Cronin M, Meier Y, Larroque S, Roger SD & Macdougall IC. (2016). Hepcidin Response to Iron Therapy in Patients with Non-Dialysis Dependent CKD: An Analysis of the FIND-CKD Trial. *PloS one* **11,** e0157063.

Gale DP, Harten SK, Reid CD, Tuddenham EG & Maxwell PH. (2008). Autosomal dominant erythrocytosis and pulmonary arterial hypertension associated with an activating HIF2 alpha mutation. *Blood* **112,** 919-921.

Ganz T. (2003). Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* **102,** 783-788.

Ganz T & Nemeth E. (2012). Iron metabolism: interactions with normal and disordered erythropoiesis. *Cold Spring Harbor perspectives in medicine* **2,** a011668.

Ganz T, Olbina G, Girelli D, Nemeth E & Westerman M. (2008). Immunoassay for human serum hepcidin. *Blood* **112,** 4292-4297.

Ganzoni AM. (1970). Intravenous iron-dextran: therapeutic and experimental possibilities. *Schweizerische medizinische Wochenschrift* **100,** 301-303.

Garvican LA, Lobigs L, Telford R, Fallon K & Gore CJ. (2011). Haemoglobin mass in an anaemic female endurance runner before and after iron supplementation. *International Journal of Sports Physiology and Performance* **6,** 137-140.

Geisser P & Banke-Bochita J. (2010). Pharmacokinetics, safety and tolerability of intravenous ferric carboxymaltose: a dose-escalation study in volunteers with mild iron-deficiency anaemia. *Arzneimittel-Forschung* **60,** 362-372.

Ghosh MC, Zhang DL, Jeong SY, Kovtunovych G, Ollivierre-Wilson H, Noguchi A, Tu T, Senecal T, Robinson G, Crooks DR, Tong WH, Ramaswamy K, Singh A, Graham BB, Tuder RM, Yu ZX, Eckhaus M, Lee J, Springer DA & Rouault TA. (2013). Deletion of iron regulatory protein 1 causes polycythemia and pulmonary hypertension in mice through translational derepression of HIF2alpha. *Cell Metabolism* **17,** 271-281.

Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP & Stewart DJ. (1993). Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *The New England Journal of Medicine* **328,** 1732-1739.

Goirand F, Bardou M, Guerard P, Dumas JP, Rochette L & Dumas M. (2003). ETA, mixed ETA/ETB receptor antagonists, and protein kinase C inhibitor prevent acute hypoxic pulmonary vasoconstriction: influence of potassium channels. *Journal of Cardiovascular Pharmacology* **41,** 117-125.

Gonzalez C, Almaraz L, Obeso A & Rigual R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiological Reviews* **74,** 829-898.

Goodnough LT. (2012). Iron deficiency syndromes and iron-restricted erythropoiesis (CME). *Transfusion* **52,** 1584-1592.

Gordeuk VR, Miasnikova GY, Sergueeva AI, Niu X, Nouraie M, Okhotin DJ, Polyakova LA, Ammosova T, Nekhai S, Ganz T & Prchal JT. (2011). Chuvash polycythemia *VHL*^{R200W} mutation is associated with down-regulation of hepcidin expression. *Blood* **118,** 5278-5282.

Gordeuk VR, Sergueeva AI, Miasnikova GY, Okhotin D, Voloshin Y, Choyke PL, Butman JA, Jedlickova K, Prchal JT & Polyakova LA. (2004). Congenital disorder of oxygen sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood* **103,** 3924-3932.

Gore CJ, Hopkins WG & Burge CM. (2005). Errors of measurement for blood volume parameters: a meta-analysis. *Journal of Applied Physiology* **99,** 1745-1758.

Grammer TB, Kleber ME, Silbernagel G, Pilz S, Scharnagl H, Tomaschitz A, Konig W & Marz W. (2014). Hemoglobin, iron metabolism and angiographic coronary artery disease (The Ludwigshafen Risk and Cardiovascular Health Study). *Atherosclerosis* **236,** 292-300.

Groves BM, Droma T, Sutton JR, Mccullough RG, Mccullough RE, Zhuang JG, Rapmund G, Sun SF, Janes C & Moore LG. (1993). Minimal hypoxic pulmonary hypertension in normal Tibetans at 3,658m. *Journal of Applied Physiology* **74,** 312-318.

Groves BM, Reeves JT, Sutton JR, Wagner PD, Cymerman A, Malconian MK, Rock PB, Young PM & Houston CS. (1987). Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *Journal of Applied Physiology* **63,** 521-530.

Grunig E, Mereles D, Hildebrandt W, Swenson ER, Kubler W, Kuecherer H & Bartsch P. (2000). Stress Doppler echocardiography for identification of susceptibility to high altitude pulmonary edema. *Journal of the American College of Cardiology* **35,** 980-987.

Gupta A, Pratt RD & Crumbliss AL. (2016). Ferrous iron content of intravenous iron formulations. *Biometals : an International Journal on the role of metal ions in biology, biochemistry, and medicine* **29,** 411-415.

Gupta ML, Rao KS, Anand IS, Banerjee AK & Boparai MS. (1992). Lack of smooth muscle in the small pulmonary arteries of the native Ladakhi. Is the Himalayan highlander adapted? *The American Review of Respiratory disease* **145,** 1201-1204.

Hackett PH, Reeves JT, Reeves CD, Grover RF & Rennie D. (1980). Control of breathing in sherpas at low and high altitude. *Journal of Applied Physiology* **49,** 374-379.

Haddad F, Couture P, Tousignant C & Denault AY. (2009). The right ventricle in cardiac surgery, a perioperative perspective: II. Pathophysiology, clinical importance, and management. *Anesthesia and Analgesia* **108,** 422-433.

Harari S. (2012). Out-of-proportion pulmonary hypertension: a paradigm for rare diseases. *Chest* **142,** 1087-1088.

Heeney MM & Finberg KE. (2014). Iron-refractory iron deficiency anemia (IRIDA). *Hematology/oncology clinics of North America* **28,** 637-652.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G & Schaper F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochemical Journal* **374,** 1-20.

Hickey MM, Lam JC, Bezman NA, Rathmell WK & Simon MC. (2007). von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis. *The Journal of Clinical Investigation* **117,** 3879-3889.

Hirshman CA, McCullough RE & Weil JV. (1975). Normal values for hypoxic and hypercapnic ventilaroty drives in man. *Journal of Applied Physiology* **38,** 1095-1098.

Ho S & Nihoyannopoulos P. (2006). Anatomy, echocardiography, and normal right ventricular dimensions. *Heart* **92 Suppl 1,** i2-13.

Hoiland RL, Foster GE, Donnelly J, Stembridge M, Willie CK, Smith KJ, Lewis NC, Lucas SJ, Cotter JD, Yeoman DJ, Thomas KN, Day TA, Tymko MM, Burgess KR & Ainslie PN. (2015). Chemoreceptor responsiveness at sea level does not predict the pulmonary pressure response to high altitude. *Chest* **148,** 219-225.

Holloway C, Cochlin L, Codreanu I, Bloch E, Fatemian M, Szmigielski C, Atherton H, Heather L, Francis J, Neubauer S, Robbins P, Montgomery H & Clarke K. (2011). Normobaric hypoxia impairs human cardiac energetics. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **25,** 3130-3135.

Howard LS, Barson RA, Howse BP, McGill TR, McIntyre ME, O'Connor DF & Robbins PA. (1995). Chamber for controlling end-tidal gas tensions over sustained periods in humans. *Journal of Applied Physiology* **78,** 1088-1091.

Howard LS & Robbins PA. (1995). Alterations in respiratory control during 8 h of isocapnic and poikilocapnic hypoxia in humans. *Journal of Applied Physiology* **78,** 1098-1107.

Howard LS, Watson GM, Wharton J, Rhodes CJ, Chan K, Khengar R, Robbins PA, Kiely DG, Condliffe R, Elliott CA, Pepke-Zaba J, Sheares K, Morrell NW, Davies R, Ashby D, Gibbs JS & Wilkins MR. (2013). Supplementation of iron in pulmonary hypertension: Rationale and design of a phase II clinical trial in idiopathic pulmonary arterial hypertension. *Pulmonary Circulation* **3,** 100-107.

Hu CJ, Wang LY, Chodosh LA, Keith B & Simon MC. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Molecular and Cellular Biology* **23,** 9361-9374.

Huez S, Retailleau K, Unger P, Pavelescu A, Vachiery JL, Derumeaux G & Naeije R. (2005). Right and left ventricular adaptation to hypoxia: a tissue Doppler imaging study. *American Journal of Physiology Heart and Circulatory Physiology* **289,** H1391-1398.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS & Kaelin WG, Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* **292,** 464-468.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY & Semenza GL. (1998). Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes & Development* **12,** 149-162.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW & Ratcliffe PJ. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* **292,** 468-472.

Jacob M, Annaheim S, Boutellier U, Hinske C, Rehm M, Breymann C & Krafft A. (2012). Haematocrit is invalid for estimating red cell volume: a prospective study in male volunteers. *Blood Transfusion* **10,** 471-479.

Jankowska EA, Kasztura M, Sokolski M, Bronisz M, Nawrocka S, Oleskowska-Florek W, Zymlinski R, Biegus J, Siwolowski P, Banasiak W, Anker SD, Filippatos G, Cleland JG & Ponikowski P. (2014). Iron deficiency defined as depleted iron stores accompanied by unmet cellular iron requirements identifies patients at the highest risk of death after an episode of acute heart failure. *European Heart Journal* **35,** 2468-2476.

Jankowska EA, Rozentryt P, Witkowska A, Nowak J, Hartmann O, Ponikowska B, Borodulin-Nadzieja L, von Haehling S, Doehner W, Banasiak W, Polonski L, Filippatos G, Anker SD & Ponikowski P. (2011). Iron deficiency predicts impaired exercise capacity in patients with systolic chronic heart failure. *Journal of Cardiac Failure* **17,** 899-906.

Jelkmann W. (1992). Erythropoietin: structure, control of production, and function. *Physiological Reviews* **72,** 449-489.

Jiang BH, Semenza GL, Bauer C & Marti HH. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *The American Journal of Physiology* **271,** C1172-1180.

Jones RD & Morice AH. (1998). The ETA antagonist CI-1020 inhibits hypoxic pulmonary vasoconstriction in small isolated rat pulmonary arteries. *Pulmonary Pharmacology & Therapeutics* **11,** 177-181.

Kaelin WG. (2007). Von Hippel-Lindau disease. *Annual Review of Pathology* **2,** 145-173.

Kaelin WG, Jr. & Ratcliffe PJ. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Molecular Cell* **30,** 393-402.

Karkouti K, McCluskey SA, Ghannam M, Salpeter MJ, Quirt I & Yau TM. (2006). Intravenous iron and recombinant erythropoietin for the treatment of postoperative anemia. *Canadian Journal of Anesthesia* **53,** 11-19.

Kautz L, Jung G, Nemeth E & Ganz T. (2014a). Erythroferrone contributes to recovery from anemia of inflammation. *Blood* **124,** 2569-2574.

Kautz L, Jung G, Valore EV, Rivella S, Nemeth E & Ganz T. (2014b). Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nature Genetics* **46,** 678-684.

Kent BD, Mitchell PD & McNicholas WT. (2011). Hypoxemia in patients with COPD: cause, effects, and disease progression. *International Journal of Chronic Obstructive Pulmonary Disease* **6,** 199-208.

Kerbaul F, Van der Linden P, Pierre S, Rondelet B, Melot C, Brimioulle S & Naeije R. (2004). Prevention of hemodilution-induced inhibition of hypoxic pulmonary vasoconstriction by N-acetylcysteine in dogs. *Anesthesia and Analgesia* **99,** 547-551.

Kiencke S, Bernheim A, Maggiorini M, Fischler M, Aschkenasy SV, Dorschner L, Debrunner J, Bloch K, Mairbaurl H & Rocca HPBL. (2008). Exercise-induced pulmonary artery hypertension - A rare finding? *Journal of the American College of Cardiology* **51,** 513-514.

Kim WY & Kaelin WG. (2004). Role of VHL gene mutation in human cancer. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* **22,** 4991-5004.

Kline DD, Peng YJ, Manalo DJ, Semenza GL & Prabhakar NR. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 821-826.

Klip IT, Comin-Colet J, Voors AA, Ponikowski P, Enjuanes C, Banasiak W, Lok DJ, Rosentryt P, Torrens A, Polonski L, van Veldhuisen DJ, van der Meer P & Jankowska EA. (2013). Iron deficiency in chronic heart failure: an international pooled analysis. *American Heart Journal* **165,** 575-582 e573.

Knowles HJ, Mole DR, Ratcliffe PJ & Harris AL. (2006). Normoxic stabilization of hypoxia-inducible factor-1alpha by modulation of the labile iron pool in differentiating U937 macrophages: effect of natural resistance-associated macrophage protein 1. *Cancer Research* **66,** 2600-2607.

Knowles HJ, Raval RR, Harris AL & Ratcliffe PJ. (2003). Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Research* **63,** 1764-1768.

Koch TA, Myers J & Goodnough LT. (2015). Intravenous iron therapy in patients with iron deficiency anemia: dosing considerations. *Anemia* **2015,** 763576.

Kodama K, Noguchi A, Adachi H, Hebiguchi M, Yano M & Takahashi T. (2014). Novel mutation in the *TMPRSS6* gene with iron-refractory iron deficiency anemia. *Pediatrics International : Official Journal of the Japan Pediatric Society* **56,** e41-44.

Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H & Kohler G. (1994). Impaired immune and acute-phase responses in interleukin-6-deficient mice. *Nature* **368,** 339-342.

Kossaify A. (2015). Echocardiographic assessment of the right ventricle, from the conventional approach to speckle tracking and three-dimensional imaging, and insights into the "right way" to explore the forgotten chamber. *Clinical Medicine Insights Cardiology* **9,** 65-75.

Krayenbuehl PA, Battegay E, Breymann C, Furrer J & Schulthess G. (2011). Intravenous iron for the treatment of fatigue in nonanemic, premenopausal women with low serum ferritin concentration. *Blood* **118,** 3222-3227.

Kulnigg S, Rumyantsev V, Stoinov S, Simanenkov V, Levchenko E, Karnafel W, Garcia LC, D'haens G & Gasche C. (2007). A novel intravenous iron formulation for treatment of anemia in IBD: The ferinject randomized, controlled trial. *Gastroenterology* **132,** A501-A502.

Kulnigg S, Stoinov S, Simanenkov V, Dudar LV, Karnafel W, Garcia LC, Sambuelli AM, D'Haens G & Gasche C. (2008). A novel intravenous iron formulation for treatment of anemia in inflammatory bowel disease: the ferric carboxymaltose (FERINJECT) randomized controlled trial. *The American Journal of Gastroenterology* **103,** 1182-1192.

Kuo KL, Hung SC, Lee TS & Tarng DC. (2014). Iron sucrose accelerates early atherogenesis by increasing superoxide production and upregulating adhesion molecules in CKD. *Journal of the American Society of Nephrology : JASN* **25,** 2596-2606.

Kupari M & Rapola J. (2012). Reversible pulmonary hypertension associated with vitamin C deficiency. *Chest* **142,** 225-227.

Kuragano T, Shimonaka Y, Kida A, Furuta M, Nanami M, Otaki Y, Hasuike Y, Nonoguchi H & Nakanishi T. (2010). Determinants of hepcidin in patients on maintenance hemodialysis: role of inflammation. *American Journal of Nephrology* **31,** 534-540.

Lam CS, Borlaug BA, Kane GC, Enders FT, Rodeheffer RJ & Redfield MM. (2009). Age-associated increases in pulmonary artery systolic pressure in the general population. *Circulation* **119,** 2663-2670.

LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, Switzer R, 3rd, Grinberg A, Love P, Tresser N & Rouault TA. (2001). Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. *Nature Genetics* **27,** 209-214.

Lenfant C & Sullivan K. (1971). Adaptation to high altitude. *The New England Journal of Medicine* **284,** 1298-1309.

Lewis CS, Jr., Samuels AJ, Daines MC & Hecht HH. (1952). Chronic lung disease, polycythemia and congestive heart failure; cardiorespiratory, vascular and renal adjustments in cor pulmonale. *Circulation* **6,** 874-887.

Liao D & Johnson RS. (2007). Hypoxia: A key regulator of angiogenesis in cancer. *Cancer and Metastasis Reviews* **26,** 281-290.

Lin L, Valore EV, Nemeth E, Goodnough JB, Gabatan V & Ganz T. (2007). Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. *Blood* **110,** 2182-2189.

Lindstrom K & Edler I. (1969a). Ultrasonic Doppler technique used in heart disease: an experimental study *Ultrasono Graphia Medica***,** 447-454.

Litton E, Xiao J & Ho KM. (2013). Safety and efficacy of intravenous iron therapy in reducing requirement for allogeneic blood transfusion: systematic review and meta-analysis of randomised clinical trials. *British Medical Journal* **347,** f4822.

Liu C, Smith TG, Balanos GM, Brooks J, Crosby A, Herigstad M, Dorrington KL & Robbins PA. (2007). Lack of involvement of the autonomic nervous system in early ventilatory and pulmonary vascular acclimatization to hypoxia in humans. *The Journal of Physiology* **579,** 215-225.

Liu JQ, Sham JS, Shimoda LA, Kuppusamy P & Sylvester JT. (2003). Hypoxic constriction and reactive oxygen species in porcine distal pulmonary arteries. *American Journal of Physiology Lung Cellular and Molecular Physiology* **285,** L322-333.

Liu Q, Davidoff O, Niss K & Haase VH. (2012). Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. *The Journal of Clinical Investigation* **122,** 4635-4644.

Lloyd BB, Jukes MG & Cunningham DJ. (1958). The relation between alveolar oxygen pressure and the respiratory response to carbon dioxide in man. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences* **43,** 214-227.

Loboda A, Jozkowicz A & Dulak J. (2010). HIF-1 and HIF-2 transcription factors-similar but not identical. *Molecules and Cells* **29,** 435-442.

Loria G & Vassura G. (1919). *Opere di Evangelista Torricelli [Works of Evangelista Torricelli]*, vol. III. Faenza, Italy.

Lukashev D, Ohta A & Sitkovsky M. (2007). Hypoxia-dependent anti-inflammatory pathways in protection of cancerous tissues. *Cancer and Metastasis Reviews* **26,** 273-279.

Lyseng-Williamson KA & Keating GM. (2009). Ferric carboxymaltose: a review of its use in iron-deficiency anaemia. *Drugs* **69,** 739-756.

Mackay EH, Banks J, Sykes B & Lee G. (1978). Structural basis for the changing physical properties of human pulmonary vessels with age. *Thorax* **33,** 335-344.

Madden JA, Vadula MS & Kurup VP. (1992). Effects of hypoxia and other vasoactive agents on pulmonary and cerebral artery smooth muscle cells. *The American Journal of Physiology* **263,** L384-393.

Maggiorini M, Melot C, Pierre S, Pfeiffer F, Greve I, Sartori C, Lepori M, Hauser M, Scherrer U & Naeije R. (2001). High-altitude pulmonary edema is initially caused by an increase in capillary pressure. *Circulation* **103,** 2078-2083.

Makino Y, Cao RH, Svensson K, Bertilsson GR, Asman M, Tanaka H, Cao YH, Berkenstam A & Poellinger L. (2001). Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature* **414,** 550-554.

Mannheimer PD. (2007). The light-tissue interaction of pulse oximetry. *Anesthesia & Analgesia* **105,** S10-17.

Mastrogiannaki M, Matak P, Delga S, Deschemin JC, Vaulont S & Peyssonnaux C. (2012). Deletion of HIF-2alpha in the enterocytes decreases the severity of tissue iron loading in hepcidin knockout mice. *Blood* **119,** 587-590.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER & Ratcliffe PJ. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399,** 271-275.

Maynor L & Brophy DF. (2007). Risk of infection with intravenous iron therapy. *Annals of Pharmacotherapy* **41,** 1476-1480.

McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, Mudaly M, Richardson C, Barlow D, Bomford A, Peters TJ, Raja KB, Shirali S, Hediger MA, Farzaneh F & Simpson RJ. (2001). An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science* **291,** 1755-1759.

McLean E, Cogswell M, Egli I, Wojdyla D & de Benoist B. (2009). Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. *Public Health Nutrition* **12,** 444-454.

Melis MA, Cau M, Congiu R, Sole G, Barella S, Cao A, Westerman M, Cazzola M & Galanello R. (2008). A mutation in the TMPRSS6 gene, encoding a transmembrane serine protease that suppresses hepcidin production, in familial iron deficiency anemia refractory to oral iron. *Haematologica* **93,** 1473-1479.

Mims MP, Guan Y, Pospisilova D, Priwitzerova M, Indrak K, Ponka P, Divoky V & Prchal JT. (2005). Identification of a human mutation of DMT1 in a patient with microcytic anemia and iron overload. *Blood* **105,** 1337-1342.

Mole DR. (2010). Iron homeostasis and its interaction with prolyl hydroxylases. *Antioxidants & Redox Signaling* **12,** 445-458.

Montandon SV, Fajt ML & Petrov AA. (2016). A safe and novel desensitization protocol with Ferric Carboxymaltose to treat iron deficiency anemia. *Current Drug Safety* **11,** 145-148.

Moore LG, Armaza F, Villena M & Vargas E. (2000). Comparative aspects of high-altitude adaptation in human populations. *Advances in Experimental Medicine and Biology* **475,** 45-62.

Moretti D, Goede JS, Zeder C, Jiskra M, Chatzinakou V, Tjalsma H, Melse-Boonstra A, Brittenham G, Swinkels DW & Zimmermann MB. (2015). Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women. *Blood* **126,** 1981-1989.

Mosso A. (1898). Life of Man on the High Alps. *London: T Fisher Unwin*.

Moudgil R, Michelakis ED & Archer SL. (2005). Hypoxic pulmonary vasoconstriction. *Journal of Applied Physiology* **98,** 390-403.

Moudgil R, Michelakis ED & Archer SL. (2006). The role of K^+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: Implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. *Microcirculation* **13,** 615-632.

Muchnik E & Kaplan J. (2011). HIF prolyl hydroxylase inhibitors for anemia. *Expert Opinion on Investigational Drugs* **20,** 645-656.

Muckenthaler M, Roy CN, Custodio AO, Minana B, deGraaf J, Montross LK, Andrews NC & Hentze MW. (2003). Regulatory defects in liver and intestine implicate abnormal hepcidin and Cybrd1 expression in mouse hemochromatosis. *Nature Genetics* **34,** 102-107.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG. (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nature Neuroscience* **7,** 1360-1369.

Murray-Kolb LE & Beard JL. (2007). Iron treatment normalizes cognitive functioning in young women. *The American Journal of Clinical Nutrition* **85,** 778-787.

Nadrous HF, Pellikka PA, Krowka MJ, Swanson KL, Chaowalit N, Decker PA & Ryu JH. (2005). The impact of pulmonary hypertension on survival in patients with idiopathic pulmonary fibrosis. *Chest* **128,** 616S-617S.

Naito Y, Hosokawa M, Sawada H, Oboshi M, Hirotani S, Iwasaku T, Okuhara Y, Morisawa D, Eguchi A, Nishimura K, Soyama Y, Fujii K, Mano T, Ishihara M, Tsujino T & Masuyama T. (2016). Transferrin receptor 1 in chronic hypoxia-induced pulmonary vascular remodeling. *American Journal of Hypertension* **29,** 713-718.

Nanas JN, Matsouka C, Karageorgopoulos D, Leonti A, Tsolakis E, Drakos SG, Tsagalou EP, Maroulidis GD, Alexopoulos GP, Kanakakis JE & Anastasiou-Nana MI. (2006). Etiology of anemia in patients with advanced heart failure. *Journal of the American College of Cardiology* **48,** 2485-2489.

Nath J, Foster E & Heidenreich PA. (2004). Impact of tricuspid regurgitation on long-term survival. *Journal of the American College of Cardiology* **43,** 405-409.

Nathan SD, Noble PW & Tuder RM. (2007). Idiopathic pulmonary fibrosis and pulmonary hypertension: connecting the dots. *American Journal of Respiratory and Critical Care Medicine* **175,** 875-880.

Nelin LD & Dawson CA. (1993). The effect of N omega-nitro-L-arginine methylester on hypoxic vasoconstriction in the neonatal pig lung. *Pediatric Research* **34,** 349-353.

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK & Ganz T. (2004a). IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *The Journal of Clinical Investigation* **113,** 1271-1276.

Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T & Kaplan J. (2004b). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306,** 2090-2093.

Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A & Ganz T. (2003). Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* **101,** 2461-2463.

Nickol AH, Frise MC, Cheng HY, McGahey A, McFadyen BM, Harris-Wright T, Bart NK, Curtis MK, Khandwala S, O'Neill DP, Pollard KA, Hardinge FM, Rahman NM, Armitage AE, Dorrington KL, Drakesmith H, Ratcliffe PJ & Robbins PA. (2015). A cross-sectional study of the prevalence and associations of iron deficiency in a cohort of patients with chronic obstructive pulmonary disease. *British Medical Journal Open* **5,** e007911.

Nicolas G, Chauvet C, Viatte L, Danan JL, Bigard X, Devaux I, Beaumont C, Kahn A & Vaulont S. (2002). The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *The Journal of Clinical Investigation* **110,** 1037-1044.

Nicolas G, Viatte L, Lou DQ, Bennoun M, Beaumont C, Kahn A, Andrews NC & Vaulont S. (2003). Constitutive hepcidin expression prevents iron overload in a mouse model of hemochromatosis. *Nature Genetics* **34,** 97-101.

Nwogu JI, Geenen D, Bean M, Brenner MC, Huang X & Buttrick PM. (2001). Inhibition of collagen synthesis with prolyl 4-hydroxylase inhibitor improves left ventricular function and alters the pattern of left ventricular dilatation after myocardial infarction. *Circulation* **104,** 2216-2221.

O'Kennedy R, Byrne M, O'Faigain & Berns G. (1990). A review of enzyme-immunoassay and a description of a competitive enzyme-linked immunosorbent assay for the detection of immunoglobulin concentrations. *Biochemical Education* **18**.

Oliver RM, Peacock AJ, Challenor VF, Fleming JS & Waller DG. (1991). The effect of acute hypoxia on right ventricular function in healthy adults. *International Journal of Cardiology* **31,** 235-241.

