IRON DEFICIENCY AND HUMAN HYPOXIA PHYSIOLOGY

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF OXFORD

MATTHEW C FRISE

ST JOHN'S COLLEGE

MICHAELMAS TERM 2016

To Charlotte

Wounds are for the desperate, blows are for the strong,
Balm and oil for weary hearts all cut and bruised with wrong.
I forgive thy treason – I redeem thy fall –
For Iron – Cold Iron – must be master of men all!

Cold Iron, Rudyard Kipling

ABSTRACT: IRON DEFICIENCY AND HUMAN HYPOXIA PHYSIOLOGY

Matthew C Frise, St John's College *DPhil Thesis, Michaelmas Term 2016*

This thesis is concerned with a very common disorder of iron homeostasis: iron deficiency. The specific focus is the manner in which iron deficiency influences physiological responses to hypoxia in humans. This work is predicated on observations made over many decades *in vitro* and *in vivo*, suggesting that variations in the bioavailability of iron have important consequences for certain biological processes known to depend on oxygen availability.

Three separate but related studies together form the basis for this thesis. The first two, *Study A* and *Study B*, adopt a similar approach in recruiting healthy volunteers who differ according to iron status, yielding iron-deficient and iron-replete groups in both cases. In *Study A*, the behaviour of the pulmonary circulation is investigated during a sustained hypoxic exposure, before and after an intravenous infusion of iron. In *Study B*, skeletal muscle metabolism is explored, both at the level of high-energy phosphate metabolism and the integrated physiological responses to exercise on a cycle ergometer. In the third study, *Study C*, a different approach is taken, recruiting patients with chronic obstructive pulmonary disease (COPD), and exploring the prevalence and associations of iron deficiency in this condition.

Chapters 2 and 3 describe experiments using sustained hypoxia in a normobaric chamber, during which the pulmonary circulation is assessed non-invasively using Doppler echocardiography. These reveal augmented hypoxic pulmonary vasoconstriction (HPV) in iron-deficient individuals, who also exhibit greater sensitivity to the effects of an infusion of intravenous iron. Additionally, the way in which certain circulating mediators important for iron haemostasis change over the course of these hypoxic exposures, and how iron status influences these responses, is explored.

Chapter 4 reports the findings of experiments using ^{31}P-magnetic resonance spectroscopy and cardiopulmonary exercise testing, which demonstrate abnormal whole-body metabolism in iron-deficient individuals during large muscle-mass exercise, despite the absence of a clear defect in mitochondrial oxidative phosphorylation. Intravenous iron is found to have significant effects to alter the lactate threshold in healthy individuals, but the effects are more striking in iron-deficient individuals. Collectively, these experiments imply that iron deficiency promotes a more glycolytic phenotype.

Chapter 5 explores iron deficiency in COPD, a condition in which pulmonary vascular disease, hypoxia and skeletal muscle dysfunction coexist, and examines some of the difficulties in assessing iron status in the setting of a chronic inflammatory disorder. Iron deficiency is found to be common, and unexpectedly associated with significantly more severe hypoxaemia, in patients with COPD. Possible reasons for these findings, and their clinical implications, are considered.

Chapter 6 provides a summary of the main conclusions to be drawn from the studies presented in this thesis.

Acknowledgements

It has been a privilege to study under the supervision of Professor Peter Robbins, who, despite his being Head of Department for the duration of my doctoral research, has always found time to support, encourage and guide me, even on those occasions when the coffee pot in the laboratory had run dry.

I would also like to thank Associate Professor Keith Dorrington, for his constant good humour, valuable advice, and patience when correcting numerous major errors of understanding on my part regarding human cardiopulmonary physiology.

I have very much enjoyed working with, and benefitted greatly from the assistance of, other members of the Robbins and Dorrington groups. This includes, but is not limited to, Vincent Cheng, Kate Curtis, David O'Neill, Mary Slingo, YuJin Chung and Samira Lakhal-Littleton. Nick Talbot, Tom Smith and Nayia Petousi have also frequently found time to help despite being busy Clinical Lecturers. I am conscious that I have very much 'stood on their shoulders' in conducting the work presented here.

Major David Holdsworth deserves a special mention since, without his expertise, flexibility, and unerring selection and maintenance of the aim, *Study B* might never have got off the ground. It has been a great pleasure to work with him during the latter part of my doctoral studies.

I am indebted to David O'Connor, who never failed to get pieces of equipment working in the laboratory when it appeared all hope was lost, and contributed in innumerable ways to the success of the studies described here. I must also thank Dr Annabel Nickol, who encouraged me to embark upon the one-year Research Fellowship that grew into this DPhil, conceived much of the experimental approach of the healthy volunteer studies presented here, and allowed me to become involved with *Study C*.

My research has been funded by the NIHR Oxford Biomedical Research Centre and via a British Heart Foundation Clinical Research Training Fellowship. I also received financial support from the Department of Physiology, Anatomy and Genetics, and should additionally thank the Fellows and staff of St John's College for their support during my doctoral studies in this regard.

Of course, there would be little to present in this thesis without the contributions of the very many willing volunteers who gave their time to sit in a hypoxic chamber or lay in an MRI scanner. My thanks go to them. I also owe a debt to Professor David Roberts and Dawn Goodwin at NHS Blood and Transplant, who together made it possible to recruit so many enthusiastic blood donors to *Study A* and *Study B*.

Finally, most of all, I should like to thank my wife and children, for their love and support throughout this period of study, for enduring many early mornings and late nights with me absent, and for always appearing happy to see me on the occasions when I did make it home before bedtime.

Contents

Abbreviations commonly used in this thesis

2-OG	2-oxoglutarate
6MWD	6-minute walk distance
ACD	Anaemia of chronic disease
AECOPD	Acute exacerbation of chronic obstructive pulmonary disease
AHVR	Acute hypoxic ventilatory response
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BMI	Body mass index
BMP	Bone morphogenetic protein
CHF	Chronic heart failure
CI	Confidence interval
CO	Cardiac output
COPD	Chronic obstructive pulmonary disease
CP	Chuvash polycythaemia
CPET	Cardiopulmonary exercise testing
CRP	C-reactive protein
DFO	Desferrioxamine
DL_{CO}	Diffusing capacity for carbon monoxide
DMT1	Divalent metal transporter 1
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
FBC	Full blood count
FEV_1	Forced expiratory volume in one second
FIH	Factor inhibiting hypoxia-inducible factor
FVC	Forced vital capacity
GOLD	Global initiative for chronic obstructive lung disease
HIF	Hypoxia-inducible factor
HPV	Hypoxic pulmonary vasoconstriction
ID	Iron deficient
IDA	Iron-deficiency anaemia

IDE	Iron-deficient erythropoiesis
IL-6	Interleukin-6
IQR	Interquartile range
IR	Iron replete
IRE	Iron-responsive element
IRP	Iron regulatory protein
IV	Intravenous
LTOT	Long term oxygen therapy
LV	Left ventricle
MCV	Mean cell volume
MEM	Mixed-effects modelling
MIGET	Multiple inert gas elimination technique
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MWU	Mann Whitney U
NAID	Non-anaemic iron deficiency
OSA	Obstructive sleep apnoea
$P_{a}O_2$	Arterial partial pressure of oxygen
$P_{A}O_2$	Alveolar partial pressure of oxygen
PAP	Pulmonary artery pressure
PASMC	Pulmonary artery smooth muscle cell
PASP	Pulmonary artery systolic pressure
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
$P_{ET}CO_2$	End-tidal partial pressure of carbon dioxide
$P_{ET}O_2$	End-tidal partial pressure of oxygen
PH	Pulmonary hypertension
PHD	Prolyl hydroxylase domain enzyme
$P_{I}O_2$	Inspired partial pressure of oxygen
$P_{I}CO_2$	Inspired partial pressure of carbon dioxide
ΔP_{MAX}	Maximum systolic pressure gradient across the tricuspid valve
pVHL	von Hippel-Lindau tumour suppressor protein
PVR	Pulmonary vascular resistance
$\dot{Q}$	Pulmonary perfusion
Q_{MAX}	Maximum theoretical rate of mitochondrial ATP synthesis

RAP	Right atrial pressure
RBC	Red blood cell
RHC	Right heart catheterisation
RM-ANOVA	Repeated measures analysis of variance
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
RV	Right ventricle
RVF	Right ventricular failure
S_aO_2	Arterial oxyhaemoglobin saturation
SD	Standard deviation
SEM	Standard error of the mean
S_pO_2	Peripheral oxyhaemoglobin saturation measured by pulse oximetry
sTfR	Soluble transferrin receptor
SWT	Shuttle walk test
TfR	Transferrin receptor
TR	Tricuspid regurgitation
TSat	Transferrin saturation
$\dot{V}_A$	Alveolar ventilation
$\dot{V}O_2MAX$	Maximal oxygen uptake

Chapter 1. General introduction

1.1. Iron and oxygen

This thesis is concerned with a particular aspect of the interaction between two elements fundamental to life on our planet: iron and oxygen. Reviews addressing the biology of these elements typically stress the reactivity and therefore potential toxicity of each, and some appear to imply that the presence in higher organisms of the two in combination must be the result of some sort of catastrophic error in design, for example:

> *"Since life's addiction to iron transcends the oxygenation of the Earth's atmosphere, living things must be protected from the potentially dangerous mix of iron and oxygen. The human being possesses grams of this potentially toxic transition metal, which is shuttling through his oxygen-rich humor." (Sheftel et al. 2012)*

The potential toxicity of iron under aerobic conditions results from its propensity as a transition metal to catalyse the generation of highly-reactive free radicals, particularly through the Fenton reaction (Kell 2009, Jomova and Valko 2011). However, many of the key biochemical processes that sustain life as we know it rely on the chemical properties of iron and oxygen. In fact, life depends on their reactivity and the successful chemical interaction of the two. Perhaps the most obvious example in vertebrates is the haem moiety of the oxygen-transport protein haemoglobin, which owes its capacity reversibly to bind dioxygen to a single ferrous ion (Figure 1-1).

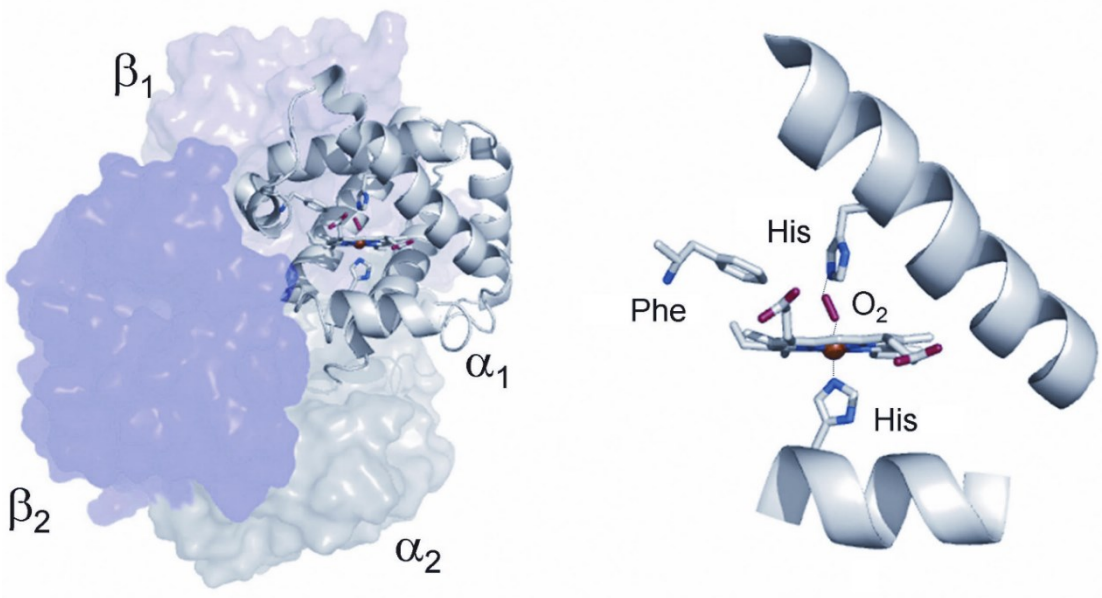

Figure 1-1. Haemoglobin structure and oxygen binding site

Left: Three-dimensional structure of a haemoglobin tetramer consisting of two α and two β chains, each of which has an associated haem molecule. *Right:* Expanded view of a single haem moiety and coordinating residues in the polypeptide chain. The single ferrous ion is represented as a red sphere at the centre of the haem molecule. Two histidine (His) residues and a phenylalanine (Phe) residue, of particular importance for iron coordination and the allosteric properties of the protein, are also highlighted. Carbon structures are grey; red portions represent oxygen atoms and blue portions indicate nitrogen atoms. Adapted from (Storz and Moriyama 2008) with permission.

One consequence of the iron dependence of haemoglobin synthesis is that much of our understanding of human iron homeostasis is couched in terms of its importance for erythropoiesis. Whilst this is entirely understandable, it does unfortunately lead to the frequent conflation of two pathological conditions that, though often found in combination, are entirely different: iron deficiency and anaemia. Though the former is an extremely common cause of the latter (Zimmermann and Hurrell 2007), this thesis will aim to make abundantly clear that despite its dominance in our understanding of human iron homeostasis, erythropoiesis is not the sole process in which iron is of importance. Similarly, when considering the consequences of iron deficiency for human health, a reduction in the oxygen-carrying capacity of the blood as a consequence of anaemia is generally assumed to be the most, and sometimes the only, significant pathophysiological process. Again, we shall see that this view is likely to be misguided.

The focus of this work is the interaction between iron homeostasis and oxygen sensing, specifically the manner in which deficiency of iron affects integrated physiological responses to hypoxia in humans. The rationale for pursuing this line of investigation is the recognition that iron is an essential cofactor for a major group of enzymes central to human oxygen sensing, coupled with observations made in humans regarding the effects of manipulating iron bioavailability on hypoxia-sensitive organ systems.

1.2. Hypoxia, euoxia and hyperoxia

Aerobic respiration allows for considerably more effective utilisation of energy from dietary fuels than does anaerobic respiration. Anaerobic metabolism of a glucose molecule to lactate is sufficient for the synthesis only of two adenosine triphosphate (ATP) molecules (Burton and Krebs 1953). The very existence of metazoans depends on oxidative

phosphorylation, which allows at least a 15-fold greater amount of ATP to be synthesised per single molecule of glucose (Hinkle *et al.* 1991). However, the reactivity of oxygen means that its supply to the tissues must be carefully regulated; there should be enough for the processes that require it but not so much that toxic effects are encountered. Again, just as with iron, this latter point is often stressed in the literature:

> *"Oxygen is addicting; in its grip are all the mitochondria-rich eukaryotes who learned to depend on it during the past 1.4 billion years…Oxygen is toxic. It rusts a person in a century or less…If introduced today, this gas might have difficulty getting approved by the Food and Drug Administration." (Severinghaus and Astrup 1986)*

It is important to be clear from the outset about the meaning of the various terms used when discussing oxygen availability. References to *anoxia* (from the Greek, literally *without oxygen*) are encountered early in the twentieth century. Barcroft quotes J. S. Haldane's famous aphorism at the outset of his 1920 Presidential Address to the Physiological Section of the British Association for the Advancement of Science:

> *"Anoxaemia not only stops the machine but wrecks the machinery…The anoxic type of anoxaemia is the most serious." (Barcroft 1920)*

Subsequently, Peters and Van Slyke stressed the distinction between a general oxygen deficit and a specific lack of oxygen in the blood:

> *"Anoxia literally means oxygen lack. We shall use the term to cover any condition which retards oxidation processes in the tissues...The less specific word anoxia seems preferable as a general term to cover the condition defined, 'anoxaemia' being limited to…oxygen deficit in the blood." (Peters and Van Slyke 1931)*

Under normal circumstances, in the healthy human breathing air at sea level, different tissues exist over a wide range of oxygen tensions. For example, whilst alveolar oxygen tension at sea level is approximately 100 mmHg, the actual oxygen tension achieved in the sarcoplasm of human skeletal muscle has classically been estimated at less than one tenth this value (Wittenberg and Wittenberg 1989). Newer techniques to measure more accurately *in vivo* oxygen tension in subcellular compartments suggest that the gradient may not in fact be as extreme as this (Mik 2013); nevertheless, it is clear that the *relative* hypoxia of many body tissues compared with the oxygen tension in alveolar gas, and thus arterial blood, is physiological. For this reason, some authors advocate use of terms such as "physoxia" (McKeown 2014, Kumar and Choi 2015), which this author does not feel adds clarity. In this thesis the term *euoxia* will be used to indicate oxygen tensions appropriate for a human breathing air at normal atmospheric pressure close to sea level, *hypoxia* to indicate an oxygen tension lower than this, and *hyperoxia* a higher oxygen tension.

1.3. Human oxygen-sensing and signalling mechanisms

All nucleated human cells are sensitive to changes in oxygen availability (Semenza 2011, Clanton *et al.* 2013). Of course, the most abundant non-nucleated human cell – the erythrocyte – is itself greatly influenced by changes in oxygen tension. The amount of oxygen bound to the haemoglobin molecules within a red cell varies in a characteristic way with the partial pressure of oxygen. This relationship is described by the well-known sigmoidal oxyhaemoglobin-dissociation curve (Figure 1-2). The binding of oxygen brings about allosteric effects that alter the affinity of haemoglobin for other ligands (Jensen 2004), notably carbon dioxide – the Haldane effect. It is perhaps arguable that this phenomenon represents oxygen sensing of a rudimentary sort, though it clearly does not engender the type of downstream effects that are mediated by the various organs specialised

for coordinating integrated physiological responses to hypoxia, which we shall now consider.

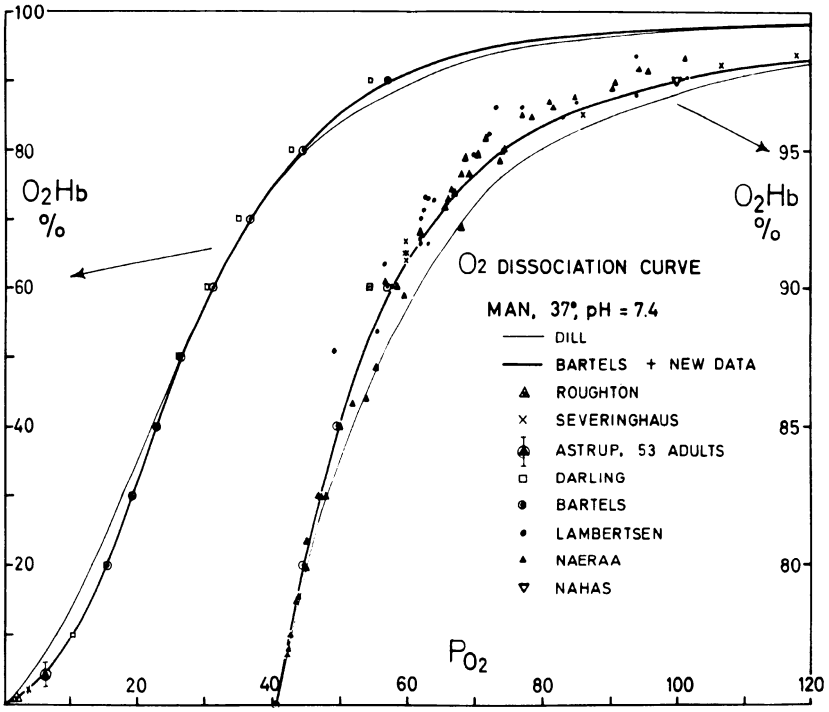

Figure 1-2. Oxyhaemoglobin dissociation curves

Data are for human haemoglobin at 37°C and pH 7.4. The left-hand curves correspond to the left ordinate and show the relationship over the full range of oxyhaemoglobin saturation. The right-hand curves correspond to the right ordinate and have the upper portion of the saturation range expanded. It is clear that a very considerable fall in oxygen tension occurs with only a small reduction in the percentage saturation. Put another way, the point of alveolar euoxia (100 mmHg) lies on a region of the curve that might be described as a plateau. The legend indicates the original sources of the data used to generate the curves. Reproduced from (Severinghaus 1966).

Though any cell may be influenced by a change in oxygen levels, humans possess a variety of tissues that are specialised for sensing local oxygen availability (Weir *et al.* 2005), as opposed simply to being affected by it. These tissues coordinate physiological responses

that aim to restore oxygen availability locally, or throughout the whole organism. An example of the former would be the smooth muscle of a systemic arteriole, which tends to relax in response to hypoxia, dilating the vessel and increasing the supply of oxygenated blood to, say, an exercising muscle (Barcroft 1972). Examples of the latter include type I cells of the carotid body (Lopez-Barneo *et al.* 1988) and pulmonary artery smooth muscle cells (PASMC) (Olschewski *et al.* 2002), which respond to hypoxia in such a way as to respectively bring about an increase in alveolar ventilation, and optimise ventilation-perfusion matching in the lung. Oxygen availability also regulates processes important in human reproduction, including placentation (Burton *et al.* 2009), and closure of the ductus arteriosus following the transition to air-breathing at birth (Michelakis *et al.* 2002).