Onken JE, Bregman DB, Harrington RA, Morris D, Buerkert J, Hamerski D, Iftikhar H, Mangoo-Karim R, Martin ER, Martinez CO, Newman GE, Qunibi WY, Ross DL, Singh B, Smith MT, Butcher A, Koch TA & Goodnough LT. (2014). Ferric carboxymaltose in patients with iron-deficiency anemia and impaired renal function: the REPAIR-IDA trial. *Nephrology, dialysis, transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association* **29,** 833-842.

Oswald-Mammosser M, Weitzenblum E, Quoix E, Moser G, Chaouat A, Charpentier C & Kessler R. (1995). Prognostic factors in COPD patients receiving long-term oxygen therapy. Importance of pulmonary artery pressure. *Chest* **107,** 1193-1198.

Page EL, Chan DA, Giaccia AJ, Levine M & Richard DE. (2008). Hypoxia-inducible factor-1alpha stabilization in nonhypoxic conditions: role of oxidation and intracellular ascorbate depletion. *Molecular Biology of the Cell* **19,** 86-94.

Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ & Simon MC. (2007). Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Molecular and Cellular Biology* **27,** 912-925.

Papandreou I, Cairns RA, Fontana L, Lim AL & Denko NC. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metabolism* **3,** 187-197.

Park CH, Valore EV, Waring AJ & Ganz T. (2001). Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *The Journal of Biological Chemistry* **276,** 7806-7810.

Pavord S, Myers B, Robinson S, Allard S, Strong J, Oppenheimer C & British Committee for Standards in H. (2012). UK guidelines on the management of iron deficiency in pregnancy. *British Journal of Haematology* **156,** 588-600.

Pearson TC, Guthrie DL, Simpson J, Chinn S, Barosi G, Ferrant A, Lewis SM & Najean Y. (1995). Interpretation of measured red cell mass and plasma volume in adults - Expert Panel on radionuclides of the International Council for Standardization in Hematology. *British Journal of Haematology* **89,** 748-756.

Peng YJ, Yuan G, Ramakrishnan D, Sharma SD, Bosch-Marce M, Kumar GK, Semenza GL & Prabhakar NR. (2006). Heterozygous HIF-1alpha deficiency impairs carotid body-mediated

systemic responses and reactive oxygen species generation in mice exposed to intermittent hypoxia. *The Journal of Physiology* **577,** 705-716.

Petousi N, Croft QP, Cavalleri GL, Cheng HY, Formenti F, Ishida K, Lunn D, McCormack M, Shianna KV, Talbot NP, Ratcliffe PJ & Robbins PA. (2014). Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *Journal of Applied Physiology* **116,** 893-904.

Peyssonnaux C, Nizet V & Johnson RS. (2008). Role of the hypoxia inducible factors HIF in iron metabolism. *Cell Cycle* **7,** 28-32.

Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V & Johnson RS. (2007). Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). *The Journal of Clinical Investigation* **117,** 1926-1932.

Pieracci FM & Barie PS. (2005). Iron and the risk of infection. *Surgical Infections* **6,** S41-46.

Pigeon C, Ilyin G, Courselaud B, Leroyer P, Turlin B, Brissot P & Loreal O. (2001). A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of Biological Chemistry* **276,** 7811-7819.

Pilon VA, Howanitz PJ, Howanitz JH & Domres N. (1981). Day-to-day variation in serum ferritin concentration in healthy subjects. *Clinical Chemistry* **27,** 78-82.

Pisarcik S, Maylor J, Lu W, Yun X, Undem C, Sylvester JT, Semenza GL & Shimoda LA. (2013). Activation of hypoxia-inducible factor-1 in pulmonary arterial smooth muscle cells by endothelin-1. *American Journal of Physiology Lung Cellular and Molecular Physiology* **304,** L549-561.

Ponikowska B, Suchocki T, Paleczny B, Olesinska M, Powierza S, Borodulin-Nadzieja L, Reczuch K, von Haehling S, Doehner W, Anker SD, Cleland JG & Jankowska EA. (2013). Iron status and survival in diabetic patients with coronary artery disease. *Diabetes Care* **36,** 4147-4156.

Ponikowski P, van Veldhuisen DJ, Comin-Colet J, Ertl G, Komajda M, Mareev V, McDonagh T, Parkhomenko A, Tavazzi L, Levesque V, Mori C, Roubert B, Filippatos G, Ruschitzka F & Anker SD. (2015). Beneficial effects of long-term intravenous iron therapy with ferric carboxymaltose in patients with symptomatic heart failure and iron deficiency. *European Heart Journal* **36,** 657-668.

Powell FL, Milsom WK & Mitchell GS. (1998). Time domains of the hypoxic ventilatory response. *Respiration Physiology* **112,** 123-134.

Prabhakar NR & Semenza GL. (2012a). Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiological Reviews* **92,** 967-1003.

Prabhakar NR & Semenza GL. (2012b). Gaseous messengers in oxygen sensing. *Journal of Molecular Medicine* **90,** 265-272.

Priestley J. (1776). Experiments and Observations on Different Kinds of Air. **Vol. II.**

Prommer N & Schmidt W. (2007). Loss of CO from the intravascular bed and its impact on the optimised CO-rebreathing method. *European Journal of Applied Physiology* **100,** 383-391.

Puchalski MD, Williams RV, Askovich B, Minich LL, Mart C & Tani LY. (2007). Assessment of right ventricular size and function: echo versus magnetic resonance imaging. *Congenital Heart Disease* **2,** 27-31.

Pugh K & Balanos G. (2013). Pulmonary vascular reactivity with ageing. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **27,** 1140.1113

Qunibi WY, Martinez C, Smith M, Benjamin J, Mangione A & Roger SD. (2011). A randomized controlled trial comparing intravenous ferric carboxymaltose with oral iron for treatment of iron deficiency anaemia of non-dialysis-dependent chronic kidney disease patients. *Nephrology, dialysis, transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association* **26,** 1599-1607.

Rankin EB, Biju MP, Liu Q, Unger TL, Rha J, Johnson RS, Simon MC, Keith B & Haase VH. (2007). Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *The Journal of Clinical Investigation* **117,** 1068-1077.

Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL & Ratcliffe PJ. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Molecular and Cellular Biology* **25,** 5675-5686.

Redfield MM, Jacobsen SJ, Burnett JC, Jr., Mahoney DW, Bailey KR & Rodeheffer RJ. (2003). Burden of systolic and diastolic ventricular dysfunction in the community: appreciating the scope of the heart failure epidemic. *The Journal of the American Medical Association* **289,** 194-202.

Reeves JT, Linehan JH & Stenmark KR. (2005). Distensibility of the normal human lung circulation during exercise. *American Journal of Physiology Lung Cellular and Molecular Physiology* **288,** L419-L425.

Ren X, Dorrington KL, Maxwell PH & Robbins PA. (2000). Effects of desferrioxamine on serum erythropoietin and ventilatory sensitivity to hypoxia in humans. *Journal of Applied Physiology* **89,** 680-686.

Reynafarje C, Lozano R & Valdivieso J. (1959). The polycythemia of high altitudes: iron metabolism and related aspects. *Blood* **14,** 433-455.

Rhodes CJ, Howard LS, Busbridge M, Ashby D, Kondili E, Gibbs JS, Wharton J & Wilkins MR. (2011a). Iron deficiency and raised hepcidin in idiopathic pulmonary arterial hypertension: clinical prevalence, outcomes, and mechanistic insights. *Journal of the American College of Cardiology* **58,** 300-309.

Rhodes CJ, Wharton J, Howard L, Gibbs JS, Vonk-Noordegraaf A & Wilkins MR. (2011b). Iron deficiency in pulmonary arterial hypertension: a potential therapeutic target. *The European Respiratory Journal* **38,** 1453-1460.

Richalet JP, Souberbielle JC, Antezana AM, Dechaux M, Le Trong JL, Bienvenu A, Daniel F, Blanchot C & Zittoun J. (1994). Control of erythropoiesis in humans during prolonged exposure to the altitude of 6,542 m. *The American Journal of Physiology* **266,** R756-764.

Rishi G, Wallace DF & Subramaniam VN. (2015). Hepcidin: regulation of the master iron regulator. *Bioscience Reports* **35**.

Roe MA, Collings R, Dainty JR, Swinkels DW & Fairweather-Tait SJ. (2009). Plasma hepcidin concentrations significantly predict interindividual variation in iron absorption in healthy men. *American Journal of Clinical Nutrition* **89,** 1088-1091.

Rouault TA. (2006). The role of iron regulatory proteins in mammalian iron homeostasis and disease. *Nature Chemical Biology* **2,** 406-414.

Rubab Z, Amin H, Abbas K, Hussain S, Ullah MI & Mohsin S. (2015). Serum hepcidin levels in patients with end-stage renal disease on hemodialysis. *Saudi Journal of Kidney Diseases and Transplantation : an Official Publication of the Saudi Center for Organ Transplantation, Saudi Arabia* **26,** 19-25.

Rudski LG, Lai WW, Afilalo J, Hua L, Handschumacher MD, Chandrasekaran K, Solomon SD, Louie EK & Schiller NB. (2010). Guidelines for the echocardiographic assessment of the right heart in adults: a report from the American Society of Echocardiography endorsed by the European Association of Echocardiography, a registered branch of the European Society of Cardiology, and the Canadian Society of Echocardiography. *Journal of the American Society of Echocardiography* **23,** 685-713.

Ruiter G, Lankhorst S, Boonstra A, Postmus PE, Zweegman S, Westerhof N, van der Laarse WJ & Vonk-Noordegraaf A. (2011). Iron deficiency is common in idiopathic pulmonary arterial hypertension. *The European Respiratory Journal* **37,** 1386-1391.

Rumsey WL, Pawlowski M, Lejavardi N & Wilson DF. (1994). Oxygen pressure distribution in the heart in vivo and evaluation of the ischemic "border zone". *The American Journal of Physiology* **266,** H1676-1680.

Sable CA, Aliyu ZY, Dham N, Nouraie M, Sachdev V, Sidenko S, Miasnikova GY, Polyakova LA, Sergueeva AI, Okhotin DJ, Bushuev V, Remaley AT, Niu X, Castro OL, Gladwin MT, Kato GJ, Prchal JT & Gordeuk VR. (2012). Pulmonary artery pressure and iron deficiency in

patients with upregulation of hypoxia sensing due to homozygous *VHL*^{R200W} mutation (Chuvash polycythemia). *Haematologica* **97,** 193-200.

Sahn DJ & Maciel BC. (1988). Physiological valvular regurgitation. Doppler echocardiography and the potential for iatrogenic heart disease. *Circulation* **78,** 1075-1077.

Salahudeen AA, Thompson JW, Ruiz JC, Ma HW, Kinch LN, Li Q, Grishin NV & Bruick RK. (2009). An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis. *Science* **326,** 722-726.

Salceda S & Caro J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *The Journal of Biological Chemistry* **272,** 22642-22647.

Sanchez M, Galy B, Muckenthaler MU & Hentze MW. (2007). Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nature Structural & Molecular Biology* **14,** 420-426.

Santamore WP & Dell'Italia LJ. (1998). Ventricular interdependence: significant left ventricular contributions to right ventricular systolic function. *Progress in Cardiovascular Diseases* **40,** 289-308.

Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M & Johnson RS. (2001). Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes & Development* **15,** 2865-2876.

Schmidt W & Prommer N. (2005). The optimised CO-rebreathing method: a new tool to determine total haemoglobin mass routinely. *European Journal of Applied Physiology* **95,** 486-495.

Schoene RB, Hackett PH, Henderson WR, Sage EH, Chow M, Roach RC, Mills WJ, Jr. & Martin TR. (1986). High-altitude pulmonary edema. Characteristics of lung lavage fluid. *The Journal of the American Medical Association* **256,** 63-69.

Schofield CJ & Ratcliffe PJ. (2004). Oxygen sensing by HIF hydroxylases. *Nature reviews Molecular cell Biology* **5,** 343-354.

Schurek HJ, Jost U, Baumgartl H, Bertram H & Heckmann U. (1990). Evidence for a preglomerular oxygen diffusion shunt in rat renal cortex. *The American Journal of Physiology* **259,** F910-915.

Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ & Garcia JA. (2003a). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *EPAS1*$^{-/-}$ mice. *Nature Genetics* **35,** 331-340.

Scortegagna M, Morris MA, Oktay Y, Bennett M & Garcia JA. (2003b). The HIF family member *EPAS1*/HIF-2alpha is required for normal hematopoiesis in mice. *Blood* **102,** 1634-1640.

Semenza GL. (2004). Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology* **19,** 176-182.

Semenza GL. (2012). Hypoxia-inducible factors in physiology and medicine. *Cell* **148,** 399-408.

Semenza GL & Wang GL. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and Cellular Biology* **12,** 5447-5454.

Shimoda LA, Fallon M, Pisarcik S, Wang J & Semenza GL. (2006). HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. *American Journal of Physiology Lung Cellular and Molecular Physiology* **291,** L941-949.

Siddiq A, Ayoub IA, Chavez JC, Aminova L, Shah S, LaManna JC, Patton SM, Connor JR, Cherny RA, Volitakis I, Bush AI, Langsetmo I, Seeley T, Gunzler V & Ratan RR. (2005). Hypoxia-inducible factor prolyl 4-hydroxylase inhibition. A target for neuroprotection in the central nervous system. *The Journal of Biological Chemistry* **280,** 41732-41743.

Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J & Camaschella C. (2008). The serine protease matriptase-2 (*TMPRSS6*) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell Metabolism* **8,** 502-511.