There are two fundamental ways in which oxygen tension could be sensed in biological systems (Sylvester *et al.* 2012). The first includes any biological process in which oxygen is consumed as a substrate; the rate of such a reaction would be expected to be inhibited by hypoxia and accelerated by hyperoxia. Given the abundance of reactions in which oxygen participates as a substrate, the number of these sorts of pathways that could fulfil an oxygen-sensing role is clearly very large indeed. A biological pathway with this sort of requirement for dioxygen would, though, best sense changes in oxygen availability if the Michaelis constant (K_m; the substrate concentration at which the reaction rate is half maximal) for the reaction were close to euoxia. A second mechanism involves oxygen acting as a ligand for another molecule, such as an ion channel, with the binding of oxygen initiating a conformational change triggering a signalling cascade.

The various sites within the cell where changes in oxygen availability could be detected and a response brought about are thus numerous: hypoxia-sensitive ion channels in the membrane of the cell or any organelle membrane; any cytosolic enzyme pathway requiring

dioxygen as a substrate; and the same within the mitochondrion – particularly since this organelle is the major site of oxygen utilisation with the cell. What follows is an overview of what is known of the various oxygen-sensing and signalling pathways in humans, with particular attention paid to the role of iron.

1.3.1. Enzyme pathways requiring dioxygen as a substrate

Of the countless biological reactions that consume oxygen, those of special interest are catalysed by members of a large family of enzymes – the 2-oxoglutarate-dependent dioxygenases. These enzymes were identified half a century ago as having a role in the post-translational modification of collagen (Hutton *et al.* 1966). In excess of sixty members of the family have now been identified, with diverse roles in human biology, including collagen biosynthesis, fatty acid metabolism, and the cellular machinery of transcription and translation; all so far identified utilise iron as a cofactor (Loenarz and Schofield 2008, Markolovic *et al.* 2015, Martinez and Hausinger 2015). The biological reactions catalysed by these enzymes utilise dioxygen and 2-oxoglutarate (2-OG, also known as α-ketoglutarate), generating CO_2, succinate and a hydroxylated (or in some cases demethylated) end product.

A very significant role of the 2-OG-dependent dioxygenases is in regulating a family of transcription factors known as hypoxia-inducible factors (HIFs), which were first characterised over two decades ago (Wang and Semenza 1995). The group of 2-OG-dependent dioxygenases that regulate HIF are collectively known as HIF-hydroxylases. The context for the discovery of HIF was considerable effort on the part of several research groups internationally to determine the mechanisms governing hypoxic regulation of expression of the gene *EPO*, encoding erythropoietin (Beck *et al.* 1991, Pugh *et al.* 1991, Semenza *et al.* 1991, Semenza and Wang 1992, Maxwell *et al.* 1993b).

Iron was understood to be of importance for hypoxia sensing long before the iron-containing HIF-hydroxylases were characterised, because of the observed effects of other metal ions, particularly Co^{2+}, on erythropoietin production. Briefly, following the demonstration of an erythropoietic effect of cobalt in animals (Waltner and Waltner 1929) it was shown that administration of cobalt to humans was able to correct some forms of anaemia (Berk *et al.* 1949). Subsequently, experiments suggested the mechanism to be direct stimulation of erythropoietin secretion (Goldwasser *et al.* 1957, Goldwasser *et al.* 1958), an observation that was ultimately confirmed in cell-culture experiments (Kurtz *et al.* 1983, Goldberg *et al.* 1987). These studies with cobaltous ions, as well as work with Ni^{2+} (Jasmin and Solymoss 1975, Morse *et al.* 1977, Sunderman *et al.* 1982), led to the expectation that the oxygen sensor responsible would be a haem protein:

> *"…cobalt, nickel, or manganese can be substituted for ferrous iron in the centre of the porphyrin ring of haem. However, cobalt haemoglobin binds oxygen with low affinity, and nickel haemoglobin is unable to bind oxygen at all...we propose the following hypothesis: The oxygen sensor for the regulation of erythropoietin production is a haem protein that is dependent on the oxygen tension to which erythropoietin-producing cells are exposed. When the oxygen tension is sufficiently low, this haem protein is in the deoxy conformation and it triggers increased expression of the erythropoietin gene. Conversely, when the oxygen tension is sufficiently high, the haem protein is in its inactive oxy conformation and does not stimulate erythropoietin production…When either cobalt or nickel is introduced into this environment, it can substitute for ferrous iron in the porphyrin ring. The resulting substituted haem protein is locked in the deoxy conformation and, like the native deoxygenated iron haem protein, acts to increase erythropoietin expression."*
> *(Goldberg et al. 1988)*

Notwithstanding the uncertainty surrounding the nature of the oxygen sensor, both iron chelation with desferrioxamine (DFO), and substitution with Ni^{2+} or Co^{2+} ions, were historically used as indicators of whether a pathway might be oxygen-regulated (Gleadle *et al.* 1995, Maxwell and Salnikow 2004, Schofield and Ratcliffe 2004). With the demonstration that iron chelation using DFO could induce HIF activity and *EPO* expression *in vitro* with a similar time course to that observed with hypoxia (Wang and Semenza 1993), and the subsequent characterisation of HIF itself (Wang *et al.* 1995, Wang and Semenza 1995), the hypothesis that a haem protein was responsible was called into question.

The HIF-protein complex is composed of two subunits designated HIF-α and HIF-β. In contrast to the HIF-β subunit, HIF-α is unstable and regulated by oxygen tension (Huang *et al.* 1996, Salceda and Caro 1997, Huang *et al.* 1998); the heterodimer is active as a transcription factor (Semenza *et al.* 1997). There are three HIF-α proteins in humans, encoded by separate genes, of which HIF-1α and HIF-2α are the paralogues that have received the most attention (Kaelin and Ratcliffe 2008). HIF-α is a target for hydroxylation at three amino-acid residues by the HIF-hydroxylases (Figure 1-3). Two specific proline residues are targeted by one group of HIF-hydroxylases known as prolyl-hydroxylase domain enzymes (PHDs) (Bruick and McKnight 2001, Epstein *et al.* 2001). Hydroxylation at either site marks HIF-α for polyubiquitination and proteasomal degradation in a process that involves the binding of von Hippel–Lindau protein (pVHL) (Maxwell *et al.* 1999, Tanimoto *et al.* 2000, Ivan *et al.* 2001, Jaakkola *et al.* 2001). Hydroxylation at an asparagine residue, by another type of HIF-hydroxylase known as factor inhibiting HIF (FIH), does not promote HIF-α degradation but instead blocks recruitment of transcriptional coactivators to the complex (Mahon *et al.* 2001, Hewitson *et al.* 2002, Lando *et al.* 2002a).

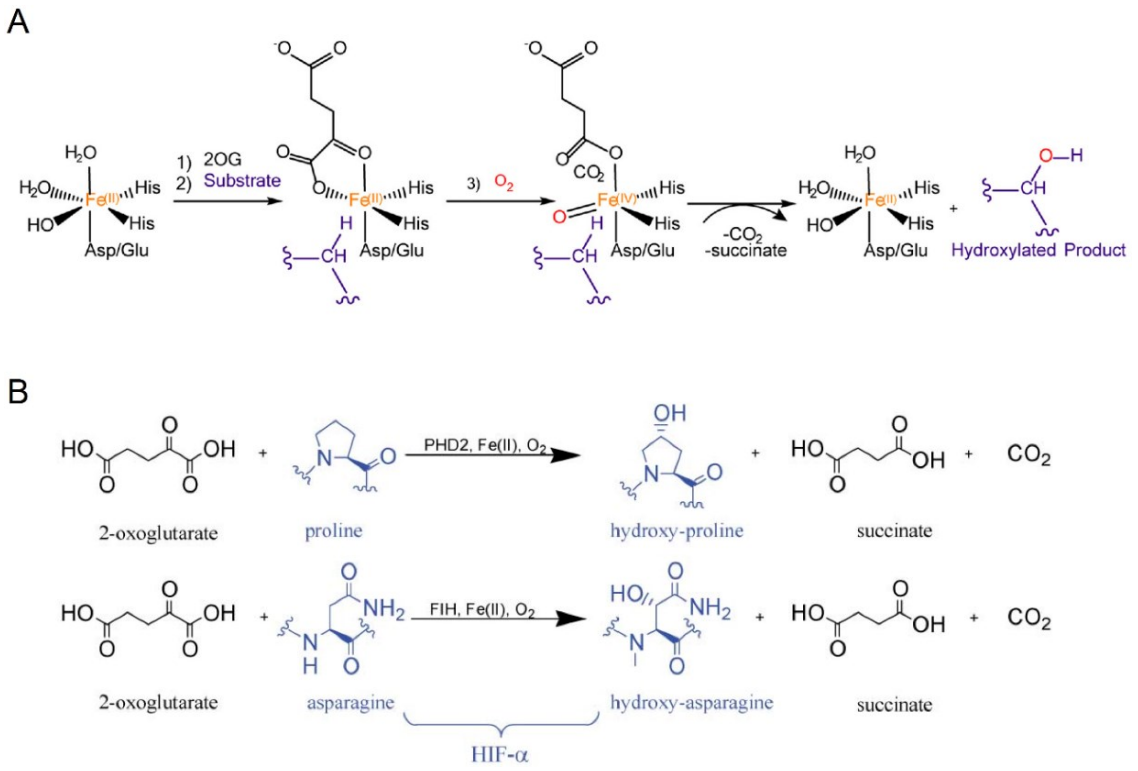

Figure 1-3. Hydroxylation reactions catalysed by iron- and 2-OG-dependent dioxygenases

(A) A single ferrous ion (orange) is bound within the enzyme active site. 2-OG displaces two water molecules as it binds and then undergoes oxidative decarboxylation generating succinate, CO_2 and a ferryl intermediate, which reacts with the substrate bringing about hydroxylation. (B) In the specific case of the HIF-hydroxylases, two proline residues and a single asparagine residue are the targets for hydroxylation. Asp, aspartate; Glu, glutamic acid; His, histidine. Adapted from (Markolovic *et al.* 2015) and with permission from (McNeill *et al.* 2005).

As 2-OG-dependent dioxygenases, the HIF-hydroxylases absolutely require dioxygen as a substrate (McNeill *et al.* 2002, Ehrismann *et al.* 2007). Accordingly, as oxygen availability falls, the rate of HIF-α prolyl-hydroxylation by the PHDs is slowed. HIF-α thus accumulates and is able to translocate to the nucleus and dimerise with HIF-β. Concurrent inhibition of asparaginyl-hydroxylation of HIF-α by FIH permits recruitment of the transcriptional coactivator p300/CBP (Lando *et al.* 2002b). As might be predicted, *in vitro* experiments suggest that HIF-α stabilisation is greatest over the range associated with

hypoxic and ischaemic events *in vivo* (Jiang *et al.* 1996b). HIF heterodimers bind to hypoxia-response elements (HREs) in the genome and control the expression of hundreds of genes (Figure 1-4), including those central to the regulation of erythropoiesis (Jiang *et al.* 1996a), angiogenesis (Forsythe *et al.* 1996), and metabolism (Kim *et al.* 2006). Thus, HIFs are central to the regulation of cellular and integrated physiological responses to variations in oxygen availability in metazoans (Kaelin and Ratcliffe 2008, Semenza 2011).

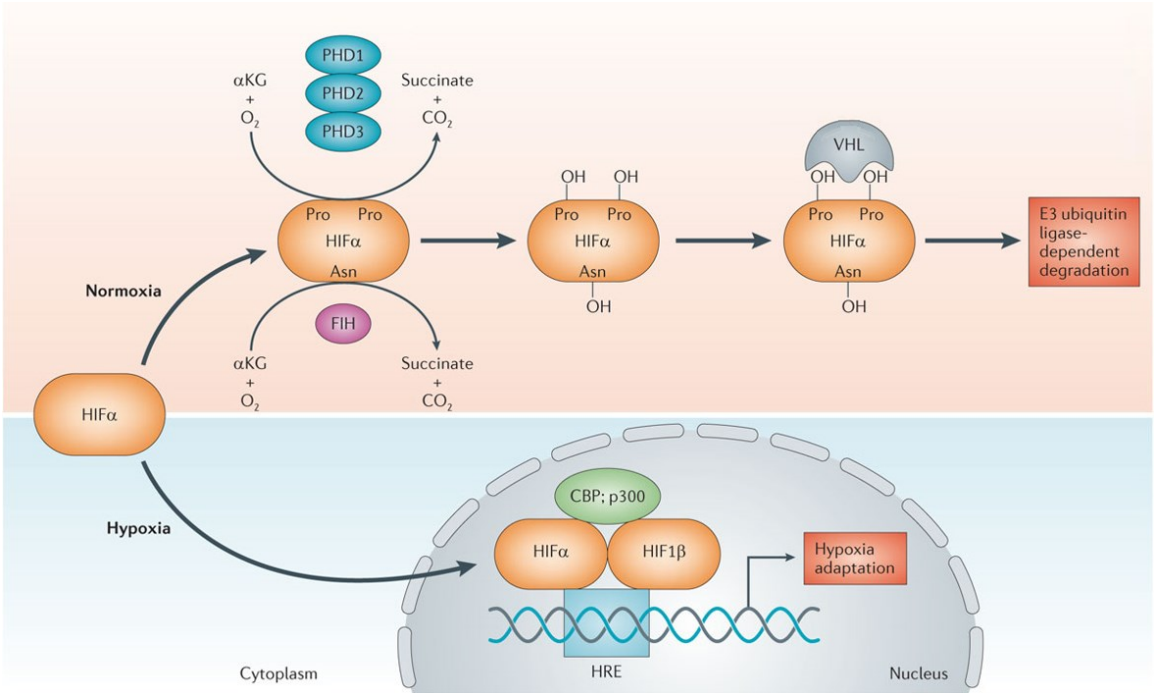

Figure 1-4. Regulation of HIF-α by oxygen availability

Prolyl hydroxylation of the HIF-α subunit under euoxic conditions leads to recruitment of the von Hippel-Lindau (VHL) protein, followed by polyubiquitination and proteasomal degradation. Under hypoxic conditions the hydroxylation reaction is inhibited; HIF-α escapes degradation, translocates to the nucleus, and binds HIF-β. Asparaginyl hydroxylation by FIH is similarly inhibited such that transcriptional coactivators can be recruited, and the complex is able to regulate transcription at hypoxia-response elements (HREs) within the genome. Pro, proline; Asn, asparagine; CBP, CREB (cAMP response element-binding protein)-binding protein; αKG, alpha-ketoglutarate (2-OG). Adapted from (Eltzschig *et al.* 2014) with permission.

We now understand that the oxygen regulation of *EPO*, as well as the host of other genes regulated by the HIF system, does not rely on haem, but instead a single ferrous ion coordinated at the active site of the HIF-hydroxylases (Epstein *et al.* 2001, Lando *et al.* 2002a, Schofield and Ratcliffe 2004, McDonough *et al.* 2006), which is essential for electron transfer (Clifton *et al.* 2006). This explains the *in vitro* observations that DFO is able to induce the paralogues HIF-1α and HIF-2α at the protein level within hours, similar to the kinetics observed with hypoxia (Wiesener *et al.* 1998). It also accounts for the finding that treatment with DFO four hours prior to introduction of hypoxia prevents binding of pVHL to HIF-1, but addition of iron chelators does not break this complex once it has been formed (Maxwell *et al.* 1999). Subsequent cell culture experiments have confirmed the effect of iron availability on HIF activity via altered HIF-hydroxylase function (Knowles *et al.* 2006, Pan *et al.* 2007, Eckard *et al.* 2010, Nandal *et al.* 2011).

Interestingly, the extent to which cobalt acts on the HIF pathway by displacing iron from the HIF-hydroxylases is unclear (Maxwell and Salnikow 2004). Other mechanisms are possible, including an action to compete with iron for cellular iron uptake by divalent metal transporter 1 (DMT1) (Gunshin *et al.* 1997), and direct binding to HIF so as to interfere with its regulation by pVHL in a manner that is independent of the action of the PHDs (Yuan *et al.* 2003).

The consequences of targeted genetic disruption of the HIF pathway in animal models demonstrate that HIF is a key regulator of cardiopulmonary physiology and metabolism (Yu *et al.* 1999, Brusselmans *et al.* 2003, Aragones *et al.* 2008, Minamishima *et al.* 2008, Moslehi *et al.* 2010, Bishop *et al.* 2013, Ghosh *et al.* 2013, Tan *et al.* 2013, Slingo *et al.* 2014, Dai *et al.* 2016, Hodson *et al.* 2016, Kapitsinou *et al.* 2016). Spontaneously occurring mutations confirm this to be the case in humans, with genetic upregulation of the pathway

resulting in polycythaemia, pulmonary arterial hypertension, abnormal ventilatory drive and metabolic abnormalities (Bushuev *et al.* 2006, Smith *et al.* 2006, Ladroue *et al.* 2008, Percy *et al.* 2008, Formenti *et al.* 2010, Formenti *et al.* 2011, Sable *et al.* 2011, McClain *et al.* 2013). Additionally, in some human populations resident for thousands of years at high altitude, there is evidence for natural selection of HIF-pathway gene variants associated with downregulation of hypoxia sensing (Beall *et al.* 2010, Lorenzo *et al.* 2014, Petousi *et al.* 2014). Many of these studies will be considered in more detail in subsequent chapters.

1.3.2. Hypoxia-sensitive ion channels

An oxygen-sensitive transcription factor system such as HIF clearly cannot operate quickly enough to bring about the sort of rapid physiological responses to changes in oxygen tension that occur in higher organisms; there must exist oxygen-sensitive pathways that do not rely on *de novo* protein synthesis. Ion channels are one of two sorts of sensors that predominate within vertebrate sensory systems capable of rapidly responding to changes in the concentration of small molecules, the other being G-protein-coupled receptors (Julius and Nathans 2012).

Much of the work on hypoxia-sensitive ion channels has focussed on the carotid body, because the ventilatory responses orchestrated by this organ in response to hypoxaemia occur extremely rapidly, suggesting that the signal transduction process must involve changes in existing proteins, rather than oxygen-regulated gene expression (Prabhakar and Semenza 2015). In 1988 an oxygen-sensitive K^+ current was identified in rabbit carotid body type I cells (Lopez-Barneo *et al.* 1988). Subsequently it was demonstrated that hypoxia brought about a rise in intracellular [Ca^{2+}] in these cells (Figure 1-5), and that this was largely due to voltage-gated Ca^{2+} entry (Buckler and Vaughan-Jones 1994).

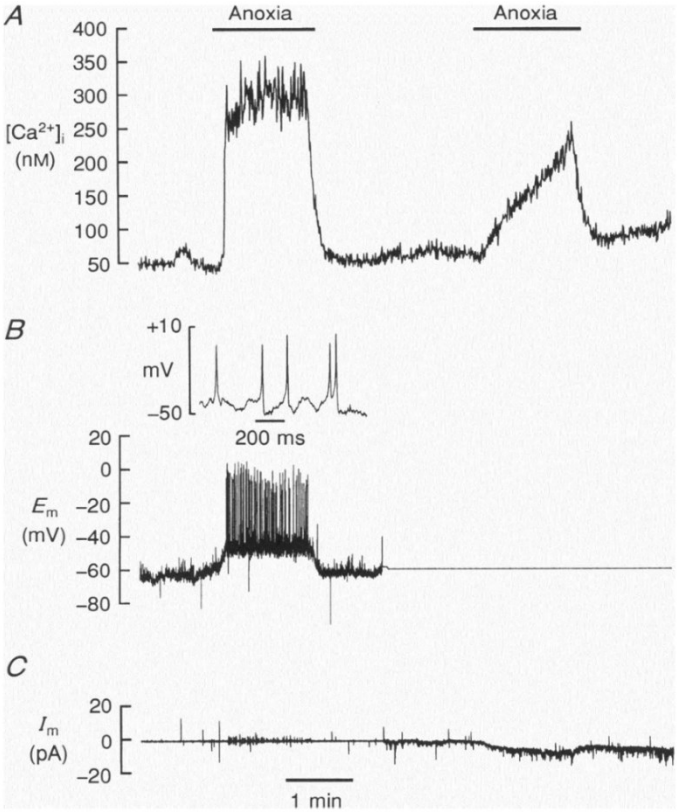

Figure 1-5. Perforated-patch recordings from a single isolated rat carotid body type I cell

(A) Intracellular calcium concentration ($[Ca^{2+}]_i$). (B) Membrane potential (E_m); the inset trace with expanded time scale illustrates individual action potentials during anoxia. (C) Current (I_m). Initially the current is clamped at zero; during the second anoxic exposure the cell is voltage clamped at approximately -60 mV. The inward shift of holding current induced by hypoxia at potentials close to the resting potential indicates either suppression of an outward K^+ current or activation of an inward Na^+ or Ca^{2+} current. Reproduced with permission from (Buckler and Vaughan-Jones 1994).

Further experiments have indicated that hypoxia depolarises type I cells by inhibiting a small voltage-insensitive background K^+ conductance (Buckler 1997), rather than by directly inhibiting voltage-gated K^+ channels. The channels responsible for this background K^+ conductance have been identified in mice as belonging to the TWIK-related acid-sensitive K^+ channel (TASK) family (Turner and Buckler 2013) (TWIK: tandem of p domains in a weak inward rectifying K^+ channel). Interestingly, murine TASK channel

behaviour is affected similarly by hypoxia and the metabolic inhibitors cyanide and FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone), suggesting that mitochondria play an important role in modulating the hypoxia-sensitivity of these channels (Buckler and Turner 2013, Buckler and Turner 2015).

There also exists a putative role for gaseous messengers, including hydrogen sulphide, nitric oxide and carbon monoxide, in mediating the hypoxia responsiveness of ion channels in the carotid body (Prabhakar and Peers 2014). Much controversy remains about the exact pathways involved, and our understanding of the role of ion channels in the oxygen-sensing behaviour of the carotid body is far from complete (Kumar and Prabhakar 2012). It is clear, though, that ion channels modulated by hypoxia, however indirectly, provide a mechanism for the acute regulation of ventilation in response to changes in oxygen availability.

A rather similar state of affairs is evident in the pulmonary circulation. It is clear that the immediate response of the pulmonary vasculature to hypoxia – hypoxic pulmonary vasoconstriction (HPV) – is so rapid that it is bound to involve hypoxia-sensitive ion channels. Several years after the first identification of a hypoxia-sensitive K^+ current in type I cells of the carotid body, a similar phenomenon was implicated in HPV by studies of PASMCs and pulmonary vascular tissue preparations (Post *et al.* 1992). Subsequently the coincidence, when pulmonary arterial smooth muscle was exposed to increasingly severe degrees of hypoxia, of graded responses in K^+ current, membrane potential, and intracellular Ca^{2+}, provided secure evidence of the importance of ion channels in HPV (Olschewski *et al.* 2002).

Other settings in which oxygen-regulated K^+ channels are implicated include the ductus arteriosus (Michelakis *et al.* 2000), fetal arteries of the placenta (Hampl *et al.* 2002) and

neuroepithelial bodies (Youngson *et al.* 1993). Just as with the carotid body and pulmonary circulation, though, in none of these settings has a directly oxygen-sensitive ion channel been convincingly shown to operate (Sylvester *et al.* 2012).

1.3.3. Mitochondria

Mitochondria have long been suspected to have a role in oxygen sensing, given how well placed they are for such a function, as the major site of oxygen consumption in the cell (Chandel and Schumacker 2000). The demonstration that the hypoxia sensitivity of certain ion channels seems to be mediated by this organelle does not of course provide an immediate answer as to how the oxygen-sensing process is actually effected. It simply moves the uncertainty to a different subcellular compartment.

One particular difficulty is that there may be significant differences between mitochondria from the various hypoxia-sensitive tissues. It would be wrong to become too diverted by the controversies surrounding these issues. However, given that several mitochondrial proteins involved in the electron transport chain (ETC) require iron as a cofactor, the organelle is clearly a site of potentially significant interactions between iron homeostasis and hypoxia sensing. This is because the ETC is highly likely to be involved, in some way, in conferring hypoxia-sensitivity on mitochondria. As Buckler and Turner explain:

> *"…it is difficult to reconcile our data with any single effect other than an inhibition of electron transport. Since all other interventions which inhibit electron transport also cause excitation in the carotid body, the most parsimonious explanation of all these data is that even if complex IV is not the primary site of oxygen sensing, modulation of electron transport is probably an integral part of the hypoxia transduction cascade." (Buckler and Turner 2013)*

Figure 1-6 illustrates three hypotheses for the mechanisms involved in oxygen sensing by mitochondria (in this particular case, those of a PASMC). The *redox hypothesis* proposes that hypoxia inhibits oxidative phosphorylation, thus promoting a more reduced cytosolic redox state, characterised by, for example, an increased abundance of the reduced form of the electron carrier nicotinamide adenine dinucleotide – NADH. Consequently, it is postulated, reactive oxygen species (ROS) generation is suppressed, which leads to closure of K^+ channels and membrane depolarisation (Archer *et al.* 1993).

The *ROS hypothesis*, in contrast, contends that hypoxia somehow brings about an increase in ROS within mitochondria (Waypa *et al.* 2006); the mechanism by which this might occur is itself the subject of much conjecture. Finally, the *energy state/AMP-kinase hypothesis* posits that during hypoxia a rise in the cellular AMP/ATP ratio activates AMP-activated protein kinase and promotes extracellular calcium entry and release from intracellular stores (Evans *et al.* 2005).

In addition to the role of the mitochondrion in mediating the behaviour of hypoxia-sensitive ion channels, mitochondrial ROS have been reported to be important for HIF-1α and HIF-2α stabilisation *in vitro* during hypoxia (Chandel *et al.* 2000, Mansfield *et al.* 2005), although others have found that an active mitochondrial ETC is not necessary for HIF-1 activation (Srinivas *et al.* 2001, Vaux *et al.* 2001).

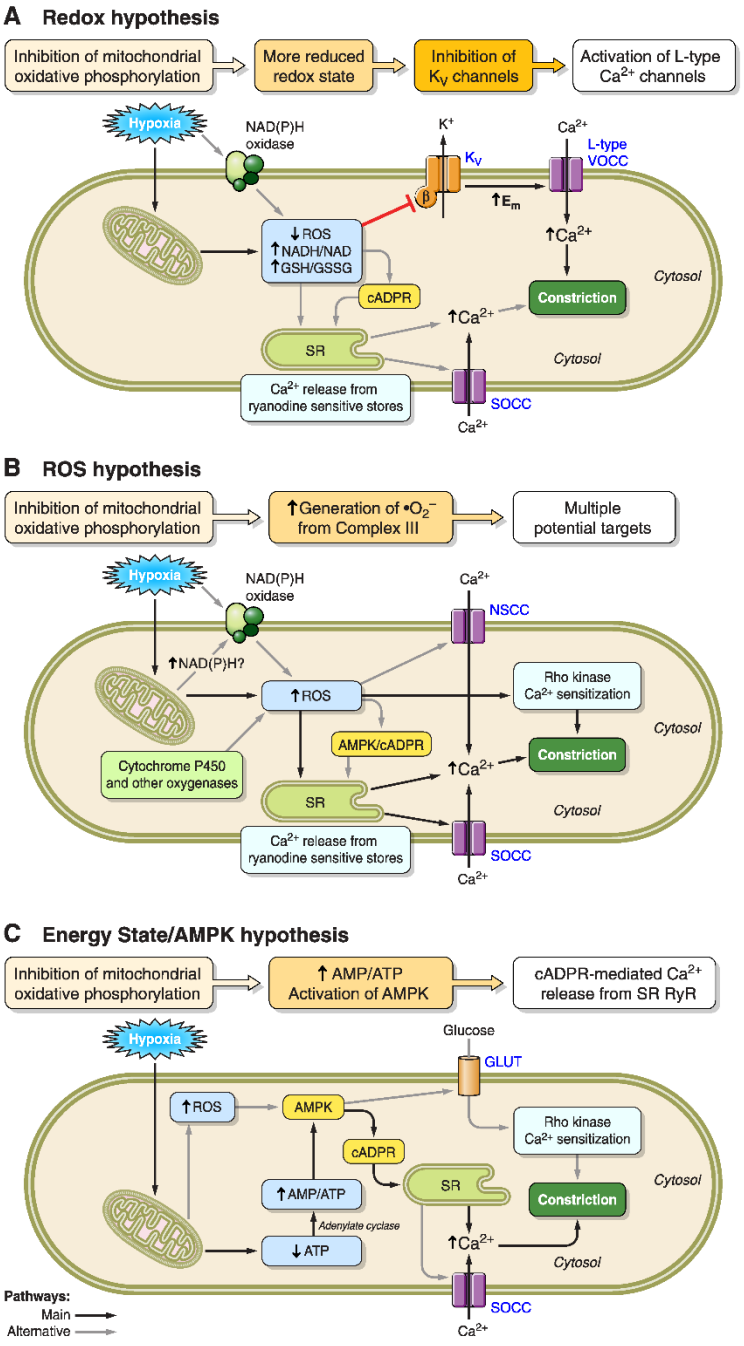

Figure 1-6. Proposed mechanisms of mitochondrial oxygen sensing in PASMCs

(A) Redox, (B) ROS, and (C) energy state hypotheses (see text). AMP, adenosine monophosphate; AMPK, AMP kinase; ATP, adenosine triphosphate; cADPR, cyclic adenosine diphosphate ribose; GLUT, glucose transporter; GSH, glutathione; GSSG, GSH disulphide; K_V, voltage-dependent K^+ channel; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; NSCC, nonselective cation channel; RyR, ryanodine receptor; SOCC, store-operated Ca^{2+} channel; SR, sarcoplasmic reticulum; VOCC, voltage-operated Ca^{2+} channel. Reproduced from (Sylvester *et al.* 2012).

1.4. Human iron homeostasis

As a transition metal, the chemistry and redox reactivity of iron confer several properties that make it particularly suited as a catalyst for biological reactions, including the capacity for oxygen binding, protein interaction and electron transfer (Aisen *et al.* 2001). Iron bioavailability and distribution must, though, be carefully regulated in order to avoid the potential toxicity of iron.

Painstaking work many decades ago using radioiron began the process of characterising human iron homeostasis in detail (Pirzio-Biroli *et al.* 1958, Finch 1959, Pollycove and Mortimer 1961) (Figure 1-7). It is important to note, though, that the majority of this research was performed in male subjects. The average adult human body contains 3-4 grams of iron, distributed between erythrocytes, storage iron (predominantly in the liver) and the bone marrow. The daily iron requirement for an adult is around 35 mg, the vast majority of which is derived from recycling within the reticuloendothelial system of senescent red cells.

Iron losses amount to around 1-2 mg per day, mainly from bleeding into, and loss of desquamated mucosal cells from, the bowel, but losses also occur from the skin and in the urine (Green *et al.* 1968). This 1-2 mg is the amount that must be absorbed daily from the gut in order to maintain neutral iron balance. Importantly, none of these processes represents a regulated pathway for the excretion of excess iron (Ganz 2013). As a result, iron uptake must instead be tightly regulated to avoid the deleterious consequences of iron accumulation and overload. In premenopausal women there is, of course, the added factor of menstrual iron loss. There exists high inter-individual variability, with iron losses of 2-79 mg per menstrual cycle considered normal (Price *et al.* 1964), which could clearly add

considerably to the daily requirement for absorption in women of reproductive age if neutral iron balance is to be maintained.

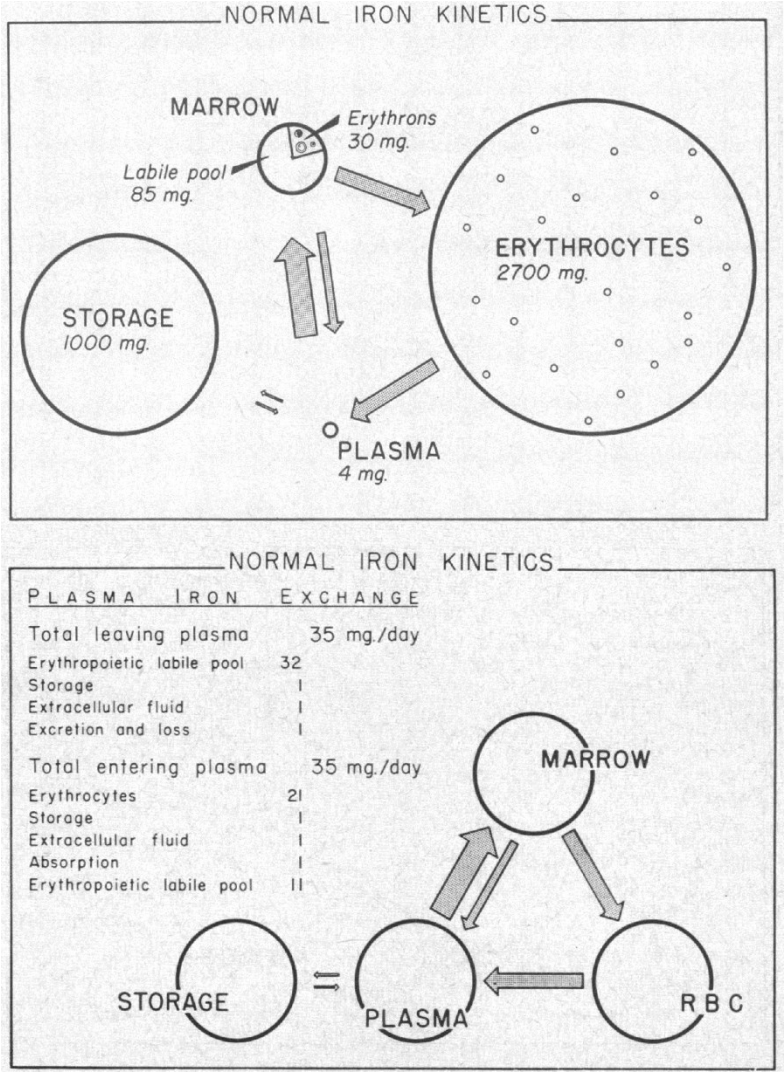

Figure 1-7. Body iron kinetics in health

Top: Compartmentalisation of body iron. The overwhelming majority is located within circulating erythrocytes. *Bottom:* Iron exchange between compartments. Though very little iron is actually to be found in the circulation outside red cells, this compartment turns over many times each day. The figures shown are based on data obtained from experiments involving mainly male volunteers. RBC, red blood cell. Reproduced with permission from (Pollycove and Mortimer 1961).

Though the quantitative movement of iron between various compartments has been reasonably well understood for some decades, the hormonal regulation of iron uptake and storage has been characterised only relatively recently. The hepatic peptide hormone hepcidin is now recognised as central to mammalian iron homeostasis (Finberg 2013, Ganz 2013). Hepcidin acts to lower serum iron by downregulating gastrointestinal absorption and promoting sequestration of iron within hepatocytes and splenic macrophages (Figure 1-8).

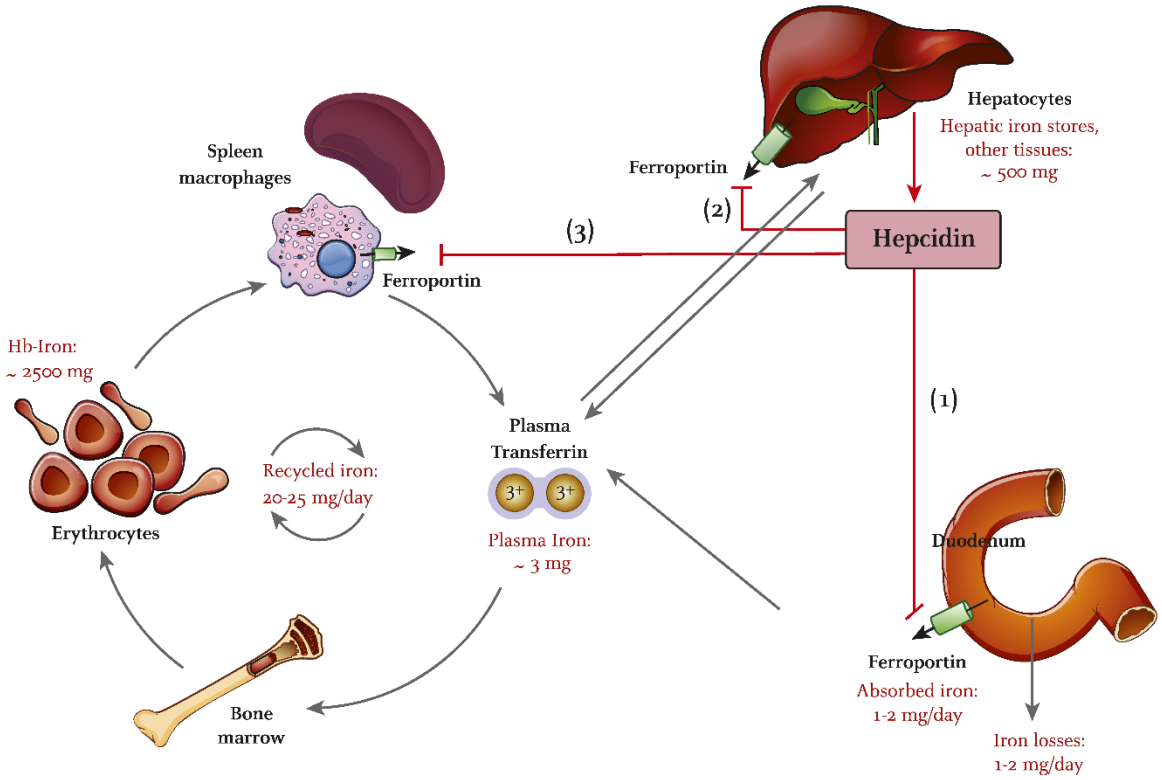

Figure 1-8. Systemic iron homeostasis

The peptide hormone hepcidin is viewed as the central regulator of iron metabolism. Inhibition of iron egress from duodenal enterocytes (1), hepatocytes (2) and splenic macrophages (3), via the action of hepcidin to promote degradation of the iron transporter ferroportin, promotes hypoferraemia. Reproduced with permission from (Evstatiev and Gasche 2012).

1.4.1. Ferritin

The major mammalian iron storage protein was discovered eighty years ago by the Czech physician and physiologist Vilém Laufberger, who gave the name ferritin to a substance – composed of 23% iron by mass – which he had isolated as a cadmium salt from horse spleen (Laufberger 1937). It is now known that ferritin exists as a protein complex composed of 24 subunits, which form an almost spherical shell of molecular weight 450 kDa that can sequester 4000-4500 iron atoms (Harrison and Arosio 1996, Arosio and Levi 2002). The iron is stored in the ferric form, and ferritin has intrinsic ferroxidase activity such that it is able to oxidise ferrous iron as it is taken up by the molecule and internalised for storage (Figure 1-9). Two different ferritin subunits exist, designated according to their electrophoretic migration as H (heavy) and L (light). Early studies reported – somewhat conveniently from the perspective of nomenclature – that ferritin isolated from liver contained mainly the L form, whilst that obtained from heart muscle was found to consist of a greater proportion of H subunits (Arosio *et al.* 1976). Comparing the amino acid sequence of H and L subunits isolated from human liver reveals 56% of residues to be identical and 81% similar or identical (Andrews *et al.* 1992).

Most storage iron is to be found within the liver, thus ferritin is an abundant intracellular hepatocyte protein. Early reports suggested that ferritin entered the circulation in certain disease states, but that it was not possible to quantify it in health (Baez *et al.* 1950, Reissmann and Dietrich 1956, Aungst 1966). Ferritin was ultimately accurately quantified in the serum of healthy individuals (and those with iron deficiency) with improvements in radioimmunoassay techniques (Addison *et al.* 1972). The authors of that work suggested, somewhat guardedly, that measurement of serum ferritin might eventually find some utility as an index of body iron storage.

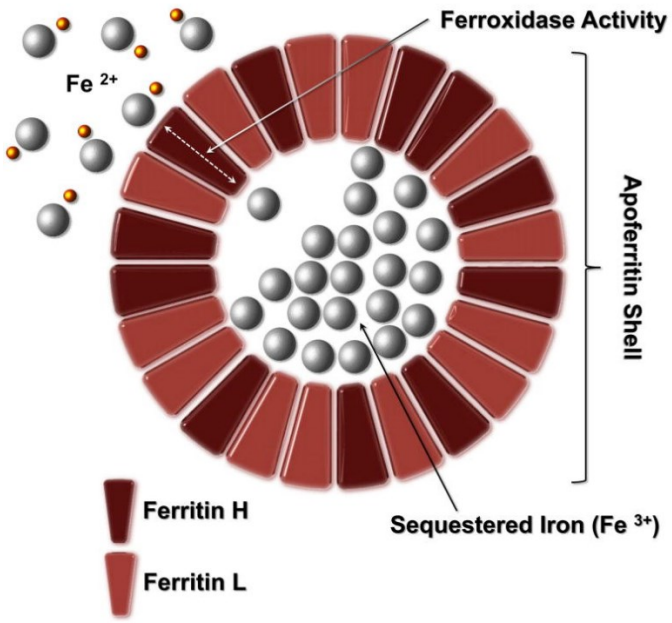

Figure 1-9. Schematic representation of a ferritin molecule

A total of twenty-four H and L subunits come together to form a near-spherical shell capable of encapsulating over 4000 iron atoms. As ferrous iron is internalised it is converted to the ferric form. Reproduced with permission from (Knovich *et al.* 2009).