Simonneau G, Gatzoulis MA, Adatia I, Celermajer D, Denton C, Ghofrani A, Gomez Sanchez MA, Krishna Kumar R, Landzberg M, Machado RF, Olschewski H, Robbins IM & Souza R. (2013). Updated clinical classification of pulmonary hypertension. *Journal of the American College of Cardiology* **62,** D34-41.

Simonson TS, Yang Y, Huff CD, Yun H, Qin G, Witherspoon DJ, Bai Z, Lorenzo FR, Xing J, Jorde LB, Prchal JT & Ge R. (2010). Genetic evidence for high-altitude adaptation in Tibet. *Science* **329,** 72-75.

Skikne BS. (2008). Serum transferrin receptor. *American Journal of Hematology* **83,** 872-874.

Skikne BS, Flowers CH & Cook JD. (1990). Serum Transferrin Receptor - a Quantitative Measure of Tissue Iron-Deficiency. *Blood* **75,** 1870-1876.

Skjaerpe T & Hatle L. (1981). Diagnosis and assessment of tricuspid regurgitation with Doppler ultrasound. *Echocardiology***,** p 299.

Skovgaard N, Abe AS, Andrade DV & Wang T. (2005). Hypoxic pulmonary vasoconstriction in reptiles: a comparative study of four species with different lung structures and pulmonary

blood pressures. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology* **289,** R1280-1288.

Slingo ME, Turner PJ, Christian HC, Buckler KJ & Robbins PA. (2014). The von Hippel-Lindau Chuvash mutation in mice causes carotid-body hyperplasia and enhanced ventilatory sensitivity to hypoxia. *Journal of Applied Physiology* **116,** 885-892.

Smith MP, Russell MJ, Wincko JT & Olson KR. (2001). Effects of hypoxia on isolated vessels and perfused gills of rainbow trout. *Comparative Biochemistry and Physiology Part A, Molecular & Integrative Physiology* **130,** 171-181.

Smith SR, Cooperman S, Lavaute T, Tresser N, Ghosh M, Meyron-Holtz E, Land W, Ollivierre H, Jortner B, Switzer R, 3rd, Messing A & Rouault TA. (2004). Severity of neurodegeneration correlates with compromise of iron metabolism in mice with iron regulatory protein deficiencies. *Annals of the New York Academy of Sciences* **1012,** 65-83.

Smith SR, Ghosh MC, Ollivierre-Wilson H, Hang Tong W & Rouault TA. (2006a). Complete loss of iron regulatory proteins 1 and 2 prevents viability of murine zygotes beyond the blastocyst stage of embryonic development. *Blood Cells, Molecules, and Diseases* **36,** 283-287.

Smith TG, Balanos GM, Croft QP, Talbot NP, Dorrington KL, Ratcliffe PJ & Robbins PA. (2008). The increase in pulmonary arterial pressure caused by hypoxia depends on iron status. *The Journal of Physiology* **586,** 5999-6005.

Smith TG, Brooks JT, Balanos GM, Lappin TR, Layton DM, Leedham DL, Liu C, Maxwell PH, McMullin MF, McNamara CJ, Percy MJ, Pugh CW, Ratcliffe PJ, Talbot NP, Treacy M & Robbins PA. (2006b). Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. *PLoS medicine* **3,** e290.

Smith TG, Talbot NP, Privat C, Rivera-Ch M, Nickol AH, Ratcliffe PJ, Dorrington KL, Leon-Velarde F & Robbins PA. (2009). Effects of iron supplementation and depletion on hypoxic pulmonary hypertension: two randomized controlled trials. *The Journal of the American Medical Association* **302,** 1444-1450.

Smollin C & Olson K. (2010). Carbon monoxide poisoning (acute). *British Medical Journal Clinical Evidence* **2010**.

Solomons NW & Schumann K. (2004). Intramuscular administration of iron dextran is inappropriate for treatment of moderate pregnancy anemia, both in intervention research on underprivileged women and in routine prenatal care provided by public health services. *The American Journal of Clinical Nutrition* **79,** 1-3.

Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, Willenborg C, Wright B, Chen L, Li M, Salo P, Voight BF, Burns P, Laskowski RA, Xue Y, Menzel S, Altshuler D, Bradley JR, Bumpstead S, Burnett MS, Devaney J, Doring A, Elosua R, Epstein SE, Erber W, Falchi M, Garner SF, Ghori MJ, Goodall AH, Gwilliam R, Hakonarson HH, Hall AS, Hammond N, Hengstenberg C, Illig T, Konig IR, Knouff CW, McPherson R, Melander O, Mooser V,

Nauck M, Nieminen MS, O'Donnell CJ, Peltonen L, Potter SC, Prokisch H, Rader DJ, Rice CM, Roberts R, Salomaa V, Sambrook J, Schreiber S, Schunkert H, Schwartz SM, Serbanovic-Canic J, Sinisalo J, Siscovick DS, Stark K, Surakka I, Stephens J, Thompson JR, Volker U, Volzke H, Watkins NA, Wells GA, Wichmann HE, Van Heel DA, Tyler-Smith C, Thein SL, Kathiresan S, Perola M, Reilly MP, Stewart AF, Erdmann J, Samani NJ, Meisinger C, Greinacher A, Deloukas P, Ouwehand WH & Gieger C. (2009). A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. *Nature Genetics* **41,** 1182-1190.

Sowter HM, Raval RR, Moore JW, Ratcliffe PJ & Harris AL. (2003). Predominant role of hypoxia-inducible transcription factor (HIF)-1alpha versus HIF-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Research* **63,** 6130-6134.

Spasic MV, Kiss J, Herrmann T, Kessler R, Stolte J, Galy B, Rathkolb B, Wolf E, Stremmel W, Hentze MW & Muckenthaler MU. (2007). Physiologic systemic iron metabolism in mice deficient for duodenal HFE. *Blood* **109,** 4511-4517.

Steensma DP, Sasu BJ, Sloan JA, Tomita DK & Loprinzi CL. (2015). Serum hepcidin levels predict response to intravenous iron and darbepoetin in chemotherapy-associated anemia. *Blood* **125,** 3669-3671.

Swenson ER, Maggiorini M, Mongovin S, Gibbs JS, Greve I, Mairbaurl H & Bartsch P. (2002). Pathogenesis of high-altitude pulmonary edema: inflammation is not an etiologic factor. *The Journal of the American Medical Association* **287,** 2228-2235.

Sylvester JT, Shimoda LA, Aaronson PI & Ward JP. (2012). Hypoxic pulmonary vasoconstriction. *Physiological Reviews* **92,** 367-520.

Talbot NP, Balanos GM, Dorrington KL & Robbins PA. (2005). Two temporal components within the human pulmonary vascular response to approximately 2 h of isocapnic hypoxia. *Journal of Applied Physiology* **98,** 1125-1139.

Talbot NP, Croft QP, Curtis MK, Turner BE, Dorrington KL, Robbins PA & Smith TG. (2014). Contrasting effects of ascorbate and iron on the pulmonary vascular response to hypoxia in humans. *Physiological Reports* **2**.

Talbot NP, Lakhal S, Smith TG, Privat C, Nickol AH, Rivera-Ch M, Leon-Velarde F, Dorrington KL, Mole DR & Robbins PA. (2012). Regulation of hepcidin expression at high altitude. *Blood* **119,** 857-860.

Talbot NP, Smith TG, Lakhal-Littleton S, Gulsever C, Rivera-Ch M, Dorrington KL, Mole DR & Robbins PA. (2016). Suppression of plasma hepcidin by venesection during steady-state hypoxia. *Blood* **127,** 1206-1207.

Talbot NP, Smith TG, Privat C, Nickol AH, Rivera-Ch M, Leon-Velarde F, Dorrington KL & Robbins PA. (2011). Intravenous iron supplementation may protect against acute mountain

sickness: a randomized, double-blinded, placebo-controlled trial. *High Altitude Medicine & Biology* **12,** 265-269.

Tan Q, Kerestes H, Percy MJ, Pietrofesa R, Chen L, Khurana TS, Christofidou-Solomidou M, Lappin TR & Lee FS. (2013). Erythrocytosis and pulmonary hypertension in a mouse model of human HIF2A gain of function mutation. *The Journal of Biological Chemistry* **288,** 17134-17144.

Tanaka T, Roy CN, Yao W, Matteini A, Semba RD, Arking D, Walston JD, Fried LP, Singleton A, Guralnik J, Abecasis GR, Bandinelli S, Longo DL & Ferrucci L. (2010). A genome-wide association analysis of serum iron concentrations. *Blood* **115,** 94-96.

Tanno T, Bhanu NV, Oneal PA, Goh SH, Staker P, Lee YT, Moroney JW, Reed CH, Luban NLC, Wang RH, Eling TE, Childs R, Ganz T, Leitman SF, Fucharoen S & Miller JL. (2007). High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nature Medicine* **13,** 1096-1101.

Tanno T, Porayette P, Sripichai O, Noh SJ, Byrnes C, Bhupatiraju A, Lee YT, Goodnough JB, Harandi O, Ganz T, Paulson RF & Miller JL. (2009). Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood* **114,** 181-186.

Tavill AS, Diseases AAftSoL, Gastroenterology ACo & Association AG. (2001). Diagnosis and management of hemochromatosis. *Hepatology* **33,** 1321-1328.

Tian H, Hammer RE, Matsumoto AM, Russell DW & McKnight SL. (1998). The hypoxia-responsive transcription factor *EPAS1* is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes & Development* **12,** 3320-3324.

Toblli JE & Angerosa M. (2014). Optimizing iron delivery in the management of anemia: patient considerations and the role of ferric carboxymaltose. *Drug Design, Development and Therapy* **8,** 2475-2491.

Toblli JE, Cao G, Giani JF, Dominici FP & Angerosa M. (2015a). Nitrosative stress and apoptosis by intravenous Ferumoxytol, Iron Isomaltoside 1000, Iron Dextran, Iron Sucrose, and Ferric Carboxymaltose in a nonclinical model. *Drug Research (Stuttg)* **65,** 354-360.

Toblli JE, Cao G, Oliveri L & Angerosa M. (2012). Comparison of oxidative stress and inflammation induced by different intravenous iron sucrose similar preparations in a rat model. *Inflammation & Allergy - Drug Targets* **11,** 66-78.

Toblli JE, Di Gennaro F & Rivas C. (2015b). Changes in echocardiographic parameters in iron deficiency patients with heart failure and chronic kidney disease treated with intravenous iron. *Heart, Lung & Circulation* **24,** 686-695.

Toblli JE, Rivas C, Cao G, Giani JF, Funk F, Mizzen L & Dominici FP. (2014). Ferric carboxymaltose-mediated attenuation of Doxorubicin-induced cardiotoxicity in an iron deficiency rat model. *Chemotherapy Research and Practice* **2014,** 570241.

Townsend A & Drakesmith H. (2002). Role of *HFE* in iron metabolism, hereditary haemochromatosis, anaemia of chronic disease, and secondary iron overload. *Lancet* **359,** 786-790.

Turner BE, Hodkinson PD, Timperley AC & Smith TG. (2015). Pulmonary Artery Pressure Response to Simulated Air Travel in a Hypobaric Chamber. *Aerospace Medicine and Human Performance* **86,** 529-534.

van Beekvelt MCP, Colier WNJM, Wevers RA & van Engelen BGM. (2001). Performance of near-infrared spectroscopy in measuring local O_2 consumption and blood flow in skeletal muscle. *Journal of Applied Physiology* **90,** 511-519.

Veit F, Pak O, Brandes RP & Weissmann N. (2015). Hypoxia-dependent reactive oxygen species signaling in the pulmonary circulation: focus on ion channels. *Antioxidants & Redox Signaling* **22,** 537-552.

Verna A, Roumy M & Leitner LM. (1975). Loss of chemoreceptive properties of the rabbit carotid body after destruction of the glomus cells. *Brain Research* **100,** 13-23.

Viethen T, Gerhardt F, Dumitrescu D, Knoop-Busch S, ten Freyhaus H, Rudolph TK, Baldus S & Rosenkranz S. (2014). Ferric carboxymaltose improves exercise capacity and quality of life in patients with pulmonary arterial hypertension and iron deficiency: a pilot study. *International Journal of Cardiology* **175,** 233-239.

Vogel JA & Harris CW. (1967). Cardiopulmonary responses of resting man during early exposure to high altitude. *Journal of Applied Physiology* **22,** 1124-1128.

von Euler U & Liljestrand G. (1946). Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiologica Scandinavica*.

Wachsmuth NB, Aigner T, Volzke C, Zapf J & Schmidt WF. (2015). Monitoring recovery from iron deficiency using total hemoglobin mass. *Medicine and Science in Sports and Exercise* **47,** 419-427.

Wang C, Graham DJ, Kane RC, Xie D, Wernecke M, Levenson M, MaCurdy TE, Houstoun M, Ryan Q, Wong S, Mott K, Sheu TC, Limb S, Worrall C, Kelman JA & Reichman ME. (2015). Comparative risk of anaphylactic reactions associated with intravenous iron products. *The Journal of the American Medical Association* **314,** 2062-2068.

Wang GL, Jiang BH, Rue EA & Semenza GL. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 5510-5514.

Wang GL & Semenza GL. (1993). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* **82,** 3610-3615.

Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL & Shimoda LA. (2006). Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca^{2+} in pulmonary arterial smooth muscle cells. *Circulation Research* **98,** 1528-1537.

Wang RH, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L & Deng CX. (2005). A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metabolism* **2,** 399-409.

Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, Bouxsein ML, Faugere MC, Guldberg RE, Gerstenfeld LC, Haase VH, Johnson RS, Schipani E & Clemens TL. (2007). The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. *The Journal of Clinical Investigation* **117,** 1616-1626.

Ward JP & Robertson TP. (1995). The role of the endothelium in hypoxic pulmonary vasoconstriction. *Experimental Physiology* **80,** 793-801.

Wartig M, Tesan S, Gabel J, Jeppsson A, Selimovic N, Holmberg E & Dellgren G. (2014). Tricuspid regurgitation influences outcome after heart transplantation. *The Journal of Heart and Lung Transplantation* 829-835.

Waypa GB, Chandel NS & Schumacker PT. (2001). Model for hypoxic pulmonary vasoconstriction involving mitochondrial oxygen sensing. *Circulation Research* **88,** 1259-1266.

Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT & Schumacker PT. (2002). Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. *Circulation Research* **91,** 719-726.

Weaver LK, Howe S, Hopkins R & Chan KJ. (2000). Carboxyhemoglobin half-life in carbon monoxide-poisoned patients treated with 100% oxygen at atmospheric pressure. *Chest* **117,** 801-808.

Weil JV & Zwillich CW. (1976). Assessment of ventilatory response to hypoxia: methods and interpretation. *Chest* **70,** 124-128.

Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JI & Andrews NC. (2002). Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood* **100,** 3776-3781.

Weir EK & Archer SL. (1995). The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **9,** 183-189.

Weiss G, Theurl I, Eder S, Koppelstaetter C, Kurz K, Sonnweber T, Kobold U & Mayer G. (2009). Serum hepcidin concentration in chronic haemodialysis patients: associations and effects of dialysis, iron and erythropoietin therapy. *European Journal of Clinical Investigation* **39,** 883-890.

Weisse AB, Moschos CB, Frank MJ, Levinson GE, Cannilla JE & Regan TJ. (1975). Hemodynamic effects of staged hematocrit reduction in patients with stable cor pulmonale and severely elevated hematocrit levels. *The American Journal of Medicine* **58,** 92-98.

Weitzenblum E, Schrijen F, Mohan-Kumar T, Colas des Francs V & Lockhart A. (1988). Variability of the pulmonary vascular response to acute hypoxia in chronic bronchitis. *Chest* **94,** 772-778.

West JB. (2015). Early history of high-altitude physiology. *Annals of the New York Academy of Sciences.*

Whalen WJ, Ganfield R & Nair P. (1970). Effects of breathing O_2 or O_2 +CO_2 and of the injection of neurohumors on the PO_2 of cat cerebral cortex. *Stroke* **1,** 194-200.

Whalen WJ & Nair P. (1975). Some factors affecting tissue Po_2 in the carotid body. *Journal of Applied Physiology* **39,** 562-566.

Whitman EM, Pisarcik S, Luke T, Fallon M, Wang J, Sylvester JT, Semenza GL & Shimoda LA. (2008). Endothelin-1 mediates hypoxia-induced inhibition of voltage-gated K^+ channel expression in pulmonary arterial myocytes. *American Journal of Physiology Lung Cellular and Molecular Physiology* **294,** L309-318.

Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH & Eckardt KU. (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **17,** 271-273.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ & Maxwell PH. (1998). Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* **92,** 2260-2268.

Wilkinson N & Pantopoulos K. (2013). IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood* **122,** 1658-1668.

Wood HE, Fatemian M & Robbins PA. (2008). Non-dimensional quantification of the interactions between hypoxia, hypercapnia and exercise on ventilation in humans. *Advances in Experimental Medicine and Biology* **605,** 245-248.

Yan L, Colandrea VJ & Hale JJ. (2010). Prolyl hydroxylase domain-containing protein inhibitors as stabilizers of hypoxia-inducible factor: small molecule-based therapeutics for anemia. *Expert opinion on Therapeutic Patents* **20,** 1219-1245.

Yock PG & Popp RL. (1984). Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. *Circulation* **70,** 657-662.

Yoon D, Ponka P & Prchal JT. (2011). Hypoxia. 5. Hypoxia and hematopoiesis. *American Journal of Physiology Cell physiology* **300,** C1215-1222.

Yoshida T, Zhang H, Iwase T, Shen J, Semenza GL & Campochiaro PA. (2010). Digoxin inhibits retinal ischemia-induced HIF-1alpha expression and ocular neovascularization. *FASEB journal : Official Publication of the Federation of American Societies for Experimental Biology* **24,** 1759-1767.

Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K & Semenza GL. (1998). Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. *American Journal of Physiology Lung Cellular and Molecular Physiology* **275,** L818-L826.

Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT & Semenza GL. (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *The Journal of Clinical Investigation* **103,** 691-696.

Zaritsky J, Young B, Wang HJ, Westerman M, Olbina G, Nemeth E, Ganz T, Rivera S, Nissenson AR & Salusky IB. (2009). Hepcidin - a potential novel biomarker for iron status in chronic kidney disease. *Clinical Journal of the American Society of Nephrology : CJASN* **4,** 1051-1056.

Zhang H, Qian DZ, Tan YS, Lee K, Gao P, Ren YR, Rey S, Hammers H, Chang D, Pili R, Dang CV, Liu JO & Semenza GL. (2008a). Digoxin and other cardiac glycosides inhibit HIF-1alpha synthesis and block tumor growth. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 19579-19586.

Zhang HF, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ & Semenza GL. (2008b). Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *Journal of Biological Chemistry* **283,** 10892-10903.

Zhu BZ, Har-El R, Kitrossky N & Chevion M. (1998). New modes of action of desferrioxamine: scavenging of semiquinone radical and stimulation of hydrolysis of tetrachlorohydroquinone. *Free Radical Biology & Medicine* **24,** 360-369.

Zimmer M, Ebert BL, Neil C, Brenner K, Papaioannou I, Melas A, Tolliday N, Lamb J, Pantopoulos K, Golub T & Iliopoulos O. (2008). Small-molecule inhibitors of HIF-2a translation link its 5'UTR iron-responsive element to oxygen sensing. *Molecular Cell* **32,** 838-848.

Zinkernagel AS, Johnson RS & Nizet V. (2007). Hypoxia inducible factor (HIF) function in innate immunity and infection. *Journal of Molecular Medicine* **85,** 1339-1346.

APPENDIX:

PUBLISHED RESULTS OF STUDY A:

Bart, NK. *et al.* (2016) Elevation of iron storage in humans attenuates the pulmonary vascular response to hypoxia. *Journal of Applied Physiology.* **121** 537-544

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Elevation of iron storage in humans attenuates the pulmonary vascular response to hypoxia

Nicole K. Bart,[1] M. Kate Curtis,[1] Hung-Yuan Cheng,[1] Sara L. Hungerford,[2] Ross McLaren,[1] Nayia Petousi,[1,3] Keith L. Dorrington,[1] and Peter A. Robbins[1]

[1]*Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom;* [2]*Department of Medicine, Royal Berkshire NHS Foundation Trust, Reading, United Kingdom; and* [3]*Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom*

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Bart NK, Curtis MK, Cheng HY, Hungerford SL, McLaren R, Petousi N, Dorrington KL, Robbins PA. Elevation of iron storage in humans attenuates the pulmonary vascular response to hypoxia. *J Appl Physiol* 121: 537–544, 2016. First published July 14, 2016; doi:10.1152/japplphysiol.00032.2016.—Sustained hypoxia over several hours induces a progressive rise in pulmonary artery systolic pressure (PASP). Administration of intravenous iron immediately prior to the hypoxia exposure abrogates this effect, suggesting that manipulation of iron stores may modify hypoxia-induced pulmonary hypertension. Iron (ferric carboxymaltose) administered intravenously has a plasma half-life of 7-12 h. Thus any therapeutic use of intravenous iron would require its effect on PASP to persist long after the iron-sugar complex has been cleared from the blood. To examine this, we studied PASP during sustained (6 h) hypoxia on 4 separate days (*days 0*, *1*, *8*, and *43*) in 22 participants. On *day 0*, the rise in PASP with hypoxia was well matched between the iron and saline groups. On *day 1*, each participant received either 1 g of ferric carboxymaltose or saline in a double-blind manner. After administration of intravenous iron, the rise in PASP with hypoxia was attenuated by ~50%, and this response remained suppressed on both *days 8* and *43* ($P < 0.001$). Following administration of intravenous iron, values for ferritin concentration, transferrin saturation, and hepcidin concentration rose significantly ($P < 0.001$, $P < 0.005$, and $P < 0.001$, respectively), and values for transferrin concentration fell significantly ($P < 0.001$). These changes remained significant at *day 43*. We conclude that the attenuation of the pulmonary vascular response to hypoxia by elevation of iron stores persists long after the artificial iron-sugar complex has been eliminated from the blood. The persistence of this effect suggests that intravenous iron may be of benefit in some forms of pulmonary hypertension.

hypoxia inducible factor; pulmonary hypertension; pulmonary circulation

NEW & NOTEWORTHY

This study shows that a single dose of intravenous iron reduces the effects of hypoxia on the pulmonary circulation in a manner that persists for at least several weeks. This is long after the foreign iron-sugar complex has been cleared from the blood. It raises the possibility that manipulating iron stores, even in people who are not initially iron deficient, could be used for therapeutic gain in some forms of pulmonary hypertension.

IRON IS A TRANSITION ELEMENT essential to life. Healthy humans have a total iron content of ~4 g, about half of which is incorporated into hemoglobin. As both Fe^{2+} and Fe^{3+} are stable in solution, iron is a highly reactive participant in oxidation-reduction reactions. This is exploited in many naturally occurring examples of catalysis, but also means that free iron is toxic, and consequently iron needs to be stored as a complex with macromolecules.

Oxygen acts as the terminal electron acceptor for aerobic metabolism. In large multicellular organisms, the flux of oxygen generates gradients in partial pressure within the organism, and these gradients are used to direct the development, maintenance, and physiological regulation of the systems for oxygen transport. Important within these regulatory mechanisms is the hypoxia-inducible factor (HIF) transcription system, which provides a coordinated cellular response to hypoxia.

At a molecular level, the regulation of iron and oxygen is tightly linked. Iron is an essential cofactor for the catalytic degradation of HIF by the prolyl-hydroxylases (PHDs), and furthermore HIF2α mRNA contains an iron-response element (IRE) within its 5′ end, so that the iron-regulatory proteins (IRPs) can regulate the expression of HIF2α (24, 39). In a complimentary manner, many of the proteins involved in the regulation of iron and iron storage are themselves HIF regulated (17). At the systems level, the importance of HIF to pulmonary vascular responses to hypoxia has been demonstrated both through experimental knockdown of the HIF pathway in mice (3, 38), which leads to abrogation of the rise in pulmonary arterial pressure in response to hypoxia, and through upregulation of the HIF pathway in rare human diseases and animal models of such diseases (4, 8, 27, 34), which leads to development of pulmonary arterial hypertension.

Taken together, the above findings suggest that iron availability itself may influence the pulmonary vasculature, and indeed this is the case. In humans, like hypoxia, iron chelation produces a progressive rise in pulmonary arterial pressure over a time period of 8 h (1), as indeed it does in circulating erythropoietin (EPO), which is a classical HIF-regulated protein (20). Importantly, administration of intravenous (IV) iron in humans abrogates the progressive rise in pulmonary arterial pressure that occurs in response to 8 h of sustained hypoxia (26, 33). In general, however, these experiments have focused on the response of the pulmonary vasculature to hypoxia on the same day as the iron administration. One exception to this is a study where IV iron was administered to participants who had already been at high altitude (4,340 m) for a period of 3 days and where the reduction in pulmonary artery systolic pressure (PASP) with iron was shown to last for a subsequent 4 days (28). However, these participants (both treated and control) will have had a cellular iron demand that was greatly increased through the enhanced rate of hematopoiesis associated with

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high-altitude exposure. Overall, it remains unclear whether the effects of iron on the pulmonary vasculature are arising as a relatively transient phenomenon, perhaps associated with the presence of an artificial iron-sugar complex in the blood, or whether they are effects that persist long after the elimination of the iron-sugar complex when the iron has been fully assimilated into the body's stores. Thus, while the close interaction between iron and the pulmonary vasculature has been well documented, the duration of effect of iron is unknown. Clinically, this is of importance as it will determine whether or not the effects of iron administration on the pulmonary vasculature will persist in time.

One area where iron administration may be beneficial is in some forms of pulmonary hypertension. In idiopathic pulmonary hypertension, iron deficiency is prevalent and carries a poorer prognosis (21, 23, 29). Supplementation of iron stores has been shown to be of benefit in patients with pulmonary arterial hypertension (22, 35), and further studies are underway to further evaluate this (10). In addition, recent studies have shown that iron deficiency is common in patients with chronic obstructive pulmonary disease, that the iron-deficient patients are more hypoxemic than their iron-replete counterparts, and that the prevalence of some degree of pulmonary hypertension is twice as great in the iron-deficient patients compared with those who are iron replete (16, 18).

The purpose of the present study was to determine whether, in healthy participants, the effects of iron loading on hypoxic pulmonary vasoconstriction persist long after the iron-sugar complex has been eliminated from the blood and when the iron has been incorporated into the body's stores.