Despite a huge amount of work since the first accurate quantification of ferritin in the serum of healthy individuals, it is remarkable how poorly understood the biology of extracellular ferritin remains, a point repeatedly made in the literature:

> *"What is most surprising is that despite this long history of clinical use, fundamental aspects of the biology of serum ferritin are still unclear. For example its tissue of origin, secretory pathway, receptor interactions and cellular effects remain topics of active debate." (Wang et al. 2010)*

> *"…it is surprising that the physiological function(s) of serum ferritin and its source (that is, whether it is derived from damaged cells or actively secreted by a regulated mechanism) still remain to be defined."*
> *(Hentze et al. 2010)*

Having said this, it matters very little to the subject of this thesis if the exact mechanisms underlying the presence of ferritin in the serum, and indeed possible actions of the protein once it arrives there, are elusive. What is important is whether serum ferritin can reliably aid in the determination of an individual's iron status, as surmised at the outset (Addison *et al.* 1972). It is of interest, though, that mouse studies have suggested that the main source of serum ferritin may be secretion by macrophages, rather than a cytosolic leak from cells (Cohen *et al.* 2010).

1.4.2. Transferrin and transferrin receptor

The major mammalian iron transport protein, transferrin, consists of a single polypeptide chain of molecular weight approximately 80 kDa. The tertiary structure is of two globular, homologous lobes, each of which contains a cleft formed by two subdomains, wherein a single ferric ion may bind (Sheftel *et al.* 2012). Chelation by transferrin of iron in the circulation serves three purposes. First, it maintains ferric iron in a soluble form in the bloodstream; secondly, it aids regulated transport and cellular uptake of iron; and thirdly, it preserves ferric iron in an inert state, protecting against the generation of free radicals (Gkouvatsos *et al.* 2012). Transferrin is synthesised predominantly by the liver, but smaller amounts are expressed in the testis and brain (Zakin 1992).

Transferrin exists in three forms: apotransferrin – without any iron bound; monoferric transferrin – with a single ferric ion bound; and diferric transferrin (holotransferrin) – with both binding sites occupied. As serum iron concentrations rise the transferrin saturation (TSat) increases and holotransferrin becomes increasingly abundant. The relationship between TSat and the relative proportions of the various forms of transferrin is illustrated in Figure 1-10.

Uptake of iron from the bloodstream into cells is achieved via transferrin receptor 1 (TfR1) on the cell surface. TfR1 is widely expressed, and cells with particularly high iron demands – such as developing erythroid and intestinal epithelial cells – show the highest levels of expression (Ponka and Lok 1999, Aisen 2004). Importantly, the binding of iron to transferrin alters its affinity for TfR1 by orders of magnitude, such that holotransferrin is the most readily taken-up species. Holotransferrin binds to TfR1 with 30-fold higher affinity than monoferric transferrin, and 500-fold higher affinity than apotransferrin (Young *et al.* 1984). Once transferrin has bound to TfR1, a reasonably well understood series of events takes place to transport the iron into the cell and recycle apotransferrin back to the circulation, as illustrated in Figure 1-11.

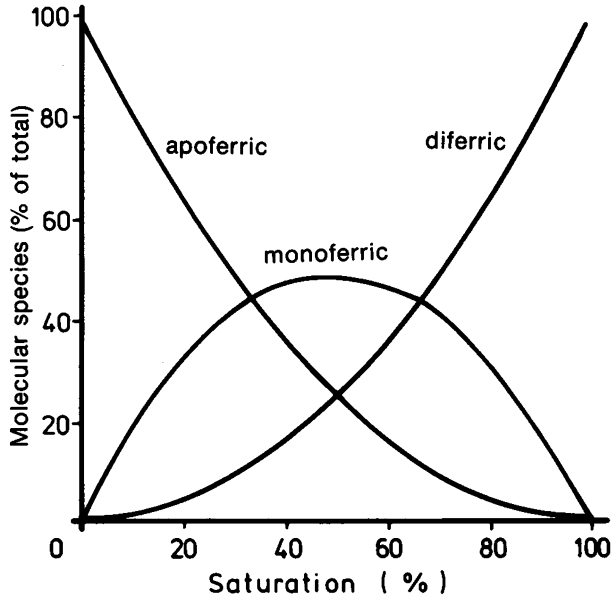

Figure 1-10. Relationship between TSat and distribution of transferrin species

Complete saturation of the iron-chelating capacity of circulating transferrin is achieved when all molecules have both binding sites occupied. Reproduced with permission from (Finch and Huebers 1982). Copyright Massachusetts Medical Society.

Though iron bound to transferrin accounts for less than 0.1% of total body iron, because in excess of two million new erythrocytes are produced every second by the bone marrow, plasma transferrin turns over more than ten times per day. In fact, it is estimated that a given ferric ion is expected to reside in the circulating transferrin pool only for approximately 90 minutes before being taken up (Cavill 2002). More than 80% of the transferrin-bound iron that circulates every day is delivered to bone marrow erythroblasts (Ponka *et al.* 1998).

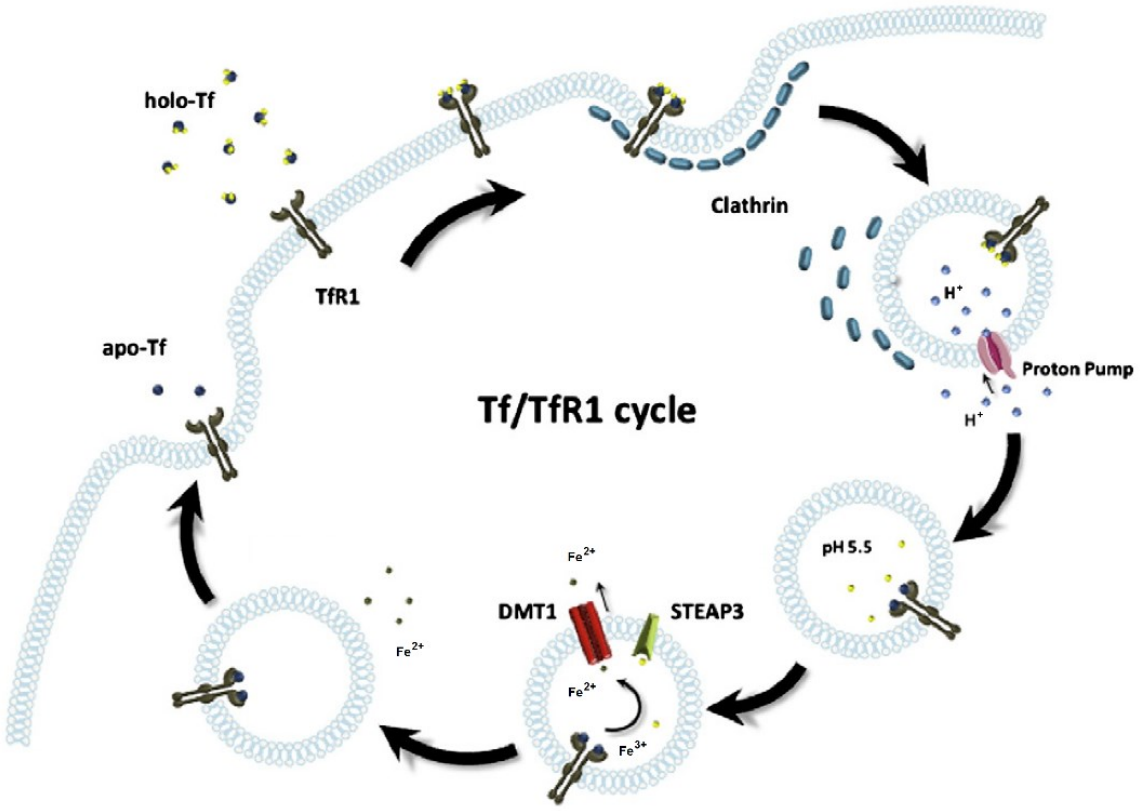

Figure 1-11. Cellular uptake of transferrin-bound iron and recycling of apotransferrin

Circulating holotransferrin binds to TfR1 (transferrin receptor 1) and the complex undergoes endocytosis via clathrin-coated pits. Acidification of the resulting endosomes causes release of ferric iron (Fe^{3+}), which is reduced to the ferrous form (Fe^{2+}) by the metalloreductase STEAP3 (six-transmembrane epithelial antigen of prostate 3). Ferrous ions traverse the endosomal membrane to the cytosol via DMT1 (divalent metal transporter 1). Apotransferrin is returned to the cell surface and released from TfR1 to allow another iron uptake cycle from the bloodstream. Modified from (Gkouvatsos *et al.* 2012) with permission.

1.4.3. Hepcidin and ferroportin

Though often described as the 'master regulator of iron homeostasis' it is important to recognise that the hormone hepcidin was identified on the basis of its antimicrobial functions (Krause *et al.* 2000, Park *et al.* 2001, Pigeon *et al.* 2001). That there should be crosstalk between iron homeostasis and the innate immune system is understandable given that iron is important for almost all microorganisms pathogenic to humans (Drakesmith and Prentice 2012). However, this brings important consequences for human iron homeostasis.

Hepcidin binds to and stimulates the internalisation and degradation of ferroportin, a transmembrane iron-transport protein (Nemeth *et al.* 2004b). Hepcidin promotes hypoferraemia via two main mechanisms. First, inhibition of ferroportin activity in the gastrointestinal tract prevents release of iron taken up by duodenal enterocytes, into the bloodstream. Secondly, inhibition of ferroportin activity in hepatocytes and splenic macrophages promotes iron sequestration within the reticuloendothelial system (Figure 1-8). Acute elevation of hepcidin will therefore alter the tissue distribution of iron but do nothing to total body stores; sustained elevation will invariably lead to a reduction in body iron as the inevitable daily losses of iron are no longer matched by gastrointestinal uptake. The factors involved in regulating hepatic hepcidin expression are shown in Figure 1-12.

Just how effective hepcidin is at promoting hypoferraemia and sequestration of iron is attested by a series of patients with glycogen storage disease who developed hepatic tumours secreting the hormone. These individuals suffered from a form of iron-deficiency anaemia (IDA) refractory to oral iron supplementation, and only partially responsive to intravenous (IV) iron therapy (Weinstein *et al.* 2002). The explanation for this observation is presumably that absorption of oral iron was blocked, and iron administered intravenously was sequestered in the liver and spleen at the expense of the bone marrow.

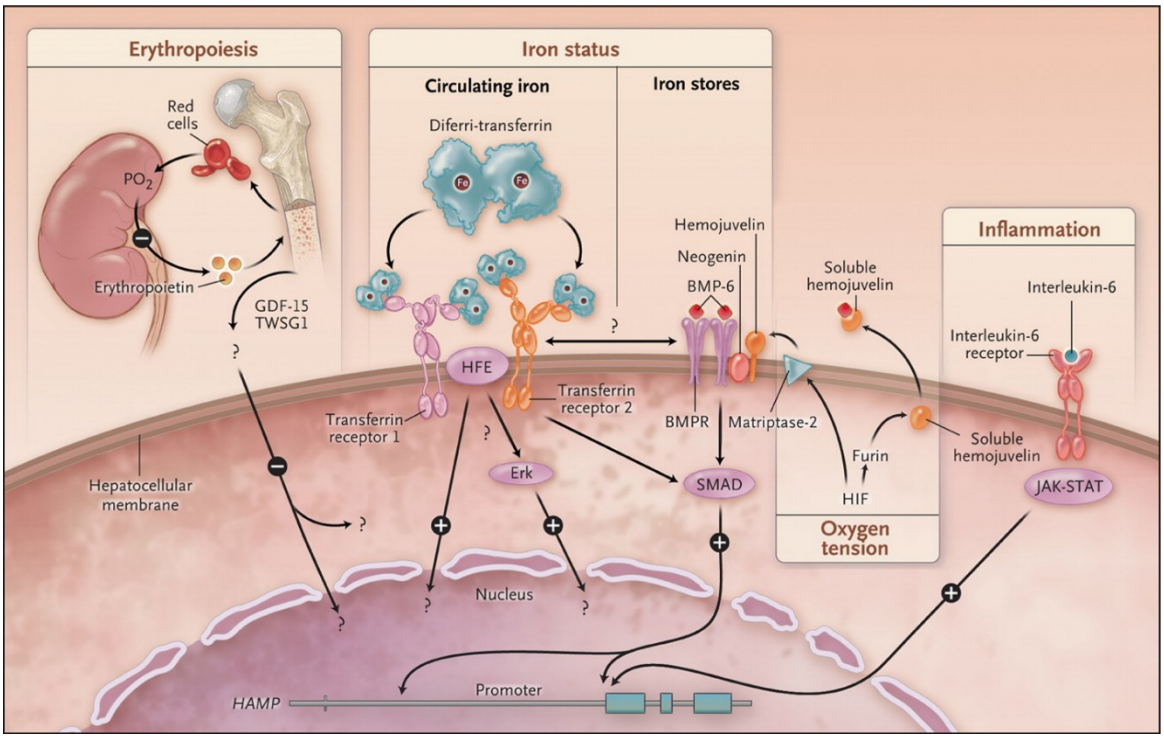

Figure 1-12. Functionally distinct regulators of hepatocyte hepcidin expression

Erythropoiesis suppresses expression of *HAMP* (the gene encoding hepcidin) by mechanisms that are not entirely clear; growth differentiation factor 15 (GDF-15), twisted gastrulation protein homolog 1 (TWSG1) and, most recently, erythroferrone have been implicated. *Iron status* influences hepcidin expression through two mechanisms: TSat and a storage iron signal provided by bone morphogenetic protein 6 (BMP-6). Binding of holotransferrin to TfR1 displaces human hemochromatosis protein (HFE), which interacts with TfR2 to upregulate hepcidin, possibly via Erk (a mitogen-activated protein kinase). Binding of BMP-6 to its receptor (BMPR) at the cell membrane activates the SMAD (Mothers Against Decapentaplegic Homolog) pathway; haemojuvelin functions as a BMP coreceptor augmenting the signal. *Hypoxia* increases the expression of two HIF-regulated genes, encoding matriptase-2 and furin. Matriptase-2 cleaves haemojuvelin from the cell surface, perhaps facilitated by neogenin, preventing its function. Furin cleaves haemojuvelin during processing to produce a soluble form that acts as a BMP-6 decoy. *Inflammation* increases hepcidin expression via interleukin-6 (IL-6) and the JAK-STAT (Janus-associated kinase-signal transducers and activators of transcription) pathway. Reproduced with permission from (Fleming and Ponka 2012). Copyright Massachusetts Medical Society.

Much about the regulation of hepcidin expression is incompletely understood (Finberg 2013) and there remains doubt about exactly how the proteins involved interact (D'Alessio *et al.* 2012, Rishi *et al.* 2013). Despite the uncertainties, key points that have been established include that a second form of transferrin receptor, TfR2, identified some time after TfR1 (Kawabata *et al.* 1999) and with more restricted tissue expression, is important in this process. HFE and various components of the BMP pathway are also crucial (Lin *et al.* 2007, Wu *et al.* 2014). The clinical consequences of mutations in the genes ending these proteins are particularly informative (Camaschella 2005). Notably, nearly all forms of human haemochromatosis have in common circulating hepcidin levels that are inappropriately low given iron bioavailability, the exception being mutations conferring on ferroportin resistance to hepcidin (Mayr *et al.* 2010).

1.4.4. Iron-responsive elements and their binding proteins

Intracellular responses to iron are regulated through iron-responsive elements (IREs) and the iron regulatory proteins (IRPs) that bind to them (Hentze *et al.* 2010). Unlike HREs, which transcriptionally regulate gene expression, IREs are found in the 5' and 3' untranslated regions of various mRNAs and exert translational control over a number of genes important in iron homeostasis. The action of a given IRE can be to promote or inhibit translation, and they are found, notably, in the mRNAs encoding ferritin (Aziz and Munro 1987, Hentze *et al.* 1987), TfR1 (Casey *et al.* 1989), DMT1 (Gunshin *et al.* 2001) and ferroportin (Lymboussaki *et al.* 2003).

The two related IRPs, IRP1 and IRP2, are iron-regulated in significantly different ways (Anderson *et al.* 2012). IRP1 has dual functions, operating as a high affinity IRE-binding protein in its apoprotein form, but when an iron-sulphur cluster binds, this activity is lost and the complex functions instead as a cytosolic aconitase. IRP2 does not have this latter

function, but is instead primarily regulated by iron-mediated proteasomal degradation (Guo *et al.* 1995, Iwai *et al.* 1995). Studies in genetically modified animals initially suggested that IRP2 was dominant, with marked and widespread disruption of iron homeostasis found in animals with targeted deletion of IRP2 (Cooperman *et al.* 2005, Galy *et al.* 2005) but not those lacking IRP1, in whom the phenotype was reported to be more tissue-restricted (Meyron-Holtz *et al.* 2004).

However, subsequent work has uncovered an important role for IRP1 in coordinating iron homeostasis, oxygen sensing and erythropoiesis (Anderson *et al.* 2013), which arises for two reasons. First, HIF-2α mRNA contains an IRE in the 5' region (Sanchez *et al.* 2007), whereas HIF-1α does not. The effect of IRP1 binding to this IRE in HIF-2α is inhibitory, meaning that under conditions where intracellular iron is scarce and IRP1 is an apoprotein, it is able to bind HIF-2α mRNA and repress its translation. Thus, the post-translational control of HIF-2α degradation by the iron-dependent HIF-hydroxylases, and the translational regulation of HIF-2α protein synthesis by IRP1, represent two opposing regulatory influences of iron on the HIF pathway. Secondly, hypoxia itself has direct – but opposing – effects on IRP1 and IRP2 activity, apparently stabilising the latter (Hanson *et al.* 1999) but favouring the aconitase function of the former at the expense of its IRP activity (Deck *et al.* 2009).

In summary, the cellular effects of iron deficiency on HIF-2α are predicted to be inhibition, via IRP1, of HIF-2α mRNA translation, but also inhibition, via the PHDs, of HIF-2α protein degradation; *competing* effects on protein levels of HIF-2α within the cell. In the case of hypoxia, HIF-2α mRNA translation will be promoted, via impaired IRP1-mediated inhibition, as will HIF-2α protein stabilisation, via inhibition of prolyl-hydroxylation; *synergistic* effects to raise intracellular levels of HIF-2α protein (Figure 1-13).

IRP1 is the predominant IRP in renal interstitial fibroblasts and pulmonary endothelial cells (Zhang *et al.* 2014a). As will be discussed in due course, with respect to both erythropoietin synthesis and HPV, HIF-2α appears to play a much greater role than HIF-1α. Accordingly, these interactions between the IRP-IRE system and the HIF pathway are likely to be of very considerable importance for some of the physiological processes examined in this thesis.

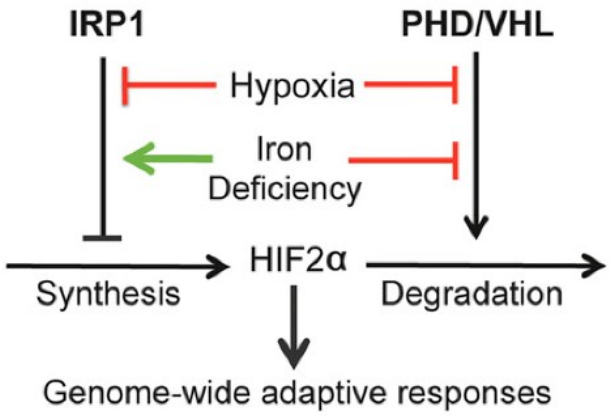

Figure 1-13. Regulation of HIF-2α protein levels by iron and oxygen via IRP1 and the PHDs

IRP1 governs HIF-2α translation, whereas the PHDs control stability of the resultant protein. Hypoxia has the same ultimate effect via both pathways – to increase HIF-2α protein abundance. Iron deficiency, in contrast, has opposing effects. Intracellular iron levels might therefore be expected to 'fine-tune' responses to hypoxia, as appropriate for prevailing iron availability at any given time. Arrows represent stimulatory effects, lines ending in bars indicate inhibitory effects. Reproduced with permission from (Anderson *et al.* 2013).

HIF-2α has been found to be translationally repressed in response to dietary iron deficiency in rats (Davis *et al.* 2011), and deletion of IRP1 in mice leads to splenomegaly and extramedullary haematopoiesis (Wilkinson and Pantopoulos 2013). If these animals are fed

an iron-deficient diet, erythropoiesis can paradoxically be stimulated, as a result of further augmentation of erythropoietin synthesis (Ghosh *et al.* 2013). In some ways, therefore, a loss of IRP1 function may be considered to be similar to a HIF-2α gain of function (Anderson *et al.* 2013). It has been suggested, based on such experiments, that IRP1 is the principal regulator of HIF-2α mRNA translation *in vivo*, and that this mechanism is likely to dominate PHD-mediated post-translational regulation of HIF-2α stability (Wilkinson and Pantopoulos 2013). The studies in this thesis will be informative with respect to the extent to which the integrated physiological responses to hypoxia seen in iron-deficient humans support this model.

1.5. Iron deficiency

1.5.1. Definitions

In order to determine whether or not there exists an effect of iron deficiency on human hypoxia physiology it seems obvious that a clear definition of the former will be necessary. Here a difficulty is immediately encountered, which is well articulated by a pioneering group reporting their efforts to study iron deficiency in Wales in the 1960's:

> *"The prevalence of iron deficiency is necessarily an artificial concept depending on the diagnostic criteria adopted. Iron deficiency exists in different grades of severity and if depletion of body iron stores alone is accepted as an early manifestation of the process then iron deficiency is very common in some sections of the population." (Jacobs et al. 1969)*

From this statement it is clear that, even prior to the development of a reliable assay for serum ferritin, a distinction was being drawn between *deficiency* and *depletion* of iron

within the human body. Clement Finch, widely regarded as the father of the field of human iron biology (Watts 2010), suggested the following definitions:

> *"**Iron depletion** refers to a decrease in iron stores from that expected for the age and sex of the individual but intact essential body iron. **Iron deficiency** refers to a virtual exhaustion of iron stores and a deficient plasma iron supply in an individual with no significant decrease in haemoglobin concentration. **Iron deficiency anaemia** refers to the presence of iron deficiency and anaemia due to iron deficiency. **Iron-deficient erythropoiesis** refers to a deficient marrow iron supply, regardless of the iron supply to other body tissues." (Finch 1994)*

Finch's classification well illustrates the issue of the close relationship of erythropoiesis with iron status mentioned earlier. It is perfectly possible, of course, that for other physiological processes, *iron deficiency* means something quite different to the state that has been determined to have consequences for red cell production. It could be the case that in another organ system – the pulmonary circulation, say – tissue iron levels are perfectly adequate for those processes that require iron long after reduced iron supply has begun to have effects on erythropoiesis. On the other hand, it is equally possible that the production of erythrocytes is of such importance for the wellbeing of the organism that some other tissues are preferentially drained of their iron before erythropoiesis is significantly compromised.

A further challenge is that different types of iron deficiency are recognised. The most straightforward of these to understand is *absolute* iron deficiency, of the sort described in the quote above by Finch: absent iron stores. However, the level of storage iron, except in

states of overload, may not be the parameter of primary interest from a physiological perspective:

> *"Iron in stores is metabolically inactive and is not only unavailable for immediate use but may be difficult to bring into use at all. The real clinical issue lies in active iron metabolism, the movement of iron from effete red cells and into further generations of developing red cells." (Thomas et al. 2013)*

Despite sufficient or even abundant body storage iron there may still exist a state of *functional* iron deficiency. This phenomenon was highlighted when recombinant erythropoietin entered clinical use for the treatment of renal anaemia. In this situation, despite a normal or raised serum ferritin concentration and stainable iron in the bone marrow, erythropoiesis could nevertheless generally be augmented by IV iron supplementation (Macdougall *et al.* 1989). This observation implied that iron-deficient erythropoiesis (IDE) was present, and that the rate of mobilisation of iron from stores could not keep pace with the demands of the marrow when stimulated by erythropoietin. A very similar situation is seen on ascent to high altitude (Gassmann and Muckenthaler 2015). Finch himself used the term *relative* iron deficiency to describe this situation in the context of haemolytic anaemias (Finch and Huebers 1982).

Another term that is used to describe a setting in which total body iron stores are apparently adequate, but supplies of iron appear scarce, is iron *sequestration* (Goodnough *et al.* 2010). Here the iron seems to be 'locked away' and unavailable for a particular physiological process. This state of affairs is most commonly seen in patients with chronic inflammatory conditions, in whom hepcidin expression is stimulated by inflammatory signals (Thomas *et al.* 2013).

These distinctions may not hold up well in clinical practice. For example, chronic renal disease is often accompanied by significant systemic inflammation, so the iron deficiency seen will rarely be purely functional. Also, as already noted, long-standing upregulation of hepcidin can reasonably be expected to lead to absolute iron deficiency, if duodenal iron uptake is blocked for a sufficient period of time.

1.5.2. Diagnosing iron deficiency

1.5.2.1. Histological assessment of tissue iron content

Direct examination of a sample of bone marrow for stainable iron (haemosiderin) is viewed as the gold standard for the diagnosis of absolute iron deficiency (Zimmermann 2008), but a major downside of this approach is its invasiveness, discomfort and associated risk of complications. Additionally, it has been pointed out that the risk of underestimating iron stores and even falsely reporting absent stainable iron is not insignificant with this approach, due to technical factors (Barron *et al.* 2001, Hughes *et al.* 2004).

When considering studies that have used bone marrow examination to determine iron status, the problem of ascertainment bias arises, since bone marrow will more often have been sampled in individuals suspected of having iron deficiency as a cause of an anaemia, or a haematological disorder of some sort for which examination of the marrow was undertaken in pursuit of a diagnosis. Very few studies provide data on the prevalence of absent bone marrow stainable iron in the population without IDE or IDA, and it does not seem likely that further studies aimed at filling this gap in our knowledge would find many volunteers. Having said this, the studies that have been performed do allow us to say with some confidence how indices obtained from a venous blood sample correlate with iron stores, albeit with this important caveat.

1.5.2.2. Ferritin

Soon after a reliable assay had been developed, it was reported that a serum ferritin concentration < 10 µg/L was associated with a low TSat (< 16%) and IDE (Jacobs *et al.* 1972); this study did not involve any further assessment of body iron stores. Next, it was shown using serial phlebotomies that serum ferritin correlated very strongly with mobilisable iron stores; it was estimated that 1 µg/L serum ferritin represented approximately 8 mg stored iron (Walters *et al.* 1973). Thereafter, a study using radioiron absorption as an index of iron stores reported a very strong inverse correlation between serum ferritin and oral radioiron uptake from the gut (Cook *et al.* 1974).

The conclusions of these three studies were based primarily on observations in healthy individuals. Subsequently, a study of 250 hospitalised patients with anaemia or disordered iron homeostasis, a third of whom underwent bone marrow examination, reported a geometric mean serum ferritin of 4 µg/L in patients with uncomplicated iron deficiency (Lipschitz *et al.* 1974). This study also highlighted the effects of various chronic disease states on serum ferritin, including chronic inflammatory conditions and liver disease, both of which significantly elevated serum ferritin. The authors noted, though, that serum ferritin in patients with these conditions increased progressively with levels of stainable iron – a relationship between bone marrow iron and serum ferritin still persisted (Figure 1-14). In a review of published studies of uncomplicated IDA, including a total of 154 patients, only 2.6% of individuals with ferritin levels greater than 12 µg/L had no stainable iron in the bone marrow (Cook 1982), attesting the extremely high sensitivity of the test in this setting.

Two decades after these studies, a systematic review concluded that serum ferritin determination was an extremely powerful test for the diagnosis of IDA (Guyatt *et al.* 1992). These authors calculated that in patients suspected of having iron deficiency as a cause of

anaemia, a serum ferritin < 15 µg/L increased the likelihood of iron deficiency by a factor of over 50; effectively, a serum ferritin < 15 µg/L could be taken as diagnostic. This work has been highly influential, even being described as a "landmark overview" (Szoke and Panteghini 2012), but it should be pointed out that it addresses the challenge of determining whether the cause of an individual's *anaemia* is iron deficiency, not whether a given individual is iron deficient. Furthermore, the timing of this review was such that it did not examine the diagnostic use of soluble TfR1 (sTfR), which is considered below.

Figure 1-14. Relationship between plasma ferritin and bone marrow iron stores

Data are from bone marrow examinations in 87 patients. Bone marrow haemosiderin was arbitrarily graded as absent, minimal, moderate or increased, for each of three states: normal, inflammation and liver disease. The relationships are similar, but for any given level of marrow iron, the ferritin level is higher in the disease states. Thus, in the setting of inflammation of any cause, determining whether a circulating ferritin within the normal range is indicative of adequate iron stores becomes less straightforward. Reproduced from (Finch *et al.* 1986).

Only two conditions other than iron deficiency are known to lower serum ferritin; hypothyroidism and ascorbate deficiency (Finch *et al.* 1986). Even when present, they do not appear significantly to confound interpretation of serum ferritin (Knovich *et al.* 2009).

1.5.2.3. Transferrin saturation

Given that TSat represents the balance between iron extraction from and delivery to the plasma, it is reasonable to expect that this parameter would have some utility in the diagnosis of iron status. Specifically, one would expect that in the situation where iron stores were deplenished, but uptake by developing erythrocytes and other cells that required iron continued, the TSat would eventually fall. This certainly seems to be the case, with the figure of a TSat < 16% appearing in the early literature on ferritin as indicative of the level at which IDE occurs (Jacobs *et al.* 1972, Walters *et al.* 1973).

A study in patients with IDA, prior to the availability of the ferritin immunoassay, found that a TSat < 16% implied an inadequate supply of iron to the erythroid marrow, associated in time with a hypochromic, microcytic anaemia (Bainton and Finch 1964). One of the few studies that subjected a large number of healthy individuals to bone marrow aspiration and examination was also performed in the pre-ferritin era. This recruited over one hundred female North American college students and found that a low TSat was a good predictor of reduced marrow iron stores, particularly if the value was < 16% on two separate occasions, in which case all individuals studied had absent iron stores (Scott and Pritchard 1967). Having said this, only a third of the individuals without any bone marrow stainable iron were detected on the basis of TSat alone; one might speculate that measurement of ferritin would have identified many of those that measurement of TSat in isolation missed.

That many contemporary clinical guidelines continue to recommend measurement of TSat alongside ferritin in the assessment of iron status has been criticised as lacking a firm evidence base (Szoke and Panteghini 2012). It may be the case that the utility of this composite measure, which depends on serum iron and transferrin levels, is compromised by the long-recognised high within-subject variability in serum iron levels over time (Dale *et al.* 2002). In support of this assertion, some studies have reported that measurement of transferrin alone may be almost as good as ferritin in reflecting iron status (Guyatt 1992, Hawkins 2007). This is perhaps not surprising when one considers that hepatic transferrin synthesis is directly regulated by intracellular iron levels via the IRP-IRE pathway.

1.5.2.4. Soluble transferrin receptor

The pathway by which cells take up transferrin-bound iron via TfR1 is illustrated in Figure 1-11. In the course of the recycling of apotransferrin to the cell surface, a proportion of the TfR1 molecules are lost from the cell membrane. These can be detected in a freely circulating form, sTfR, levels of which were reported to increase early in the course of tissue iron deficiency (Skikne *et al.* 1990), presumably in proportion to the level of tissue expression. Since the release of sTfR is not increased by inflammation, it was hoped that circulating levels could be exploited to identify iron deficiency in settings where other markers no longer accurately reflect iron stores (Ferguson *et al.* 1992). However, the clinical role of sTfR has not yet been clearly established (Mast *et al.* 1998, Siebert *et al.* 2003, Koulaouzidis *et al.* 2009, Infusino *et al.* 2012). sTfR is probably best understood as an index of the balance between iron supply and demand in the marrow (Siebert *et al.* 2003). Importantly, pathologies that suppress marrow activity will lead to a low sTfR, and those that greatly stimulate that marrow a high sTfR, independent of the sufficiency or otherwise of the iron supply for erythropoiesis.

1.5.2.5. Hepcidin

At present, measurement of plasma hepcidin is not in routine clinical use, and a single diagnostic assay standard has not been established (van der Vorm *et al.* 2016). Instead, hepcidin is used as an experimental tool, and in the investigation by specialist centres of rare heritable anaemias, such as iron-refractory iron-deficiency anaemia (Camaschella 2015). Whether measurement of hepcidin adds significantly to the information provided by routine determination of serum ferritin will be considered in Chapter 5.

1.5.2.6. Haemoglobin and red cell indices

Given that formation of new red blood cells is dependent on iron supply, it might be supposed that measurement of [Hb] would provide a convenient way of assessing iron status. From what has gone before it will be clear that this is unlikely to be the case, for several reasons. First, a similar fall in [Hb] could result from either a reduction in the supply of iron to the marrow despite plentiful iron stores or from exhaustion of the stores themselves. Secondly, [Hb] will fall in an individual with acute blood loss without any immediate accompanying change in storage iron. Thirdly, [Hb] might not be low in situations where erythropoiesis is stimulated, for example by hypobaric hypoxia, despite limited iron stores. Fourthly, a fall in [Hb] could be brought about by deficiency of some other factor (such as vitamin B12 or folic acid) or indeed marrow failure of any aetiology. Again, this issue has been recognised for some decades:

> *"Iron deficiency has only a low correlation with haemoglobin concentration and it is commonly found in subjects with a normal haemoglobin concentration by conventional standards."*
> *(Jacobs et al. 1969)*

It is therefore regrettable that examples of confused thinking on this issue are to be found relatively easily in the contemporary literature, for example:

> *"As almost two thirds of the iron in the human body is found in haemoglobin, low haemoglobin means low available iron."*
> *(Lee et al. 2011)*

Though absolute [Hb] may be of little use in determining iron status, various other parameters exist that may be of assistance. A low mean cell volume (MCV) or mean cell haemoglobin concentration are both indicators of IDE, but being derived from the entire circulating red cell mass, they change slowly, and neither is capable of differentiating between absolute and functional iron deficiency (Thomas *et al.* 2013).

1.5.3. A clinical perspective on iron deficiency

> *"Why, then, is the human race beset by such difficulties in maintaining iron balance? Why are there estimated to be more than 500 million persons throughout the world with iron deficiency, and perhaps 20 million in this country? Why are we also the only species with parenchymal iron overload sufficient to produce tissue damage? The apparent explanation for these difficulties is our uniquely limited iron absorption and excretion." (Finch and Huebers 1982)*

The World Health Organisation considers iron deficiency to be the underlying aetiology in the vast majority of the two billion individuals worldwide today suffering from anaemia (World Health Organisation 2016). A state exists in individuals in negative iron balance where iron stores are depleted but significant anaemia has not yet developed; Finch would have called this simply *iron deficiency*, today the condition is sometimes referred to as non-anaemic iron deficiency (NAID) or iron deficiency without anaemia (IDWA). It follows that NAID must be even more common globally than IDA. That is, more than a third of the

world's population must be iron deficient. If correct, this is an astonishingly high prevalence.

The most recent British Society for Gastroenterology guidance on IDA acknowledges that despite the high prevalence of NAID – perhaps three times that of IDA in westernised countries – there is no consensus or high grade evidence regarding how the condition should be investigated (Goddard *et al.* 2011). Notably, the emphasis in these guidelines is very much on the appropriateness of excluding a sinister underlying cause for iron deficiency, rather than addressing the pathophysiological consequences that arise as a result in these iron-deficient individuals.

1.6. Aim of this thesis

The aim of this thesis is to determine whether the interactions described between iron homeostasis and oxygen sensing translate into a significant effect of iron deficiency on human physiology. This will be achieved using two separate interventional physiology studies in healthy humans. *Study A* examines the effect of iron deficiency on the pulmonary vascular response to hypoxia. *Study B* explores the consequences for skeletal muscle metabolism of iron deficiency. In both cases, IV infusion of iron is used as a device to provide further evidence with respect to the role of iron bioavailability. Finally, *Study C*, a cross-sectional observational study in patients with chronic obstructive pulmonary disease (COPD), investigates the role of iron deficiency in a chronic inflammatory condition in which hypoxia, pulmonary vascular disease and skeletal muscle dysfunction are all features.

Chapter 2. Iron status and hypoxic pulmonary vascular responses

2.1. Introduction

This chapter will explore the consequences of iron deficiency for the behaviour of the pulmonary vasculature during hypoxia. At one level, an argument could be made for viewing the pulmonary circulation simply as a highly hypoxia-responsive system that depends to a great extent on the HIF pathway, which is exploited to provide a convenient biological index of the effects of iron on hypoxia sensing and signalling. However, there are several objections to this approach, not least that it would ignore much about the physiology of human gas exchange that is of interest in its own right and, moreover, important when considering the clinical implications of this work. Also, the HIF pathway is not the sole possible target for an action of variations in iron bioavailability on pulmonary physiology. Additionally, there is much of interest in a historical perspective on work

examining the pulmonary circulation and oxygen sensing; it is, after all, some small continuation of these efforts that the work reported herein represents.

Accordingly, this chapter begins with an overview of the relevant anatomy and physiology, with a specific focus on HPV, the role of the HIF pathway in the pulmonary circulation, and what is known from previous pulmonary studies including those involving iron chelation and supplementation.

2.1.1. Basic anatomy and evolutionary considerations

The lungs are generally not well conserved in fossilised organisms, which leads to considerable uncertainty about how exactly the human lung as we know it today arose, and makes necessary a comparative anatomical approach with other living species (Farmer 1999). The mammalian lung may represent a highly evolved form of the gasbladder of primitive fish; alternatively, the swimbladder and lungs could represent two divergent paths for adaptation of a single common primitive organ arising from the gut, depending on the particular environmental demands (Liem 1988). An aqueous environment prone to drought has been postulated as the factor exerting the selection pressure to confer a survival advantage on organisms able to utilise atmospheric oxygen (Barrell 1916).

A putative sequence for the evolution of air-breathing organs in vertebrates envisages 'breathing' as preceding the emergence of a lung as it would be recognised today (Perry *et al.* 2001). In this model, a central nervous oscillator of some sort initially drives a suitable motor pattern, with subsequent development of gas-exchange surfaces and an appropriate blood supply; ultimately a cyclical respiratory mechanism emerges. However the current arrangement was reached, the requirement for a very large gas exchange surface providing contact between the air and blood leads to the basic structure of the human lung resembling

three well-matched interdigitating trees – airways, arteries and veins – with gas-exchange units surrounding terminal portions of an extensively branched airway tree (Liem 1988) (Figure 2-1).

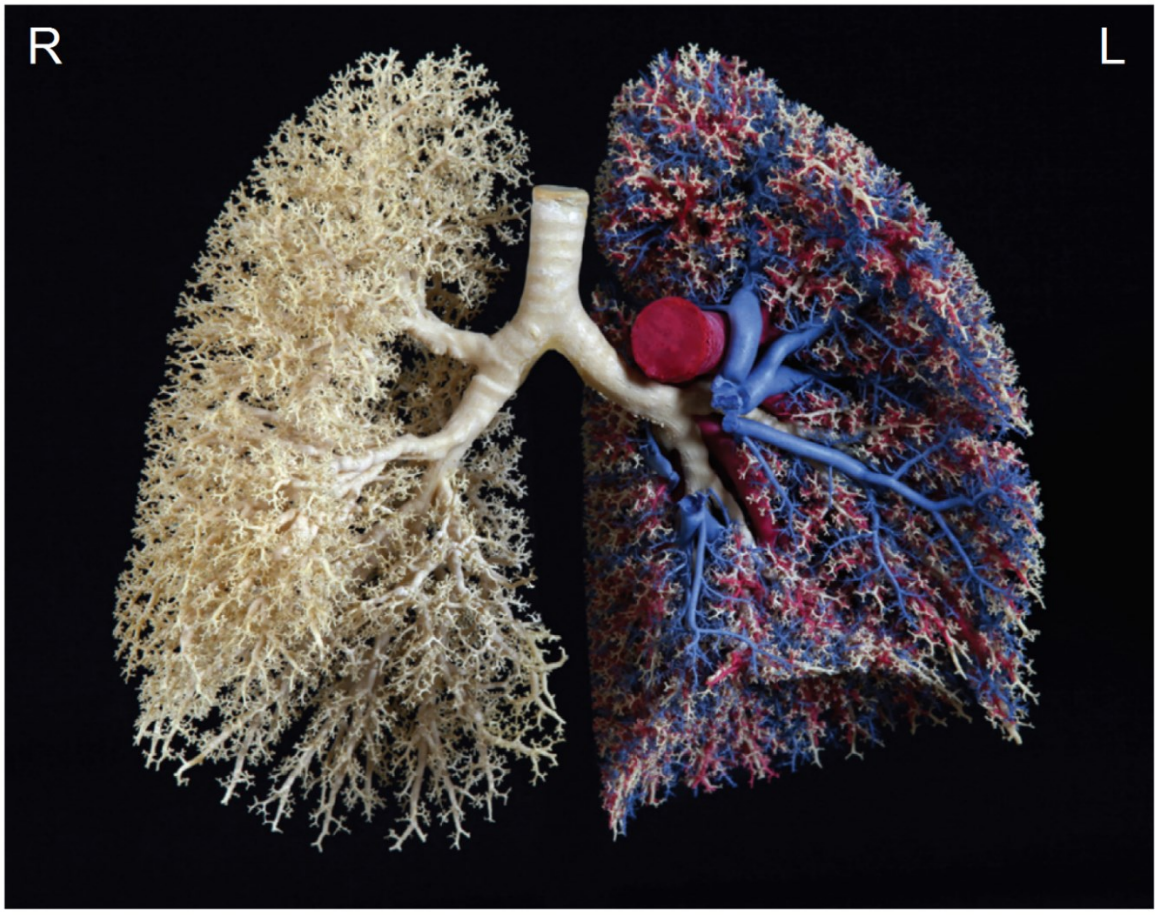

Figure 2-1. Post mortem cast of the human tracheobronchial tree

Airways are shown in beige, pulmonary arteries in red, and pulmonary veins in blue. Physiologically speaking, these last two colours ought to be reversed. On the right, only the airways are shown. Modified from (Hsia *et al.* 2016). Copyright© American Physiological Society; reproduced with permission of John Wiley and Sons Limited (Journals) via PLSClear.