METHODS

The study protocol is illustrated in Fig. 1. Participants were block randomized (1:1) between two groups who were to receive either iron or saline infusions, respectively. There were 4 full study days (*days 0*, *1*, *8*, and *43*) in each limb of the study. *Day 0* was a baseline measurement day and occurred at least 3 days before *day 1*. On *day 0*, control measurements were made of the pulmonary vascular responses to a sustained (6 h) exposure to isocapnic hypoxia. On *day 1*, participants received either a saline infusion (NaCl 0.9%) or an infusion of 1 g of ferric carboxymaltose. Following this infusion, participants underwent a second 6-h exposure to sustained hypoxia to assess their pulmonary vascular response. These measurements were repeated again at 1 wk (*day 8*) and 6 wk (*day 43*) after the saline or iron infusion.

The study was conducted in a double-blind manner. The investigator who randomized the participants, administered the infusions, and collated the blood results was not involved in any other part of the study. The participants were blind to the nature of the infusion as they wore a blindfold during the infusion and the infusion port was kept out of sight. The recruitment process, the experimental measurements, and data analysis were conducted by an investigator who was blind to the nature of the infusion.

Participants. Twenty-two healthy participants were studied so as to be able to detect a difference of ~4 mmHg in pulmonary artery pressure between the two groups at a significance level of $P < 0.05$ with a power of 80%. Participants were recruited by advertisement. Inclusion criteria required participants to be healthy and iron replete (ferritin 20–300 μg/l, transferrin saturation 20–50%) and to have detectable physiological tricuspid regurgitation on echocardiography to allow for measurement of PASP. Exclusion criteria were any significant comorbidity that may affect hematology parameters or iron status, or pulmonary vascular or ventilatory responses. Participants with recent exposure to an altitude >2,500 m or air travel >4 h within the week prior to any experimental day were also excluded.

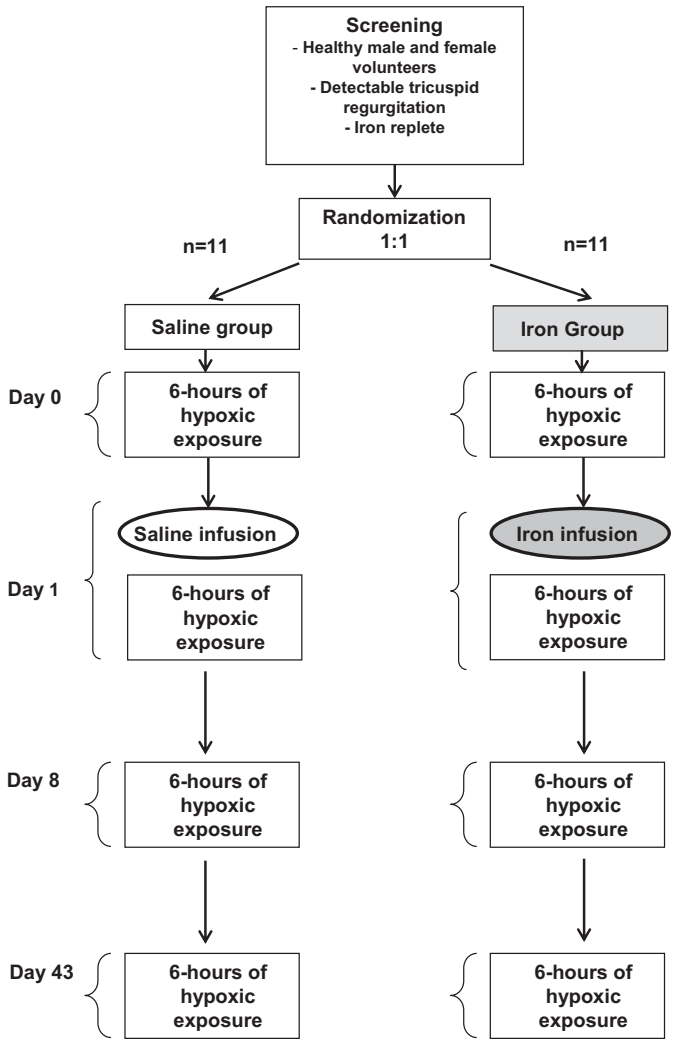

Fig. 1. Protocol for the study. Twenty-two participants were recruited. Participants were block randomized 1:1 to iron or saline. Participants were exposed to sustained isocapnic hypoxia for 6 h on *days 0*, *1*, *8*, and *43*. *Day 0* was a baseline measurement day that was undertaken at least 3 days before *day 1*. An infusion of iron or saline was given on *day 1*.

Each participant gave informed written consent. The study was approved by the National Research Ethics Service Committee South Central–Oxford A (REC 12/SC/0586) and performed in accordance with the Declaration of Helsinki.

Iron infusion. On *day 1*, participants received either 50 ml of sterile 0.9% m/V NaCl (placebo group) or 1 g of iron as ferric carboxylmaltose (Ferinject; Vifor Pharma, Glattbrugg, Switzerland) diluted in 50-ml sterile 0.9% m/V NaCl (iron group), administered over 15 min via a syringe infusion pump (Graseby 3100 Syringe Pump; Smiths Medical International, Ashford, UK). Ferric carboxymaltose has a half-life of 7.4–12.1 h (5).

Blood tests. Venous blood samples were taken on each experimental day prior to any other interventions. A full blood count together with associated parameters was obtained through a hospital laboratory. Similarly, iron markers including the concentration of ferritin and transferrin, as well as transferrin saturation, were determined by a clinical biochemistry laboratory. In addition, enzyme-linked immunosorbent assays (ELISA) were performed in duplicate on serum to measure the concentration of EPO and interleukin 6 (IL-6) (Quan-

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Table 1. *Participant demographics and baseline venous blood analyses*

Variable (Normal Range)	Saline Group (n = 11)	Iron Group (n = 11)	P Value
Age, yr	26.3 ± 8.6	33.5 ± 9.9	0.09
BMI, kg/m^2	24.3 ± 1.6	24.9 ± 2.1	0.51
Sex	Female, n = 4; male, n = 7	Female, n = 4; male, n = 7	
Hemoglobin (12.0–17.0), g/dl	13.9 ± 1.3	14.0 ± 0.9	0.81
Hematocrit (0.36–0.50)	0.42 ± 0.04	0.42 ± 0.03	0.77
Mean cell volume (83–105), fl	93.3 ± 3.8	90.8 ± 5.0	0.20
Erythropoietin, IU/l	8.1 ± 3.4	9.3 ± 3.0	0.38
Serum ferritin (10–300), μg/l	66 ± 40	55 ± 37	0.52
Serum transferrin (1.8–3.6), g/l	2.6 ± 0.4	2.7 ± 0.3	0.44
Serum transferrin saturation (16–50), %	31.3 ± 11.3	29.3 ± 12.7	0.70
Hepcidin, μg/l	51.5 ± 57.9	40.2 ± 34.2	0.58
sTfR, nmol/l	20.6 ± 5.9	21.5 ± 6.1	0.73
Interleukin-6, fg/l	0.56 ± 0.41	0.56 ± 0.4	0.97
C-reactive protein (0–8), mg/l	0.8 ± 0.6	2.5 ± 3.2	0.30

Values are means ± SD. An unpaired two-tailed Student's t-test was used to calculate the P value. BMI, body mass index; sTfR, soluble transferrin receptor.

tikine IVD human EPO and Quantikine high-sensitivity human IL-6; R&D Systems, Abingdon, UK) and on plasma to measure soluble transferrin receptor concentration (sTfR) (Quantikine IVD human sTfR; R&D Systems) and hepcidin concentration (Bachem, Peninsula Laboratories, Torrance, CA) according to the manufacturers' instructions.

Hypoxic exposures. The 6-h isocapnic hypoxic exposures were conducted using a purpose-built chamber (9). The target end-tidal P_{O_2} was 55 mmHg, and the target end-tidal P_{CO_2} was the naturally occurring air-breathing value for each participant, as assessed at the beginning of each experimental day. Respired gas was sampled continuously via a fine nasal catheter held in place at the opening of the participant's nostril. The samples were continually analyzed for CO_2 and O_2, and the chamber gas composition was automatically adjusted at 5-min intervals so as to minimize the difference between the desired and measured end-tidal values for P_{CO_2} and P_{O_2}. Partici-

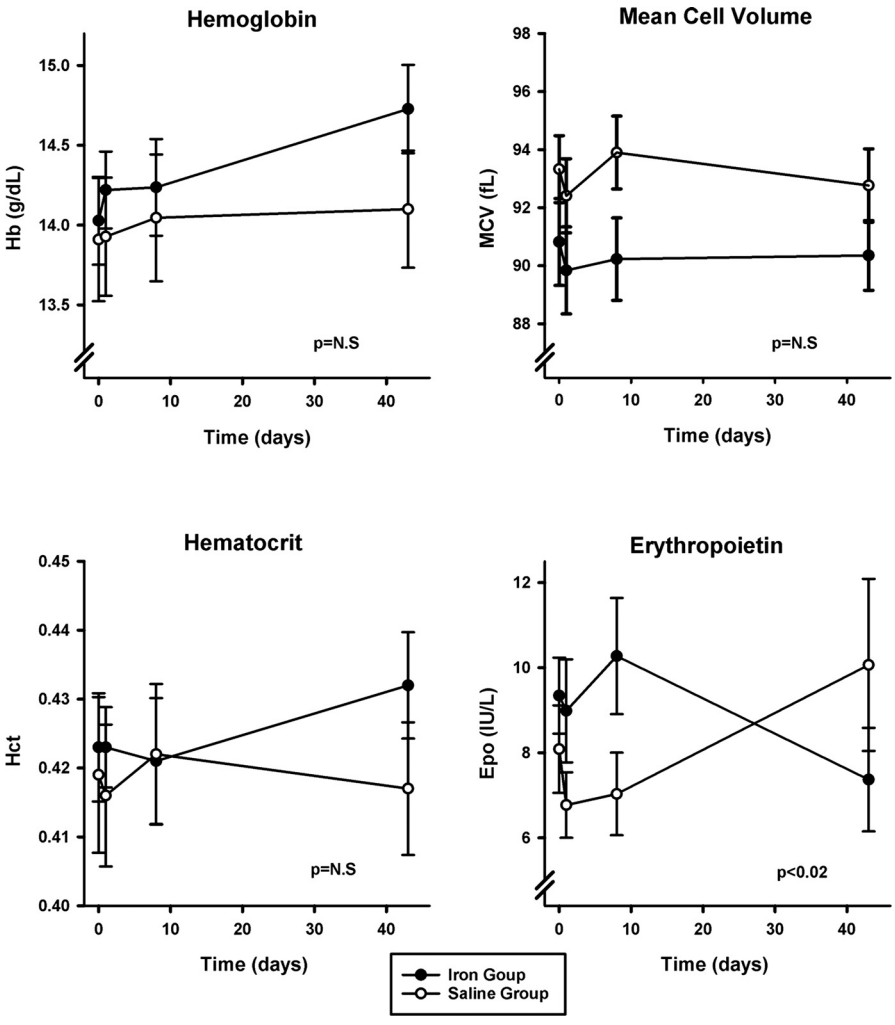

Fig. 2. Hematology results. No significant effects of infusion of 1 g of iron were detected in hemoglobin concentration (Hb), hematocrit (Hct), or mean cell volume (MCV). Erythropoietin concentration (EPO) appeared mildly elevated at *day 8* following iron infusion compared with controls. Values and error bars are means ± SE. P values refer to the interactive term for the change over time between groups and are calculated using linear mixed-effects modeling. N.S, not significant.

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pants spent no longer than 10 min in total outside of the chamber, e.g., for toilet breaks, over the course of a visit.

Assessment of PASP. PASP was estimated using standard echocardiographic techniques (27, 31) and equipment Vivid i (GE Healthcare, Chalfont St Giles, UK). In brief, the minor (physiological) degree of tricuspid regurgitation present in our participants resulted in a retrograde jet of blood from the right ventricle to the right atrium during systole. The velocity of this jet of blood was measured using Doppler ultrasound, and from this the peak pressure gradient across the tricuspid valve was determined and PASP was calculated using the modified Bernoulli's equation, with a value of 5 mmHg assumed for right atrial pressure (7). PASP was measured hourly during the hypoxic exposures. One blinded investigator obtained the echocardiographic images used for PASP measurements and analyzed the data. A second blinded investigator separately analyzed a sample of the images to check consistency of the analysis.