The challenges posed by this arrangement, in comparison to the situation that is to be found in the avian lung, have been highlighted by West:

> *"A major difference is that the ventilation of the gas-exchanging tissue has a flow-through pattern in the bird but is reciprocating in the mammal. The result is that mammals have a reduced alveolar and arterial oxygen tension, a potential for uneven ventilation, and relatively large terminal air spaces…This in turn means that the pulmonary capillaries are poorly supported compared with the bird…One of the most fundamental differences between the structure of bird and mammalian lung is the separation of the gas-exchange function from the ventilatory function in the former…The bioengineering requirements of tissues for these two functions are so dissimilar that it is truly remarkable that the mammalian lung has combined the two functions." (West et al. 2007)*

In terms of the pulmonary capillaries, therefore, it is clear that there are two competing requirements. They need to be very thin so as not to impede gas exchange with their adjacent alveoli, but their walls must at the same time be strong enough to avoid stress failure. Thus, an important vertebrate developmental advance was the separation of the pulmonary circulation from the systemic circulation, such that the two could operate at different pressures (West 2011b). In this way the pulmonary circulation is, in health, protected from high capillary wall stress.

2.1.2. Hypoxic pulmonary vasoconstriction: a brief history

It might be anticipated that the lung, as the primary organ of gas exchange, would somehow be constructed so as to optimise the matching of pulmonary perfusion ($\dot{Q}$) with alveolar ventilation ($\dot{V}_A$). That is, there should be some means of signalling that pulmonary blood flow might usefully be directed away from poorly ventilated, poorly oxygenated alveoli,

towards other regions of the lung. Nearly a century ago, J. S. Haldane recognised that the lungs ought to employ such a mechanism to match delivery of deoxygenated blood to well-ventilated lung regions:

> *"It seems probable that by some means at present unknown to us a fair adjustment is maintained normally between air supply and blood supply." (Haldane 1922)*

Interest in measuring intravascular pressures in live animals had existed for some time prior to Haldane's suggestion, and it seems likely that the first measurements of pulmonary arterial pressure (PAP) were made in the cat, dog and rabbit in the middle of the previous century (Beutner 1850). Almost a hundred years afterwards, the specific effects of hypoxia (and indeed hypercapnia) on the pulmonary circulation in anaesthetised cats were reported by von Euler and Liljestrand. These investigators concluded that:

> *"The experiments seem to warrant the conclusion, that the regulation of the pulmonary blood flow is mainly mediated by a local action of the blood and alveolar gases leading to an adequate distribution of the blood through the various parts of the lungs according to the efficiency of aeration." (von Euler and Liljestrand 1946)*

In fact, the tendency of pulmonary vessels to vasoconstrict in response to hypoxia had probably been reported much earlier, but by investigators whose primary interest lay in the innervation of the pulmonary circulation (Bradford and Dean 1894). The behaviour of the canine pulmonary and systemic circulations during asphyxia, particularly as the point of cardiac arrest was neared, led to the following insightful observations:

> *"…a greater effect is obtained on the pulmonary circulation by asphyxia than by any other mode of excitation…We think that…the rise of pressure in the pulmonary artery during asphyxia is of mixed origin. In the first place there is an active constriction of the pulmonary vessels producing an early rise of pressure synchronous with the accompanying systemic rise. The persistent and final pulmonary rise has however a different origin, being produced mechanically by a transmission (back through the heart cavities) of the increased systemic tension, owing probably to dilatation and failure of the left ventricle, so that the blood is no longer pumped out into the aorta but accumulates in the heart cavities, and more especially on the right side."*
> *(Bradford and Dean 1894)*

Whilst it is true that asphyxia will raise alveolar carbon dioxide tension, so the stimulus in these experiments will not have been pure alveolar hypoxia, it is clear that this early discussion not only correctly recognises the existence of HPV in all but name, but also raises the important issue of the pressure in the right heart reflecting not only the behaviour of the pulmonary vasculature but also that of the left heart.

Fifty years after the work of Bradford and Dean, Beyne produced a brief report of observations in anaesthetised dogs (Beyne 1942), which were broadly similar. Following this, and what is now generally considered to be the definitive report of HPV by von Euler and Liljestrand, there was something of a rekindling of interest in the field (Fishman 1976a), and others sought to characterise the hypoxic behaviour of the pulmonary circulation in humans. First, pulmonary haemodynamics were assessed using right heart catheterisation (RHC) in five healthy unanaesthetised males breathing 10% oxygen in nitrogen for around 15-20 minutes (Motley *et al.* 1947). A rise in PAP was seen to be induced quickly, with only a small concomitant rise in systemic arterial pressure; PAP rapidly returned to normal on resumption of air breathing. However, direct measurements

of pulmonary vascular resistance (PVR) were not made in this study, which others took to preclude the conclusion that pulmonary vasoconstriction was definitely the cause of the rise in PAP. Subsequently, this was confirmed to be the case in a similarly invasive study of 32 individuals, this time sedated, a subset of whom had chronic cardiopulmonary diseases of various sorts (Westcott *et al.* 1951). This study was additionally notable for the demonstration that inhalation of pure oxygen in subjects with pulmonary disease and elevated PAP when breathing air, led to a fall in mean PAP (mPAP). This prompted the observation that:

> *"…anoxic elevation of pulmonary arteriolar resistance may be a contributing and reversible factor in the production and maintenance of at least some types of chronic pulmonary hypertension."*
> *(Westcott et al. 1951)*

In the time since these seminal studies, a huge amount of research effort has been directed towards understanding HPV. A review only a few years ago on the topic (Sylvester *et al.* 2012) cited over two thousand references, and it has been estimated that there are well in excess of three thousand publications on the subject (Swenson 2013). Despite this body of work, there is still much that is not well understood. Debate continues not only about the cellular mechanisms underlying the process, as already discussed, but also about its significance for human health and disease.

2.1.3. Characteristics of hypoxic pulmonary vasoconstriction

2.1.3.1. Site of HPV

Much effort has been directed at determining the anatomical location of HPV. Early work suggested that the small muscular pulmonary arteries of diameter < 500 μm were the main site of resistance in the pulmonary circulation, and HPV (Kato and Staub 1966, Bergofsky

et al. 1968, Hakim *et al.* 1982, Marshall and Marshall 1983). Subsequently, cultured PASMCs were shown to be intrinsically responsive to hypoxia, contracting and relaxing with variations in oxygen tension (Murray *et al.* 1990, Madden *et al.* 1992). The larger pulmonary arteries also exhibit HPV, but it is thought that this is not manifest in intact animals owing to the supply of bronchial arterial blood – at systemic arterial P_{O_2} – that reaches these larger vessels via their *vasa vasorum*. As a result, concomitant systemic arterial hypoxaemia is required for HPV to be clearly evident in these vessels (Heistad *et al.* 1986, Marshall *et al.* 1991, Marshall *et al.* 1994c). Though PASMCs are themselves directly responsive to hypoxia, it appears that the pulmonary vascular endothelium plays an important role in modulating HPV. Isolated pulmonary artery preparations from several species show significant impairment of HPV if denuded (Dipp and Evans 2001, Liu *et al.* 2001, Robertson *et al.* 2003).

2.1.3.2. Stimulus for HPV

A further question relates to what it is that is actually sensed to bring about vasoconstriction. Is alveolar P_{O_2} the key stimulus or does the oxygen tension of mixed venous blood contribute? Careful experiments in animals, varying independently the oxygen tension of perfusate and alveolar gas, have suggested that the contribution from the mixed venous blood is ~ 40% and that from the alveolar gas ~ 60% (Marshall and Marshall 1988, Marshall *et al.* 1994a). It is important not to overlook this contribution made by the mixed venous oxygen saturation (S_{vO_2}). For example, in physiological studies where alveolar hypoxia is induced, the systemic arterial hypoxaemia that results may lead to a lower S_{vO_2}, which will influence HPV in its own right. Equally, a state not associated with alveolar hypoxia but that tends to reduce S_{vO_2} as a consequence of increased oxygen extraction by the tissues, such as exercise, will stimulate HPV.

2.1.3.3. Quantification of HPV in human physiological studies

Before considering further what is known of HPV in adult humans we must address one question that arises surrounding the assessment of the process: which variable ought to be measured when quantifying HPV? Is it more appropriate to measure PAP, or is PVR preferable? Neither of these variables is a direct measure of pulmonary vascular smooth muscle tone, which is arguably of most interest physiologically. An added complication is that the change in cardiac output (CO) that occurs as a result of any arterial hypoxaemia induced by alveolar hypoxia, may affect both PAP and PVR directly.

These issues have been addressed in a collection of studies using Doppler echocardiography simultaneously to measure pulmonary artery systolic pressure (PASP) and CO (Balanos *et al.* 2002, Balanos *et al.* 2005, Croft *et al.* 2013). The argument, in essence, is as follows. The pulmonary circulation is known to be remarkable in its ability to accommodate a several-fold increase in CO during exercise without any great change in the arteriovenous pressure difference across the lung. It must therefore be the case that PVR varies greatly as a function of CO; this will tend to limit the usefulness of PVR as an index of primary changes in pulmonary vascular smooth muscle activity in this setting. In contrast, changes in PASP during hypoxia (ΔPASP) appear to be much less affected by concomitant variations in CO, with an estimate of < 0.7 mmHg/L/min as the increase in PASP attributable to the rise in CO that occurs during alveolar hypoxia (Balanos *et al.* 2005, Croft *et al.* 2013). Thus, ΔPASP provides a more convenient and reliable index of HPV.

The gold-standard technique for investigating pulmonary haemodynamics is pulmonary artery catheterisation (Swan *et al.* 1970). Though this approach undoubtedly plays a key role in the clinical assessment of patients with pulmonary hypertension (PH) and right heart disease, and is appropriately used in such individuals in the setting of clinical trials (Howard

et al. 2013), the associated complications make it unattractive as a technique for use in contemporary physiology studies of healthy volunteers.

Much recent work examining the pulmonary circulation during hypoxia has therefore employed transthoracic echocardiography, which is non-invasive and gives measurements that correlate very closely with those obtained from RHC (Yock and Popp 1984, Currie *et al.* 1985, Skjaerpe and Hatle 1986, Stevenson 1989, Peacock *et al.* 1990, Allemann *et al.* 2000). One widely used technique involves measuring the maximum velocity of a small regurgitant jet of blood through the tricuspid valve, which occurs during systole in the majority of individuals. Using Bernoulli's equation, this velocity (*v),* measured in metres per second, can be used in conjunction with the density of blood (ρ), which is taken as 1.06 x 10^3 kg/m^3, to estimate the pressure difference (ΔP_{MAX}), measured in mmHg, between the right ventricle and right atrium:

$$\Delta P_{MAX} = \rho v^2 / 2$$

This equation lacks some of the terms to be found in that originally described by Bernoulli. Flow acceleration is omitted since it is zero at the moment of peak flow, and viscous friction is ignored since it does not appear to compromise the accuracy of the technique in practice (Skjaerpe and Hatle 1986). If we take 1 mmHg = 133.3 N/m^2, it follows that:

$$\Delta P_{MAX} = (1.06 \times 10^3 \text{ kg/m}^3) \cdot v(\text{m/s})^2 / (2 \times 133.3 \text{ kg/m}^2)$$

This simplifies to:

$$\Delta P_{MAX} = 4 \cdot v^2$$

Since right atrial pressure (RAP) is in healthy individuals not significantly altered by hypoxia (Groves *et al.* 1987), ΔP_{MAX} itself offers a convenient index of HPV, though an estimated RAP may be added to give PASP if so desired (Bossone *et al.* 1999, Lam *et al.* 2009).

Here a brief note on units of measurement for pressure is appropriate. In 1644, Evangelista Torricelli (1608-1647), an Italian physicist and mathematician, described the principle of the barometer in a letter to his friend Michelangelo Ricci, who was a mathematician and cardinal in Rome (West 2013). Torricelli recognised that atmospheric air had weight, and that it was most dense near to the Earth's surface. Historically, standard atmospheric pressure was taken to be 760 mmHg; the eponymous *Torr* was defined as a unit of pressure equal to one mmHg.

In 1954 the definition of the atmosphere was revised in Paris at the *Dixième Conférence Générale des Poids et Mesures* to the currently accepted standard: one atmosphere equals 101.325 kPa (CGPM 1954), and a Torr was then redefined accordingly as 1/760 of an atmosphere. This momentous decision has had the important consequence that 1 mmHg today equals 1.00000015001 Torr, so it is quite wrong to use Torr and mmHg interchangeably, as one could happily have done in the early part of the 20th Century. This thesis will generally use mmHg, since although it is not an SI unit, it still dominates the relevant literature. One kPa is equal to 7.5006157584 mmHg.

2.1.3.4. Time course of human HPV

The effects of changes in alveolar gas tensions on the pulmonary vasculature may be conveniently studied in awake humans using either an end-tidal forcing system (Robbins *et al.* 1982), where the participant breathes through a mouthpiece and concentrations of

inspired gas can be varied precisely breath-by-breath, or, for longer studies, a normobaric chamber (Howard *et al.* 1995). Figure 2-2A shows the time course of the rise in PASP in response to a sustained period of alveolar hypoxia in a normobaric chamber, characterised using pulmonary artery catheterisation (Dorrington *et al.* 1997). It is apparent that the pulmonary vasculature has been changed in some way as a consequence of this sustained hypoxic stimulus, since if a further hypoxic challenge is delivered subsequently during the period for which euoxic PASP remains elevated, the resulting acute response is more marked. This phenomenon is termed acclimatisation.

Further temporal resolution of the early stages of human HPV has been provided using a dynamic end-tidal forcing system and transthoracic echocardiography (Talbot *et al.* 2005). With the onset of alveolar hypoxia, a first phase of HPV is rapidly detectable and becomes maximal within a few minutes (Figure 2-2B); following this initial brisk response, a second phase can be demonstrated after approximately 40 minutes (Figure 2-2C). If the pulmonary vasculature is exposed to a brief hypoxic stimulus, less than or equal to the duration of the first phase of HPV (Figure 2-2B), then on return to euoxia, PASP quickly returns to pre-exposure levels. If, though, the hypoxic stimulus continues such that the second phase of HPV is manifest (Figure 2-2A&C), then it takes considerably longer for PASP to fall back to baseline levels (Dorrington *et al.* 1997, Balanos *et al.* 2002, Talbot *et al.* 2005, Smith *et al.* 2008a). The magnitude of this effect depends on the duration of hypoxia; when this extends beyond hours into days and weeks, pulmonary vascular remodelling occurs, such that inhalation of supplemental oxygen has little effect (Groves *et al.* 1987). The PH associated with prolonged residence at altitude can take months to years to reverse with descent to sea level, and augmentation of HPV may never completely resolve in this situation (Sime *et al.* 1971).

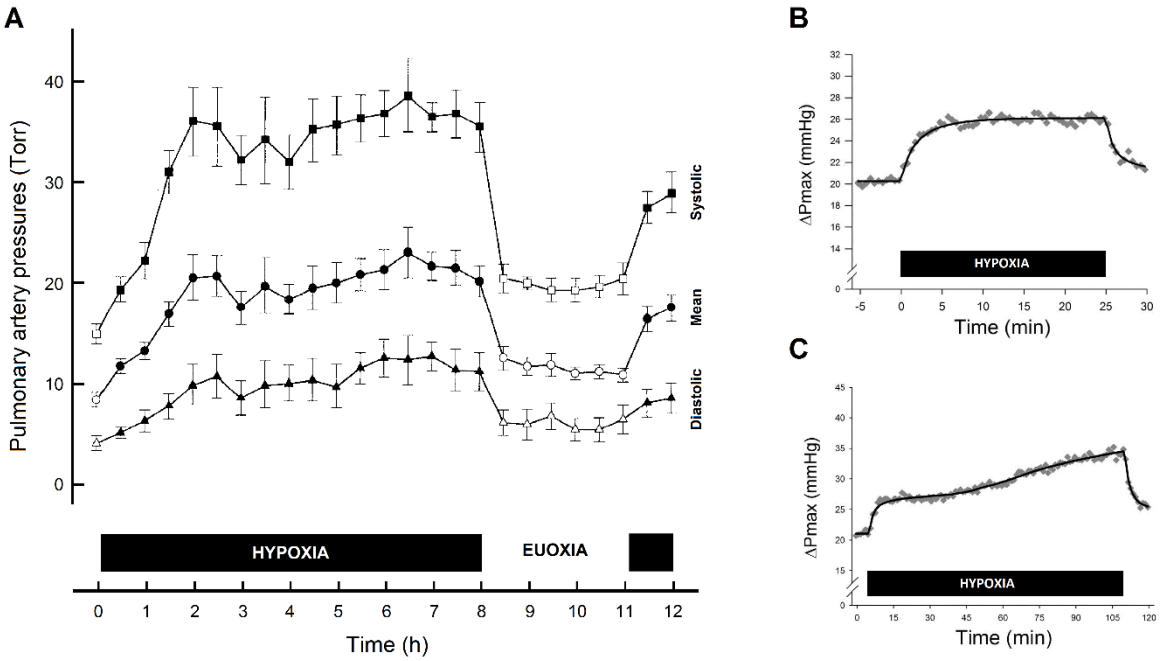

Figure 2-2. Time course of hypoxic pulmonary vasoconstriction in conscious humans

(A) Rise in PAP during an 8-hour exposure to alveolar hypoxia measured using pulmonary artery catheterisation in 6 healthy male volunteers. Open symbols indicate measurements in euoxia, filled symbols measurements in hypoxia. On return to euoxia at the 8-hour time point, PAP initially remains elevated; if a further short hypoxic challenge is given, the acute rise in PAP is considerably brisker than that observed at the beginning of the experiment. (B) Rise in ΔP_{MAX} during, and rapid return towards pre-hypoxia levels after, a 25-minute exposure to alveolar hypoxia in a group of 12 healthy individuals (6 males, 6 females). (C) Second phase of HPV seen to occur around 40 minutes into a 105-minute period of alveolar hypoxia in a group of 7 healthy individuals (5 males, 2 females). It can be seen that, in contrast to the brief hypoxic exposure in (B), ΔP_{MAX} remains elevated after cessation of hypoxia. Measurements in (B) and (C) were made using Doppler echocardiography. Each hypoxic exposure was isocapnic with an end-tidal oxygen tension ($P_{ET}O_2$) = 50 Torr. Adapted from (Dorrington *et al.* 1997) and (Talbot *et al.* 2005).

2.1.4. Hypoxic pulmonary vasoconstriction in health and disease

Figure 2-3 illustrates the regional variation in ventilation and perfusion within the human lung. It can be seen that there is approximately a nine-fold difference in perfusion between the apex and the base; the corresponding difference in regional ventilation is less marked. It is thought that much of the regional variation in perfusion is accounted for by structural aspects of the branching pulmonary vasculature and by gravity (West *et al.* 1964, Glenny *et al.* 1999, Burrowes and Tawhai 2006, Galvin *et al.* 2007).

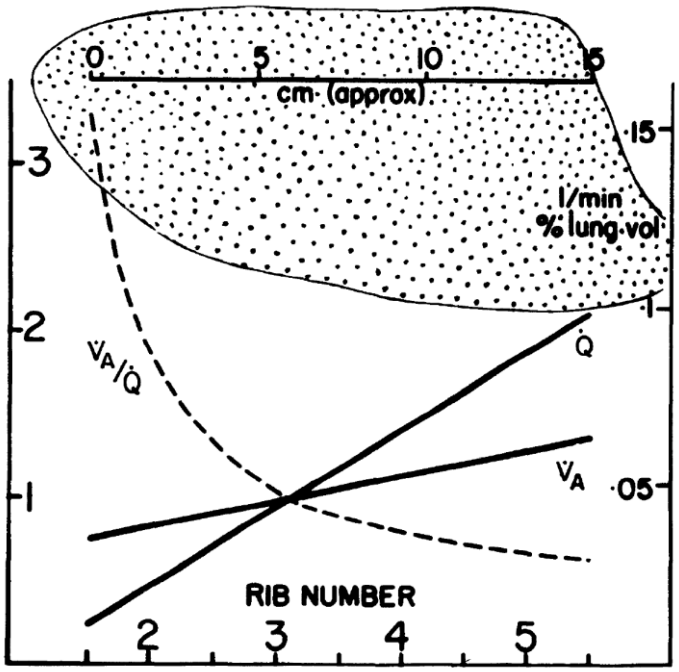

Figure 2-3. Regional variation in ventilation and perfusion at rest within the erect human lung

The data used to determine these relationships were derived from experiments in 16 healthy individuals using carbon dioxide radiolabelled with ^{15}O, and an assumed CO of 6 L/min. $\dot{Q}$ and $\dot{V}_A$ are illustrated by solid black lines, to which the scale on the right refers (L/min % lung volume). Corresponding $\dot{V}_A/\dot{Q}$ values are shown by the dashed line, to which the scale on the left refers. The dotted area represents a coronal plane though the lung to give an indication of the proportion of lung tissue operating at a given ratio. Reproduced from (West 1962).

There has been understandable interest in whether HPV plays a role in determining regional pulmonary blood flow at rest in healthy humans, but the evidence is conflicting. For example, Arai *et al.*, using pulmonary magnetic resonance imaging (MRI) and inhalation of a hyperoxic gas mixture, reported that HPV did not seem to contribute to pulmonary blood flow heterogeneity in euoxia in healthy supine humans (Arai *et al.* 2009). Subsequently, this same group apparently reached the opposite conclusion, having modified their experimental technique to include the use of inhaled nitric oxide (iNO) (Asadi *et al.* 2015).

Using the concept of the "efficiency of regulation" (the percent correction to an initial perturbation in regional alveolar gas composition generated by the pulmonary vascular response to this disturbance), Dorrington *et al.* found that pulmonary vascular responses to both CO_2 and O_2 played a substantial role in $\dot{V}_A/\dot{Q}$ matching in the healthy human lung (Dorrington *et al.* 2010). Furthermore, these investigators were able to demonstrate that HPV was the dominant mechanism operating at low $\dot{V}_A/\dot{Q}$, with responses to CO_2 prevailing at high ratios.

Until now very little has been said regarding the effects of CO_2 on the pulmonary vasculature. The use of isocapnic conditions in experiments makes it possible to study the direct effect of hypoxia without a confounding influence of changes in CO_2 tension, which inevitably occur as a consequence of hypoxia-driven increases in alveolar ventilation. It is important to mention, though, that the effects of CO_2 are, as would be expected, the opposite of those of O_2; hypercapnia promotes pulmonary vasoconstriction, and hypocapnia, vasodilatation (Dorrington and Talbot 2004). The effects of CO_2 appear to be additive to, rather than synergistic with, those of O_2 (Croft *et al.* 2013).

Of interest from a clinical perspective are situations in which there is significant alveolar hypoxia, be it regional or global. Global alveolar hypoxia inevitably arises in the healthy lung on ascent to high altitude as a consequence of the fall in barometric pressure (West *et al.* 1983). Since in this situation the alveolar oxygen tension falls considerably throughout both lungs, it is difficult to see how any HPV occurring in response could ameliorate the problem:

> *"Hypoxic pulmonary vasoconstriction appears to be a disadvantage in a number of situations, particularly ascent to high altitude when the vasoconstriction causes pulmonary hypertension, which serves no useful purpose." (West 2011b)*

In the case of regional alveolar hypoxia, perhaps the most striking illustration of the degree to which HPV operates to match $\dot{Q}$ with $\dot{V}_A$ comes from cases involving patients suffering from physical obstruction of either of the main bronchi, such that ventilation of an entire lung is almost abolished (Figure 2-4).

Morrell *et al.* investigated the effect of alveolar hypoxia limited to a single lobe in healthy humans undergoing bronchoscopy with local anaesthesia, and attempted to measure the time course and magnitude of the resultant blood flow diversion (Morrell *et al.* 1995). During lobar hypoxia (mean P_AO_2 = 38 mmHg), which was induced using a catheter-tipped balloon in the left upper lobe bronchus, blood flow to the occluded lobe fell to 53% of baseline values after 5 minutes. The degree to which such a response tends to correct the arterial hypoxaemia that would otherwise result from continued perfusion of a significant portion of unventilated lung, is shown nicely by experiments inducing sudden unilateral alveolar hypoxia in anaesthetised rabbits (Figure 2-5).

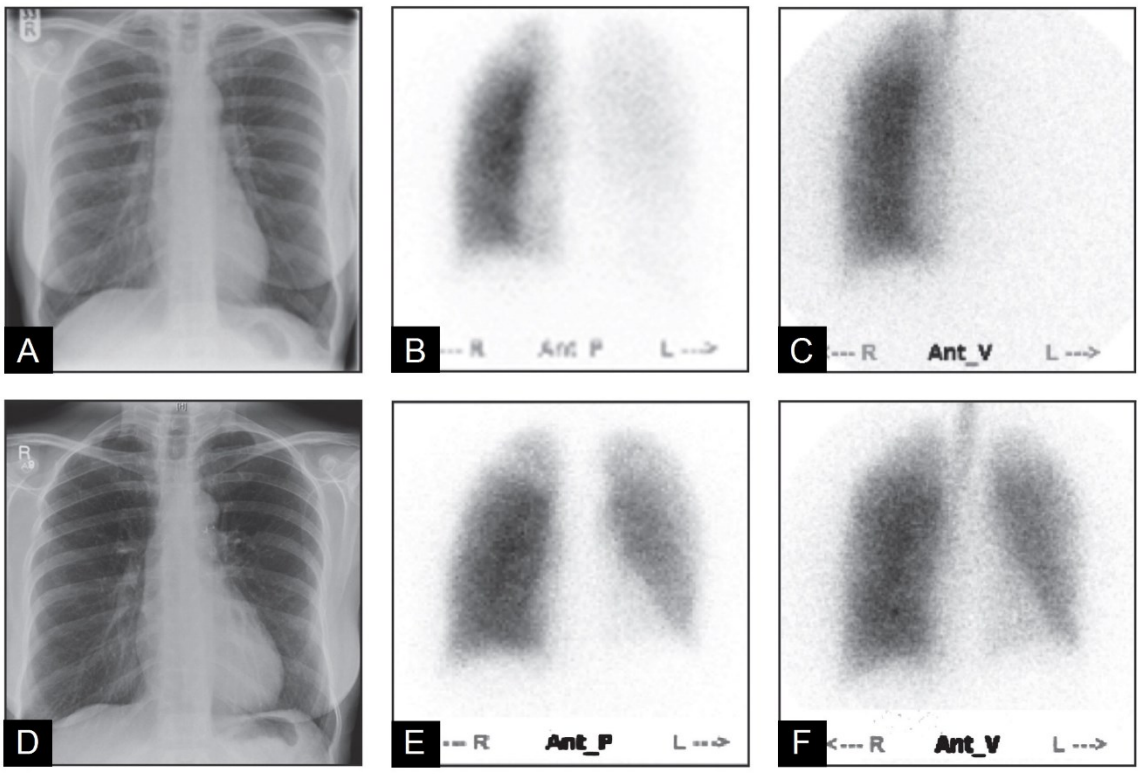

Figure 2-4. Radiological demonstration of the efficacy of hypoxic pulmonary vasoconstriction

A 50-year-old female patient with dyspnoea was found to have a carcinoid tumour of the left main bronchus, which was surgically resected. (A) Unremarkable posteroanterior (PA) chest radiograph at presentation, at which time S_pO_2 was > 95% whilst breathing air. (B) Perfusion scintigraphy at presentation demonstrating near-absent perfusion of left lung. (C) Ventilation scintigraphy at presentation demonstrating absent ventilation of left lung. (D) PA chest radiograph showing essentially normal appearances following tumour resection, save the surgical clips visible at the left hilum. (E) Corresponding perfusion and (F) ventilation scintigraphy after resection showing return of ventilation and thus perfusion of the left lung. Adapted from (Jafri *et al.* 2011) under Creative Commons license (CC BY-NC-SA 3.0).

One situation in which such marked mismatch of perfusion and ventilation of this sort occurs acutely in humans is one-lung anaesthesia for thoracic surgery, during which collapse of one lung may lead to a shunt of ~ 50%. Marshall and colleagues modelled the effects of HPV with varying degrees of lung atelectasis and found that with a degree of

shunting commensurate with that seen in one-lung anaesthesia, HPV was capable of elevating P_{aO_2} between 20 and 40 mmHg over the range of inspired oxygen fraction 0.3-1.0 (Marshall *et al.* 1994b).

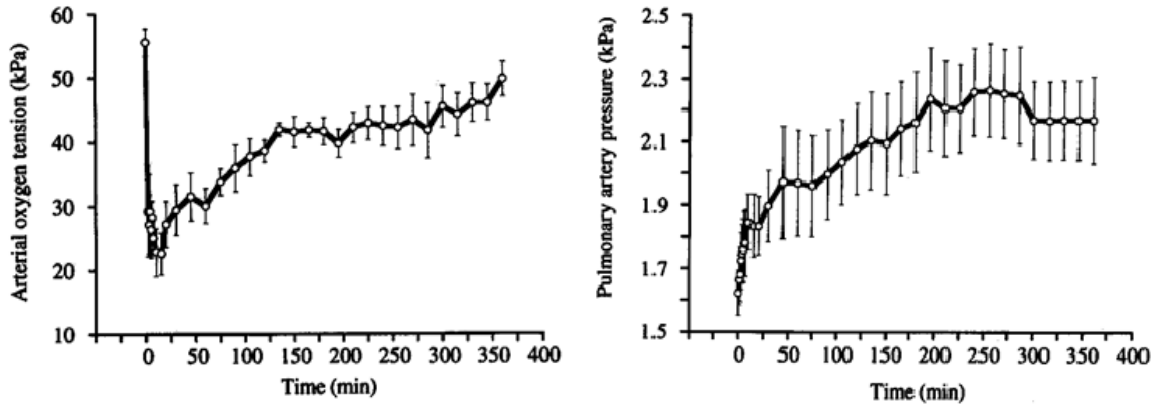

Figure 2-5. Time course of arterial hypoxaemia and PAP elevation with one-lung ventilation

Data are for six anaesthetised rabbits mechanically ventilated with oxygen, before abolition of ventilation of one lung by liquid instillation. *Left:* After six hours the arterial P_{O_2} has almost recovered to initial levels. *Right:* A progressive increase in PAP is evident – an initial brisk rise followed by a more gradual climb over several hours – reflecting the two phases of HPV already discussed. Modified from (Vejlstrup and Dorrington 1993) with permission.

In most clinical settings $\dot{V}_A/\dot{Q}$ inequality is much more heterogeneously distributed than in the situations considered thus far, and the lungs may themselves be diffusely diseased; however, HPV is no less important in such situations. In patients with respiratory failure as a consequence of COPD or acute respiratory distress syndrome (ARDS), for example, it has been estimated that HPV may act to elevate P_{aO_2} by as much as 20 mmHg (Brimioulle *et al.* 1996, Naeije and Brimioulle 2001). $\dot{V}_A/\dot{Q}$ matching in COPD is considered at length

in Chapter 5, but a brief review of experiments in patients with ARDS, using agents that relax PASMCs, will usefully illustrate some points of physiological importance.

In one such study, IV administration of diltiazem, a non-dihydropyridine calcium channel blocker, resulted in a significant fall in arterial oxygen saturation in patients with ARDS, accompanied by a decrease in PVR but no change in CO (Melot *et al.* 1987). Likewise, though PH was ameliorated by prostaglandin E1 infusion in a similar group of patients, this was at the expense of worsening pulmonary gas exchange (Melot *et al.* 1989). Comparable findings were evident with nitroglycerine infusion (Radermacher *et al.* 1989). Thus, indiscriminate pulmonary vasodilatation is unhelpful in ARDS.

However, if agents that promote pulmonary vasodilatation are delivered by *inhalation* during mechanical ventilation for ARDS, P_aO_2 tends to increase. For example, iNO has been shown to reduce PAP and improve arterial oxygenation in patients with severe ARDS (Rossaint *et al.* 1993, McIntyre *et al.* 1995). The explanation is presumably that via this route, the agents are delivered preferentially to well-ventilated lung units – those to which it is useful to increase perfusion. As an aside, it is disappointing to note that this effect has not been shown to translate into a mortality benefit in ARDS (Afshari *et al.* 2010, Adhikari *et al.* 2014), implying that in such patients, mortality is not simply a direct consequence of hypoxaemia.

2.1.5. The HIF pathway and HPV

At the simplest level, HPV involves inhibition of oxygen-sensitive potassium channels, depolarisation of PASMCs, and calcium influx through voltage-gated calcium channels causing vasoconstriction (Lumb and Slinger 2015). Though the first phase of HPV, mediated by these mechanisms, occurs very rapidly indeed, the second phase takes longer

to become evident, is slowly progressive, and brings with it a transient change in the behaviour of the pulmonary circulation – acclimatisation. These features suggest that alterations in gene expression are likely to be important during this component of the pulmonary vascular response to hypoxia. An obvious candidate for orchestrating at least some of this effect is HIF (Shimoda and Laurie 2014).

2.1.5.1. Chuvash polycythaemia and other diseases of oxygen sensing

A rare human genetic disorder, Chuvash polycythaemia (CP), provides a striking demonstration of the importance of the HIF pathway for the integrated physiology of human hypoxia sensing. As discussed earlier, when either of two proline residues in HIF-α is hydroxylated this permits binding of pVHL, an essential step in the process leading to proteasomal degradation of HIF-α (Ivan *et al.* 2001, Jaakkola *et al.* 2001). Patients with CP are homozygous for a hypomorphic mutation leading to the production of pVHL with an arginine-to-tryptophan change at amino-acid residue 200 (R200W), which does not bind hydroxylated HIF-α as strongly (Ang *et al.* 2002). Thus, as a consequence of inappropriate HIF-α stabilisation, these individuals have a HIF system signalling hypoxia under euoxic conditions. This disorder is distinct from the von Hippel-Lindau familial cancer syndrome, an autosomal dominant condition characterised by a variety of malignancies, in which grossly disturbed hypoxia physiology is not reported (Formenti *et al.* 2011).

CP is characterised by polycythaemia, PH and elevated ventilation at normal alveolar oxygen tensions. Exposure of individuals with CP even to mild degrees of alveolar hypoxia results in a marked increase in PASP and ventilation (Smith *et al.* 2006). At oxygen tensions producing appreciable effects in healthy volunteers, CP patients demonstrate very profound responses; the rise in PASP may be so great as to bring pressures close to those within the systemic circulation (Figure 2-6).

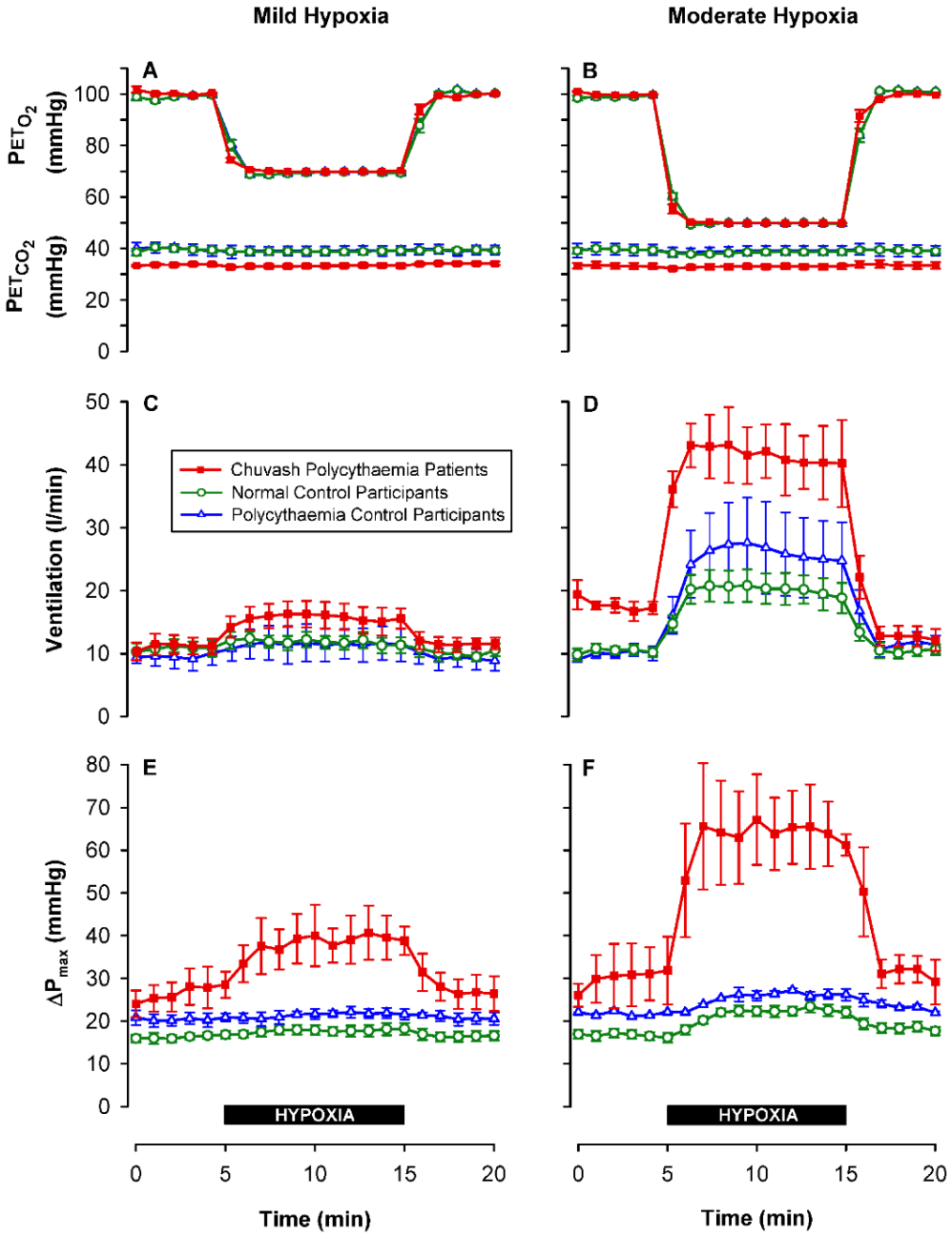

Figure 2-6. Responses to hypoxia in patients with Chuvash polycythaemia

(A & B) Partial pressures of end-tidal gases; (C &D) ventilatory responses; and (E & F) pulmonary vascular responses, during mild (A, C & E) and moderate (B, D & F) alveolar hypoxia. Control groups included healthy volunteers (green) and patients with polycythaemia *rubra vera* (blue). Mild ($P_{ET}O_2$ = 70 mmHg) and moderate ($P_{ET}O_2$ = 50 mmHg) hypoxia both provoked greater increases in ventilation and ΔPMAX in CP patients than healthy controls. Values are means; error bars represent SEM. Adapted from (Smith *et al.* 2006) under Creative Commons license (CC BY 4.0).

In many respects, patients with CP are similar to healthy individuals who have acclimatised to hypobaric hypoxia during a sojourn at altitude (Smith *et al.* 2008c). Perhaps surprisingly, they appear very dissimilar to individuals born and raised at high altitude, in whom physiological responses to hypoxia are blunted (Smith *et al.* 2008b).

Though it is tempting to view CP as a prototypical disease of oxygen sensing, some care is required when drawing conclusions about the biology of the HIF pathway from studies in patients with the disorder. One important issue to bear in mind is that pVHL is not solely involved in the degradation of HIF-α. For example, it also plays a role in the degradation of Janus Kinase 2 (JAK2) (Russell *et al.* 2011). Mutations in *JAK2* that stabilise this protein cause polycythaemia *rubra vera* (PRV) in humans, so dysregulation of pathways other than HIF is bound to contribute to the phenotype in CP. Additionally, therapeutic venesection, to the point of inducing iron deficiency, is frequently employed to lower haematocrit as part of the management of patients with CP (Smith *et al.* 2008c). Iron deficiency itself appears to contribute to PH in CP (Sable *et al.* 2011), as well as exerting a significant influence on expression of HIF-target genes in the condition (Zhang *et al.* 2014b).

2.1.5.2. Animal models

Animal models provide the opportunity to delineate the roles of the various HIF-pathway components *in vivo*, and illustrate very well the central importance of HIF in regulating the hypoxic behaviour of the pulmonary circulation. Mice heterozygous for deletion of HIF-1α demonstrate attenuated physiological sequelae of alveolar hypoxia (Yu *et al.* 1999), with delayed polycythaemia, PH and right ventricular hypertrophy compared to wild-type littermates. Animals heterozygous for deletion of HIF-2α are similarly protected, and do not show the sizeable increases in pulmonary expression of endothelin-1, nor the rise in plasma catecholamine concentration, which are evident during prolonged hypoxia in wild-

type littermates (Brusselmans *et al.* 2003). Additionally, mice heterozygous for a gain-of-function mutation that activates HIF-2α, develop PH under euoxia in a gene dose-dependent manner (Tan *et al.* 2013).

Recently, two elegant studies reported at the same time provided further clarity about the role of the HIF pathway in HPV. In the first, Dai and colleagues found that mice with inducible disruption of PHD2 expression in endothelial cells *and* hematopoietic cells exhibited spontaneous, extensive pulmonary vascular remodelling and severe PH, with progressive right ventricular failure (RVF) and death (Dai *et al.* 2016). Genetic deletion studies identified HIF-2α as the mediator of these effects. In the second study, Kapitsinou *et al.* reported that inactivation of PHD2 in murine endothelial cells *alone* led to severe PH, with abnormal muscularisation of peripheral pulmonary arteries and hypertrophy of the right ventricle (RV), but not polycythaemia (Kapitsinou *et al.* 2016). Furthermore, by concurrently inactivating either HIF-1α or HIF-2α in these animals, these latter investigators were able to show that the development of PH was dependent on HIF-2α but not HIF-1α, and that endothelial HIF-2α was required for the development of increased PAP in a model of chronic hypoxia-induced PH. The observations reinforce the important role of the pulmonary endothelium in modulating the hypoxic behaviour of PASMCs.