Statistics. Statistical analysis was conducted either using IBM SPSS Statistics version 22.0 or using the nlme package in R

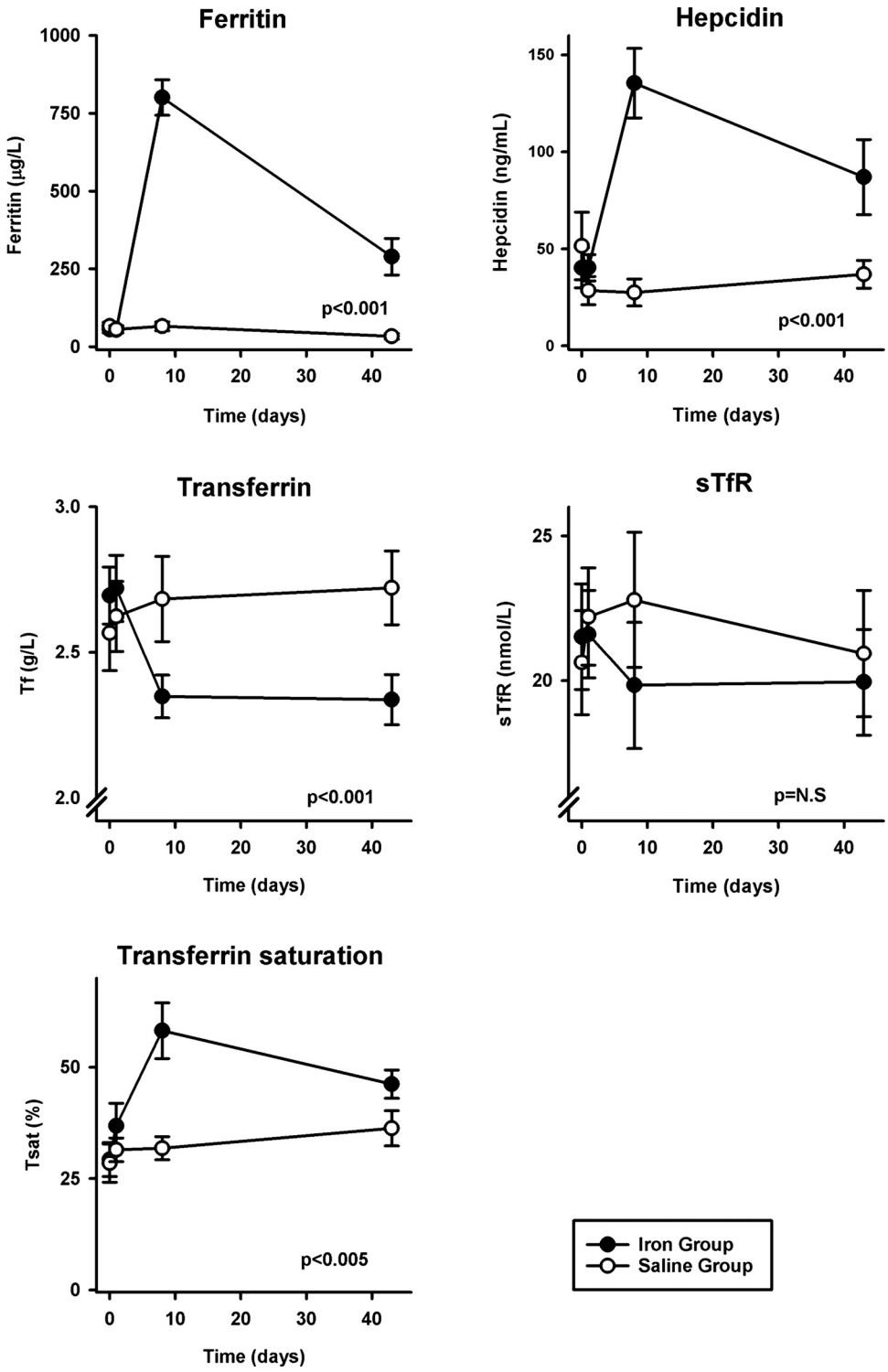

Fig. 3. Iron indexes. Following infusion of 1 g of iron, there were significant increases in the concentration of ferritin, transferrin saturation (Tsat), and hepcidin and a significant fall in transferrin concentration (Tf). No significant effects were observed in soluble transferrin receptor concentration (sTfR). Ferritin $n = 6$ for both groups at *day 43*. Values and error bars are means ± SE. *P* values refer to the interactive term for the change over time between groups and are calculated using linear mixed-effects modeling.

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(version R3.1.0; Vienna, Austria). Comparisons were drawn either by using unpaired two-tailed Student *t*-tests or by using linear mixed-effects modeling with participants as a random factor. For the blood results, where there was one value per day, the fixed effects were group (saline or iron), day, and the interactive term between the two. For the measurements of PASP, the fixed effects were group (saline or iron), day, and "state" (normoxia or sustained hypoxia) (*hours 4*, *5*, *6*, where the measurements of PASP were relatively stable), together with interactive terms. Statistical significance was assumed at $P < 0.05$.

RESULTS

All participants were healthy and iron replete, and their demographics and *day 0* venous blood analyses are given in Table 1. All infusions were well tolerated. There were no major adverse events.

Hematology variables. The two groups were well matched at *day 0* for hemoglobin concentration, hematocrit, and mean cell volume (Table 1). These did not vary differentially over

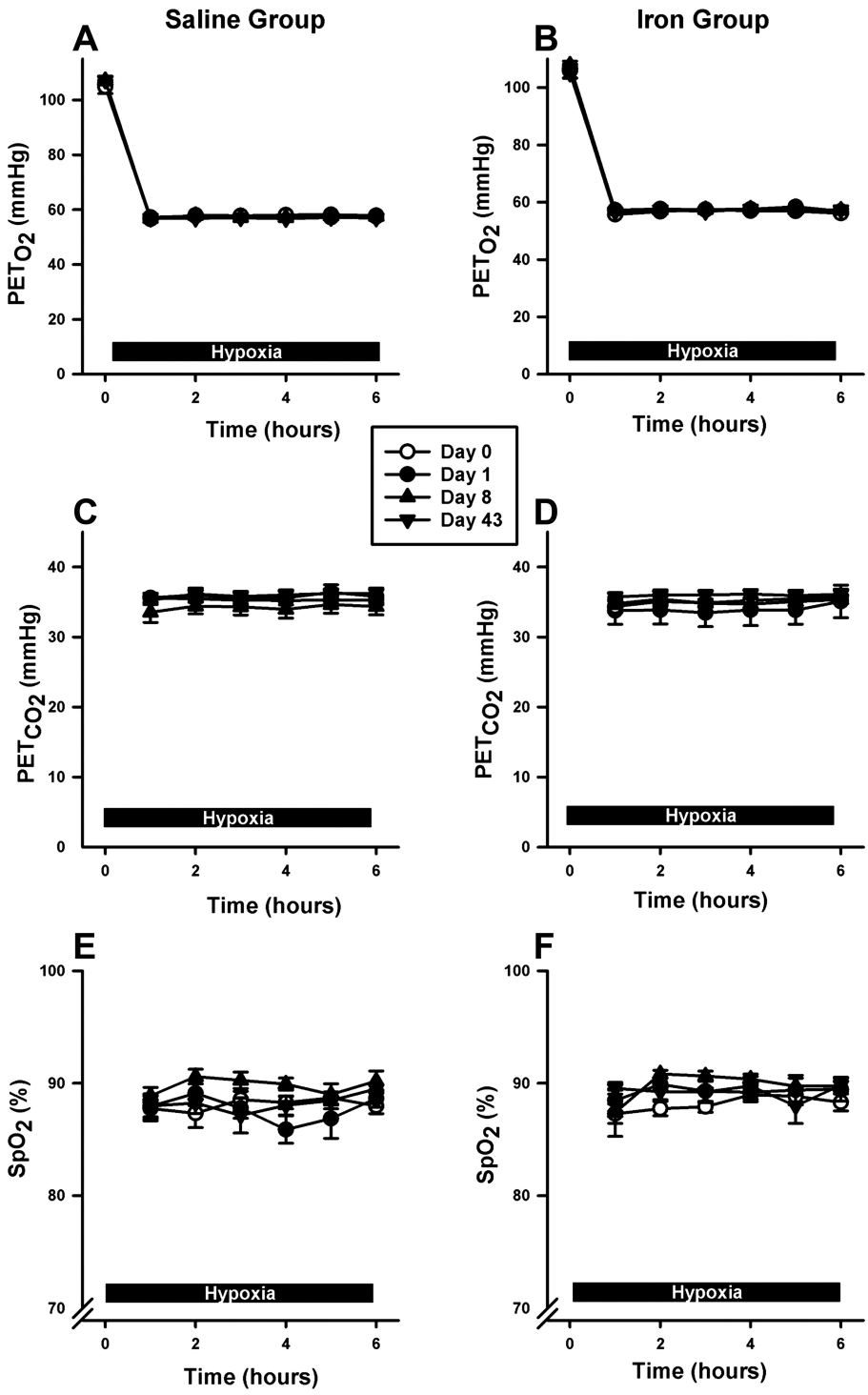

Fig. 4. Gas control during exposure to hypoxia. *A* and *B*: end-tidal oxygen ($P_{ET_{O_2}}$) levels over 6 h for saline and iron groups, respectively. *C* and *D*: end-tidal carbon dioxide ($P_{ET_{CO_2}}$) levels for saline and iron groups, respectively. *E* and *F*: arterial hemoglobin saturation (SpO_2) for saline and iron groups, respectively. Values and error bars are means at each hour of hypoxia ± SE.

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time between the saline and iron groups (Fig. 2). The groups were similarly well matched for EPO concentration at *day 0* (Table 1), but these values diverged such that EPO appeared higher at *day 8* in the iron group compared with control ($P < 0.02$) (Fig. 2).

Assessment of iron status. Values for the concentration of ferritin, transferrin, transferrin saturation, hepcidin, and sTfR were well matched between the saline and iron groups at *day 0* (Table 1). Iron loading with 1 g of iron led to an acute rise in ferritin concentration ($P < 0.001$), transferrin saturation ($P < 0.005$), and hepcidin concentration ($P < 0.001$) by *day 8* (Fig. 3). These changes remained significant at *day 43*. Transferrin concentration fell post-iron infusion and had not returned to preinfusion levels by *day 43* ($P < 0.001$) (Fig. 3). Values for sTfR concentration did not differ significantly over time between the saline and iron infusion groups (Fig. 3).

Gas control during sustained hypoxic exposures. Values for end-tidal P_{CO_2} and P_{O_2} were well matched between the two groups during the hypoxic exposures in the chamber on each experimental day (Fig. 4). Similarly, average saturation of oxygen measured by pulse oximetry was well matched between the two groups across experimental days (Fig. 4).

PASP response to hypoxia. The responses of PASP to hypoxia are illustrated in Fig. 5. A progressive rise in PASP was observed in all participants over the 6-h period of hypoxia. The interactive terms between hypoxia and group on *days 1*, *8*, and *43* were all significantly different from *day 0* (each $P < 0.001$). The interactive term between hypoxia and group for *day 0* (before iron was administered) was not significant. Thus iron significantly attenuated the PASP response to hypoxia, by ~50% on *day 1*, and the response then remained suppressed over the 6-wk study period.

DISCUSSION

The main finding of this study is that the suppression of the pulmonary vascular response to sustained hypoxia following IV administration of iron persists at 8 and 43 days after administration. This finding demonstrates that the effects of iron administration on the pulmonary vasculature are not simply due to the presence of a foreign iron-sugar complex in the blood, as this is long eliminated by the 43-day time point (5). Rather, the effect persists after the iron dose has been incorporated into the body's stores of iron.

Total body iron in a normal iron-replete individual is ~4 g (15). Therefore the dose of 1 g of IV iron represents an elevation of total body iron of ~25%. The loss of iron from the body, mainly from the gut, is ~1–2 mg/day (15), and in the absence of either further iron absorption or any additional iron loss, it will take ~2–3 yr for iron stores to return to preinfusion values. In our study, apart from sTfR (itself a marker of cellular iron demand rather than iron overload), values for the concentration of plasma ferritin, transferrin, and transferrin saturation all remained altered at 8 and 43 days post-iron infusion. What is not clear is whether any, or all, of these indexes of iron status will return to normal somewhat more rapidly than the rate at which the additional iron is lost from the body or whether they will only return to preinfusion values once the additional iron is lost.

A similar uncertainty relates to the pulmonary vascular response to hypoxia. It is uncertain whether this response is related to one or more of the circulating indexes of iron status or whether it is linked more directly to overall iron stores. From a more clinical perspective, the real interest is that effects of iron administration on the pulmonary vasculature are persistent; they last for a long time after the iron-sugar compound has been eliminated from the bloodstream. Thus the iron stores of the body could potentially be manipulated artificially to derive therapeutic gain.

Our underlying hypothesis for investigating the effects of iron on the pulmonary vasculature has been that iron availability affects the abundance of the transcription factor HIF, which itself is implicated in the pulmonary vascular response to hypoxia. While there is a very high degree of certainty both that iron modulates the HIF pathway (1, 13, 36) and that the HIF pathway affects the pulmonary vascular response to hypoxia (3, 8, 27, 38), it nevertheless cannot be concluded with equivalent certainty that the actions of iron availability on HIF are indeed the mechanism by which iron affects the pulmonary vascular response to hypoxia.

An interesting observation in our study is that the effects of iron on PASP become apparent only after the first hour of sustained hypoxia, and not at 1 h or before (Fig. 5). This finding is consistent with previous work (33). Thus iron does not appear to affect the acute (within minutes) hypoxic pulmonary vascular response, but rather the second phase of intensification of hypoxic pulmonary vasoconstriction, which be-

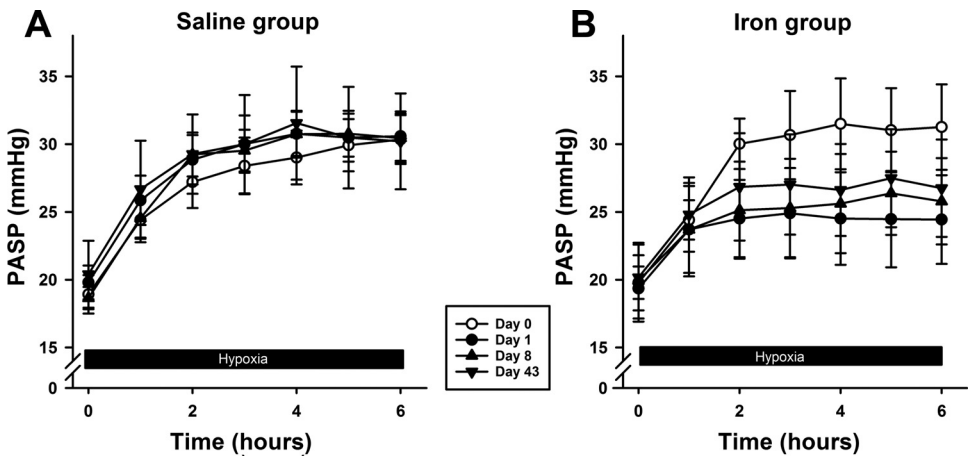

Fig. 5. Pulmonary artery systolic pressure (PASP) during exposure to hypoxia. *A* and *B:* PASP responses in saline and iron groups, respectively. Responses to sustained hypoxia were blunted following iron infusion on *day 1* and remained blunted at *days 8* and *43* ($P < 0.001$). Values and error bars are means ± SE.

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gins after ~45 min and causes a further progressive rise in PASP (32). This second slower phase of intensification has been attributed to new gene expression triggered by hypoxia (33), which may well be HIF regulated.

If indeed iron is working through HIF, then we may reasonably expect other HIF-regulated aspects of biology to be affected by manipulation of iron stores. For example, in humans it has been shown that iron chelation increases plasma EPO (20). However, in the present study, iron supplementation also appeared to increase EPO (at the *day 8* time point), perhaps via a separate IRP-related effect on HIF2α (6). Furthermore, there have been no reports of iron manipulation affecting the ventilatory response to sustained hypoxia despite the fact that HIF has been implicated in this process (12, 25, 27). This is similar to the PHD inhibitors, which have been shown to have a marked effect on erythropoiesis but to have minimal effects on the ventilatory response (2). Biologically, there are many complex issues to consider. First, manipulation of iron stores may have much greater effects on some cell types than others. Therefore the effects of manipulating the HIF pathway through iron stores may be specific to certain cell types. Second, HIF1 and HIF2 quite clearly have different actions (14, 19), and therefore it may be the case that iron has differential effects in relation to these two isoforms (11, 30). Third, although the classical effect of iron on the HIF pathway is through its involvement in the catalytic hydroxylation of HIF by the PHD enzymes, it has also been shown that cellular iron status affects the translation of HIF2α through the presence of an IRE in the 5′ end of the HIF2α mRNA (6, 24, 37). Again, this is a potential mechanism that could underlie both cell-specific and isoform-specific actions of iron on the HIF pathway.

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AUTHOR CONTRIBUTIONS

N.K.B., M.K.C., H.-Y.C., S.L.H., R.M., and K.L.D. performed experiments; N.K.B., M.K.C., H.-Y.C., R.M., and N.P. analyzed data; N.K.B., M.K.C., N.P., K.L.D., and P.A.R. interpreted results of experiments; N.K.B. and P.A.R. prepared figures; N.K.B. and P.A.R. drafted manuscript; N.K.B., M.K.C., H.-Y.C., S.L.H., R.M., N.P., K.L.D., and P.A.R. approved final version of manuscript; M.K.C., H.-Y.C., N.P., K.L.D., and P.A.R. edited and revised manuscript; P.A.R. conception and design of research.

REFERENCES

1. **Balanos GM, Dorrington KL, Robbins PA.** Desferrioxamine elevates pulmonary vascular resistance in humans: potential for involvement of HIF-1. *J Appl Physiol* 92: 2501–2507, 2002.
2. **Bishop T, Talbot NP, Turner PJ, Nicholls LG, Pascual A, Hodson EJ, Douglas G, Fielding JW, Smith TG, Demetriades M, Schofield CJ, Robbins PA, Pugh CW, Buckler KJ, Ratcliffe PJ.** Carotid body hyperplasia and enhanced ventilatory responses to hypoxia in mice with heterozygous deficiency of PHD2. *J Physiol* 591: 3565–3577, 2013.
3. **Brusselmans K, Compernolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P.** Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *J Clin Invest* 111: 1519–1527, 2003.
4. **Formenti F, Beer PA, Croft QP, Dorrington KL, Gale DP, Lappin TR, Lucas GS, Maher ER, Maxwell PH, McMullin MF, O'Connor DF, Percy MJ, Pugh CW, Ratcliffe PJ, Smith TG, Talbot NP, Robbins PA.** Cardiopulmonary function in two human disorders of the hypoxia-inducible factor (HIF) pathway: von Hippel-Lindau disease and HIF-2alpha gain-of-function mutation. *FASEB J* 25: 2001–2011, 2011.
5. **Geisser P, Banke-Bochita J.** Pharmacokinetics, safety and tolerability of intravenous ferric carboxymaltose: a dose-escalation study in volunteers with mild iron-deficiency anaemia. *Arzneimittelforschung* 60: 362–372, 2010.
6. **Ghosh MC, Zhang DL, Jeong SY, Kovtunovych G, Ollivierre-Wilson H, Noguchi A, Tu T, Senecal T, Robinson G, Crooks DR, Tong WH, Ramaswamy K, Singh A, Graham BB, Tuder RM, Yu ZX, Eckhaus M, Lee J, Springer DA, Rouault TA.** Deletion of iron regulatory protein 1 causes polycythemia and pulmonary hypertension in mice through translational derepression of HIF2alpha. *Cell Metab* 17: 271–281, 2013.
7. **Groves BM, Reeves JT, Sutton JR, Wagner PD, Cymerman A, Malconian MK, Rock PB, Young PM, Houston CS.** Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *J Appl Physiol* 63: 521–530, 1987.
8. **Hickey MM, Lam JC, Bezman NA, Rathmell WK, Simon MC, von Hippel-Lindau .** Mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis. *J Clin Invest* 117: 3879–3889, 2007.
9. **Howard LS, Barson RA, Howse BP, McGill TR, McIntyre ME, O'Connor DF, Robbins PA.** Chamber for controlling end-tidal gas tensions over sustained periods in humans. *J Appl Physiol* 78: 1088–1091, 1995.
10. **Howard LS, Watson GM, Wharton J, Rhodes CJ, Chan K, Khengar R, Robbins PA, Kiely DG, Condliffe R, Elliott CA, Pepke-Zaba J, Sheares K, Morrell NW, Davies R, Ashby D, Gibbs JS, Wilkins MR.** Supplementation of iron in pulmonary hypertension: rationale and design of a phase II clinical trial in idiopathic pulmonary arterial hypertension. *Pulm Circ* 3: 100–107, 2013.
11. **Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC.** Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 23: 9361–9374, 2003.
12. **Kline DD, Peng YJ, Manalo DJ, Semenza GL, Prabhakar NR.** Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. *Proc Natl Acad Sci U S A* 99: 821–826, 2002.
13. **Knowles HJ, Raval RR, Harris AL, Ratcliffe PJ.** Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells. *Cancer Res* 63: 1764–1768, 2003.
14. **Loboda A, Jozkowicz A, Dulak J.** HIF-1 and HIF-2 transcription factors: similar but not identical. *Mol Cells* 29: 435–442, 2010.
15. **Mole DR.** Iron homeostasis and its interaction with prolyl hydroxylases. *Antioxid Redox Signal* 12: 445–458, 2010.
16. **Nickol AH, Frise MC, Cheng HY, McGahey A, McFadyen BM, Harris-Wright T, Bart NK, Curtis MK, Khandwala S, O'Neill DP, Pollard KA, Hardinge FM, Rahman NM, Armitage AE, Dorrington KL, Drakesmith H, Ratcliffe PJ, Robbins PA.** A cross-sectional study of the prevalence and associations of iron deficiency in a cohort of patients with chronic obstructive pulmonary disease. *BMJ Open* 5: e007911, 2015.
17. **Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, Nizet V, Johnson RS.** Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). *J Clin Invest* 117: 1926–1932, 2007.
18. **Plesner LL, Schoos MM, Dalsgaard M, Goetze JP, Kjøller E, Vestbo J, Iversen K.** Iron deficiency in COPD associates with increased pulmonary artery pressure estimated by echocardiography. *Heart Lung Circ* (May 25, 2016). doi:10.1016/j.hlc.2016.04.020.

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19. **Prabhakar NR, Semenza GL.** Adaptive and maladaptive cardiorespiratory responses to continuous and intermittent hypoxia mediated by hypoxia-inducible factors 1 and 2. *Physiol Rev* 92: 967–1003, 2012.
20. **Ren X, Dorrington KL, Maxwell PH, Robbins PA.** Effects of desferrioxamine on serum erythropoietin and ventilatory sensitivity to hypoxia in humans. *J Appl Physiol* 89: 680–686, 2000.
21. **Rhodes CJ, Howard LS, Busbridge M, Ashby D, Kondili E, Gibbs JS, Wharton J, Wilkins MR.** Iron deficiency and raised hepcidin in idiopathic pulmonary arterial hypertension: clinical prevalence, outcomes, and mechanistic insights. *J Am Coll Cardiol* 58: 300–309, 2011.
22. **Rhodes CJ, Wharton J, Howard L, Gibbs JS, Vonk-Noordegraaf A, Wilkins MR.** Iron deficiency in pulmonary arterial hypertension: a potential therapeutic target. *Eur Respir J* 38: 1453–1460, 2011.
23. **Ruiter G, Lankhorst S, Boonstra A, Postmus PE, Zweegman S, Westerhof N, van der Laarse WJ, Vonk-Noordegraaf A.** Iron deficiency is common in idiopathic pulmonary arterial hypertension. *Eur Respir J* 37: 1386–1391, 2011.
24. **Sanchez M, Galy B, Muckenthaler MU, Hentze MW.** Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nature Struct Mol Biol* 14: 420–426, 2007.
25. **Slingo ME, Turner PJ, Christian HC, Buckler KJ, Robbins PA.** The von Hippel-Lindau Chuvash mutation in mice causes carotid-body hyperplasia and enhanced ventilatory sensitivity to hypoxia. *J Appl Physiol* 116: 885–892, 2014.
26. **Smith TG, Balanos GM, Croft QP, Talbot NP, Dorrington KL, Ratcliffe PJ, Robbins PA.** The increase in pulmonary arterial pressure caused by hypoxia depends on iron status. *J Physiol* 586: 5999–6005, 2008.
27. **Smith TG, Brooks JT, Balanos GM, Lappin TR, Layton DM, Leedham DL, Liu C, Maxwell PH, McMullin MF, McNamara CJ, Percy MJ, Pugh CW, Ratcliffe PJ, Talbot NP, Treacy M, Robbins PA.** Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. *PLoS Med* 3: e290, 2006.
28. **Smith TG, Talbot NP, Privat C, Rivera-Ch M, Nickol AH, Ratcliffe PJ, Dorrington KL, Leon-Velarde F, Robbins PA.** Effects of iron supplementation and depletion on hypoxic pulmonary hypertension: two randomized controlled trials. *JAMA* 302: 1444–1450, 2009.
29. **Soon E, Treacy CM, Toshner MR, MacKenzie-Ross R, Manglam V, Busbridge M, Sinclair-McGarvie M, Arnold J, Sheares KK, Morrell NW, Pepke-Zaba J.** Unexplained iron deficiency in idiopathic and heritable pulmonary arterial hypertension. *Thorax* 66: 326–332, 2011.
30. **Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL.** Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Res* 63: 6130–6134, 2003.
31. **Swenson ER, Maggiorini M, Mongovin S, Gibbs JS, Greve I, Mairbaurl H, Bartsch P.** Pathogenesis of high-altitude pulmonary edema: inflammation is not an etiologic factor. *JAMA* 287: 2228–2235, 2002.
32. **Talbot NP, Balanos GM, Dorrington KL, Robbins PA.** Two temporal components within the human pulmonary vascular response to approximately 2 h of isocapnic hypoxia. *J Appl Physiol* 98: 1125–1139, 2005.
33. **Talbot NP, Croft QP, Curtis MK, Turner BE, Dorrington KL, Robbins PA, Smith TG.** Contrasting effects of ascorbate and iron on the pulmonary vascular response to hypoxia in humans. *Physiol Rep* 2: e12220, 2014.
34. **Tan Q, Kerestes H, Percy MJ, Pietrofesa R, Chen L, Khurana TS, Christofidou-Solomidou M, Lappin TR, Lee FS.** Erythrocytosis and pulmonary hypertension in a mouse model of human HIF2A gain of function mutation. *J Biol Chem* 288: 17134–17144, 2013.
35. **Viethen T, Gerhardt F, Dumitrescu D, Knoop-Busch S, ten Freyhaus H, Rudolph TK, Baldus S, Rosenkranz S.** Ferric carboxymaltose improves exercise capacity and quality of life in patients with pulmonary arterial hypertension and iron deficiency: a pilot study. *Int J Cardiol* 175: 233–239, 2014.
36. **Wang GL, Semenza GL.** Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 82: 3610–3615, 1993.
37. **Wilkinson N, Pantopoulos K.** IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood* 122: 1658–1668, 2013.
38. **Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT, Semenza GL.** Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *J Clin Invest* 103: 691–696, 1999.
39. **Zimmer M, Ebert BL, Neil C, Brenner K, Papaioannou I, Melas A, Tolliday N, Lamb J, Pantopoulos K, Golub T, Iliopoulos O.** Small-molecule inhibitors of HIF-2a translation link its 5′UTR iron-responsive element to oxygen sensing. *Mol Cell* 32: 838–848, 2008.

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