In terms of the importance of the HIF pathway for ventilation, Bishop *et al.* have demonstrated that mice heterozygous for deficiency of PHD2 display enhanced ventilatory sensitivity to acute hypoxia, similar to that seen in wild-type animals acclimatised to sustained hypoxia for a week; enlargement of the carotid bodies is also a feature of these animals (Bishop *et al.* 2013). The same group subsequently confirmed similar findings using inducible PHD2 inactivation, and demonstrated with both approaches that specific inactivation of HIF-2α almost entirely corrected the phenotype (Hodson *et al.* 2016).

Inducible inactivation of HIF-2α, but not HIF-1α, markedly reduced ventilatory acclimatisation to hypoxia and associated carotid body cell proliferation in this latter study, indicating that in carotid body biology, as in the pulmonary vasculature, there seems to be a key role for the PHD2-HIF-2α axis.

A study of mice engineered to be homozygous for the R200W VHL mutation (as found in humans with CP) reported that PH developed independently of polycythaemia, and was associated with pulmonary features similar to those seen in humans exposed chronically to hypoxia (Hickey *et al.* 2010). Heterozygosity for HIF-2α, but not HIF-1α, suppressed polycythaemia and PH in this study, and also partially protected against vascular remodelling, again suggesting a dominant role for HIF-2α. Using the same animal model, Slingo *et al.* demonstrated CP mice to have elevated euoxic ventilation and an exaggerated acute hypoxic ventilatory response (AHVR). Immunohistochemistry of excised carotid bodies revealed marked type I cell hyperplasia, and electron microscopy showed numerous mitochondria, enlarged dense-cored vesicles, and markedly expanded rough endoplasmic reticulum – recapitulating the features seen in animals exposed chronically to hypoxia (Slingo *et al.* 2014).

2.1.5.3. Insights from high-altitude populations

The Tibetan Plateau has probably been inhabited for at least 25,000 years (Aldenderfer 2011, Qi *et al.* 2013), exposing the population resident there to a P_IO_2 of around 80 mmHg. Tibetans are therefore one population living at altitude for a period of time sufficiently long for selection pressure to have had an appreciable effect on cardiorespiratory physiology (Petousi and Robbins 2014).

Very soon after genetic approaches were applied to this population, multiple single nucleotide polymorphisms (SNPs) related to the gene encoding HIF-2α – known as *EPAS1* – were identified as having undergone positive selection (Beall *et al.* 2010, Yi *et al.* 2010). In one study, sex-adjusted mean [Hb] at altitude in Tibetan individuals homozygous for the major alleles was 0.8 g/dl lower than heterozygotes (Beall *et al.* 2010).

A study of Tibetans living in the United Kingdom (UK) confirmed significant physiological effects existed in addition to altered erythropoiesis (Petousi *et al.* 2014). When compared to an ethnically similar control group of Han Chinese individuals, Tibetans exhibited hyporeactivity of the pulmonary circulation in response to an acute hypoxic challenge, as well as diminished acclimatisation of the pulmonary vasculature during a sustained exposure to moderate hypoxia (Figure 2-7). Of note, the Tibetan and Han Chinese groups were found not to differ with respect to AHVR, and to exhibit similar early ventilatory acclimatisation to sustained isocapnic hypoxia. Interestingly, though, euoxic $P_{ET}CO_2$ was significantly lower in Tibetans, suggesting a higher ratio of pulmonary ventilation to metabolism at rest. In addition to *EPAS1*, SNPs related to the gene *EGLN1* – which encodes PHD2 – have also been identified as having undergone positive natural selection in the Tibetan population (Simonson *et al.* 2010, Yi *et al.* 2010) and are similarly associated with lower [Hb] at altitude.

It would be anticipated that the effect of these genetic differences in Tibetans is the opposite to that seen in CP patients, such that intracellular HIF-α concentrations are lower, either as a result of reduced basal transcription of HIF-α mRNA, or more rapid proteasomal degradation of the HIFα protein at a given oxygen tension. Recent work studying ethnic Tibetans resident in North America has provided evidence supporting the latter effect. Missense mutations in exon 1 of *EGLN1* give rise to a PHD2 variant with a lower K_m for

oxygen (Lorenzo *et al.* 2014). As a result, this mutant HIF-hydroxylase may promote increased HIF-α hydroxylation and degradation compared with the wild type enzyme, serving to 'damp-down' hypoxia signalling.

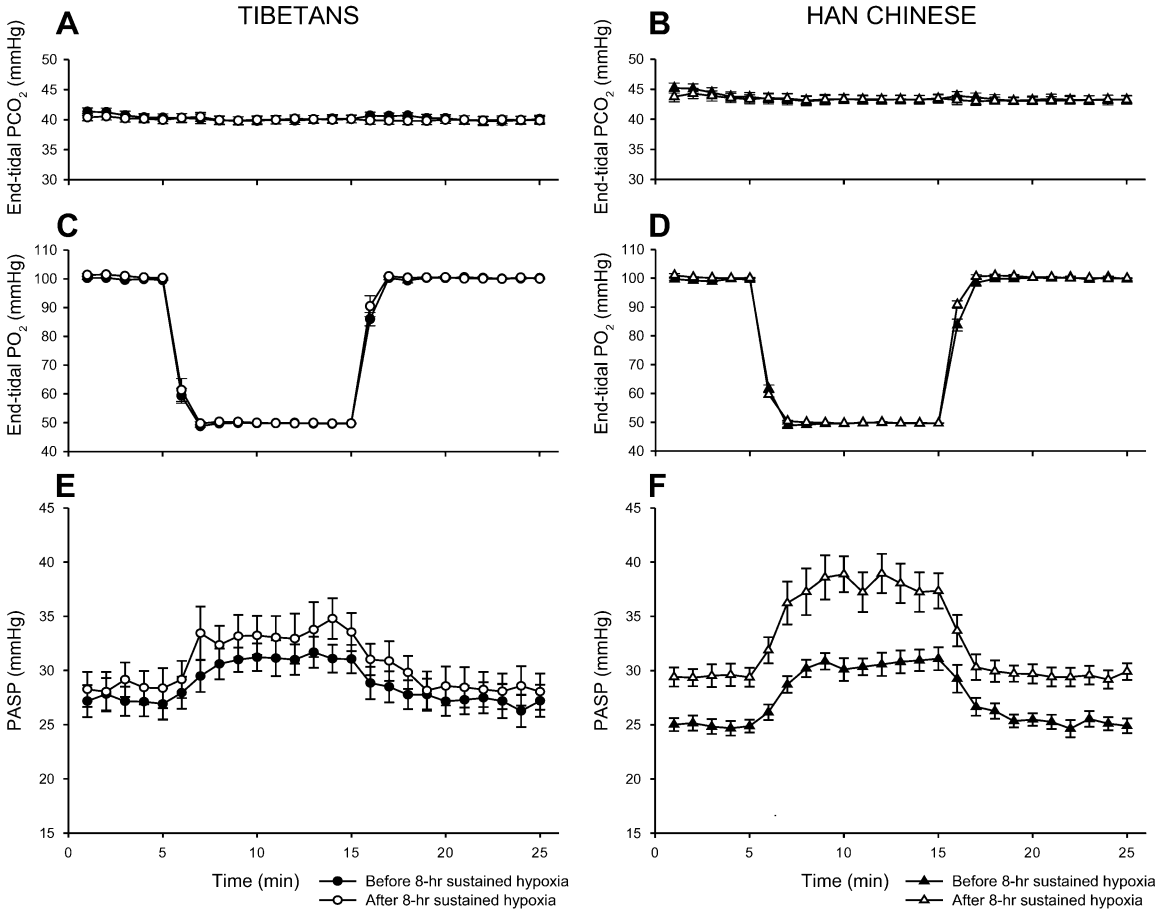

Figure 2-7. HPV in Tibetans and Han Chinese residing close to sea level

Shown are the pulmonary vascular responses to acute isocapnic hypoxia before and after an 8-hour exposure to sustained isocapnic hypoxia ($P_{ET}O_2$ = 50 mmHg). (A & B) $P_{ET}CO_2$; (C & D) $P_{ET}O_2$; and (E & F) PASP, over time. Data are for 10 Tibetan and 10 Han Chinese volunteers, respectively. Filled symbols are values obtained prior to the sustained hypoxic exposure, open symbols those afterwards. Values are means; error bars represent SEM. Reproduced from (Petousi *et al.* 2014) under Creative Commons license (CC BY 3.0).

Finally, it is of interest that a HIF-2α variant has recently been identified that predisposes to hypoxic PH in Angus cattle when herds are farmed at high altitude. There does not appear to be an associated phenotype in lowland cattle, only in animals exposed to hypobaric hypoxia (Newman *et al.* 2015).

2.1.6. Human physiology studies examining iron and HPV

The demonstration *in vitro* of the sensitivity of the HIF pathway to iron availability prompted Balanos *et al.* to administer an infusion of DFO to healthy humans to investigate whether this could provoke pulmonary vasoconstriction (Balanos *et al.* 2002). Not only was this found to be the case, but the kinetics of the rise in PASP with DFO and with hypoxia were similar, supporting the view that that both were acting through a common pathway (Figure 2-8).

In a subsequent, somewhat more complex study (Figure 2-9), the effects on the first phase of HPV of both acute iron chelation and IV iron supplementation were investigated by the same group (Smith *et al.* 2008a). This study consisted of two separate but related protocols. In the first, brief hypoxic challenges were employed to measure the responsiveness of the pulmonary circulation before and after an 8-hour period of exposure to hypoxia, a duration more than adequate to induce acclimatisation. Subsequently, around a week later, an identical assessment was performed, but participants were first given 200 mg iron-sucrose intravenously over 105 min before the initial acute hypoxic challenge. Thus, the effect of acute iron loading on acclimatisation during hypoxia was tested.

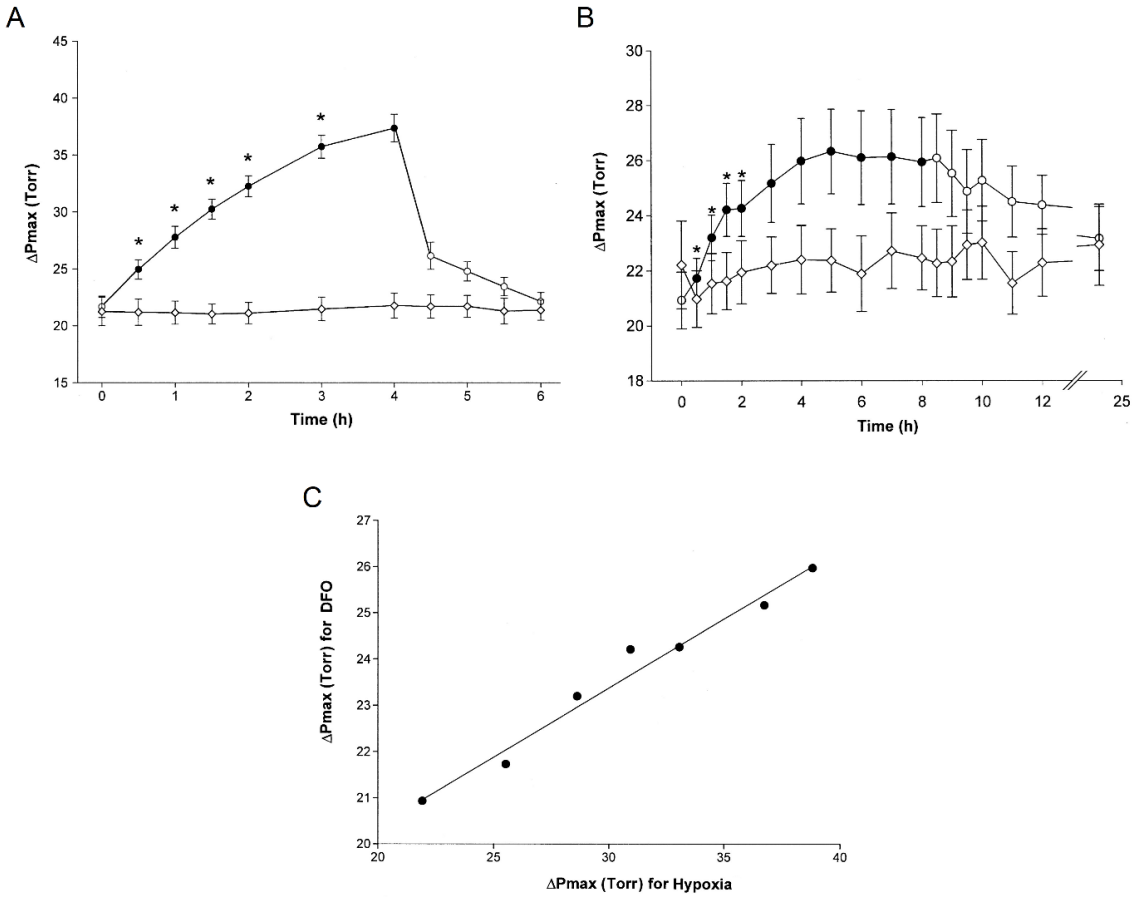

Figure 2-8. Pulmonary vasoconstriction provoked by hypoxia or by iron chelation

(A) Effect of a 4-hour exposure to isocapnic hypoxia ($P_{ET}O_2$ = 50 Torr) on ΔPMAX in 11 healthy volunteers. Filled circles, measurements made under hypoxic conditions; open circles, measurements made after cessation of hypoxia; open diamonds, control measurements made during euoxia; *, time points during hypoxia at which a progressive linear model indicated that steady state had not been reached. Values are means; error bars represent SEM. (B) Effect of an 8-hour DFO infusion (4 g/70 kg body weight) in 9 healthy volunteers. Filled circles, measurements made during DFO infusion; open circles, measurements made after cessation of infusion; open diamonds, measurements made with control infusion (0.9% saline) for first eight hours; *, time points during DFO infusion at which a progressive linear model indicated that steady state had not been reached. Values are means; error bars represent SEM. (C) ΔPMAX data from the first 4 hours of (B) plotted against those for (A), with a linear line of best fit. Adapted from (Balanos *et al.* 2002).

After the first 8-hour period of eucapnic hypoxia, healthy volunteers demonstrated an increase in euoxic PASP and an exaggerated response to the acute hypoxic challenge compared with that seen before the exposure – the hallmarks of pulmonary vascular acclimatisation. When IV iron was given in advance of the second hypoxic acclimatisation period, the usual subsequent elevation in euoxic PASP was abolished, and the response to the acute hypoxic challenge following the sustained hypoxic exposure was only slightly greater than initially – acclimatisation had been greatly attenuated (Smith *et al.* 2008a). Of itself, though, IV infusion of iron did not affect euoxic PASP or the rise in PASP in response to the initial acute hypoxic challenge.

In the second protocol, brief hypoxic challenges were again employed to measure the responsiveness of the pulmonary circulation, but an intervening 8-hour DFO infusion, or 0.9% saline control infusion, was used on this occasion rather than hypoxia. In this way, the effect on acute HPV of an acute reduction in iron bioavailability was explored. As can be seen in Figure 2-9B, acute responses to hypoxia were identical following a prolonged saline infusion, but an 8-hour DFO infusion elevated euoxic PASP and led to an exaggerated ΔPASP during acute hypoxia. In this way, iron chelation with DFO had a hypoxia-mimetic action, inducing what might be called pseudo-acclimatisation.

Echocardiographic data recorded during the sustained hypoxic exposure during the first protocol of this study were subsequently published (Talbot *et al.* 2014) and are illustrated in Figure 2-10. Following IV iron, the time course of the progressive rise in PASP during the long hypoxic exposure is quite different. It is as if IV iron abolishes the second phase of HPV, along with its consequences for the subsequent behaviour of the pulmonary vasculature on return to euoxia, whilst having very little, if any, effect on the first phase of

HPV. In some respects, therefore, IV iron loading causes the hypoxic behaviour of the pulmonary vasculature to resemble that observed in Tibetans (Figure 2-7).

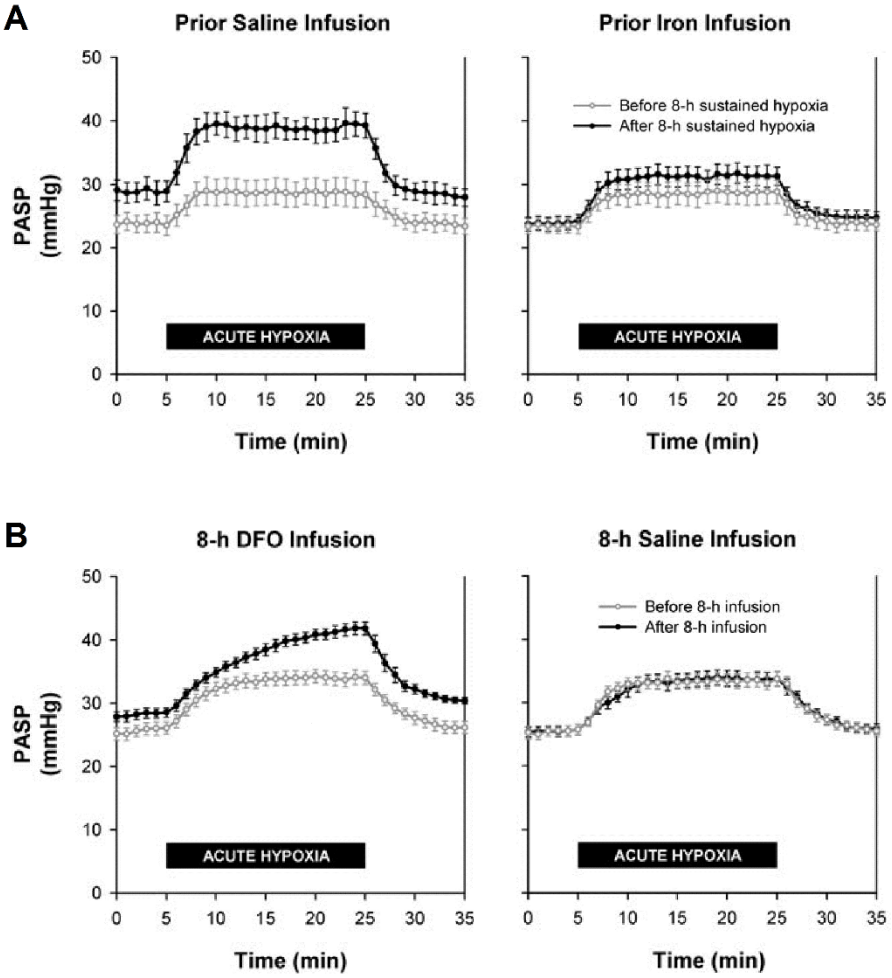

Figure 2-9. Manipulation of the first phase of HPV using sustained hypoxia, IV iron and DFO

(A) With prior saline infusion, a normal response to sustained hypoxia is evident, consisting of a rise in PASP and a sensitised PASP response to subsequent acute hypoxia. Intravenous infusion of iron prior to the initial acute hypoxic challenge prevented the increase in euoxic PASP and markedly attenuated the degree to which the pulmonary vasculature was seen to be sensitised on repeat acute hypoxic exposure. (B) 8-hour infusion of DFO increased PASP and sensitised the response to acute hypoxia, a similar effect to that seen with prolonged hypoxia and saline infusion in (A), albeit with a different shape to the acute rise in PASP. RAP was assumed to be 5 mmHg. Values are means; error bars represent SEM. Adapted from (Smith *et al.* 2008a) with permission.

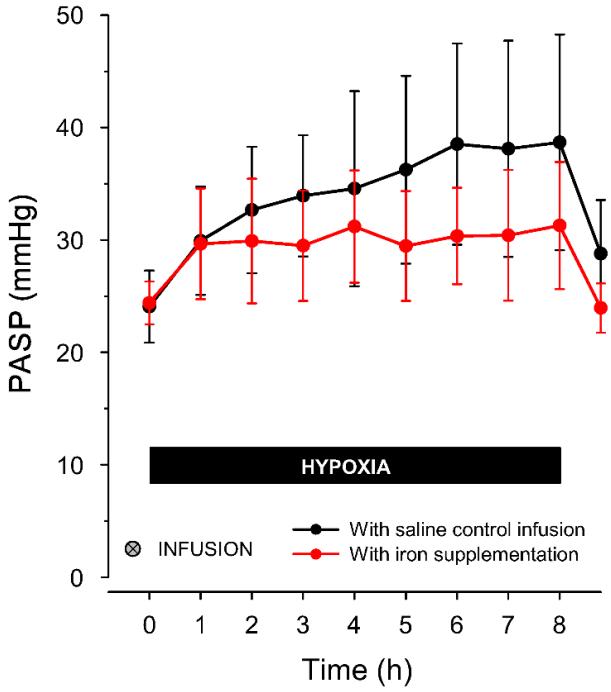

Figure 2-10. Time course of HPV during isocapnic hypoxia with and without prior iron loading

The first phase of HPV is similar with (red) or without (black) prior iron loading, but the progressive rise in PASP with continued hypoxia is abolished by iron. With prior iron infusion PASP is also seen to fall back to initial levels more rapidly upon return to euoxia. RAP was assumed to be 5 mmHg. Values are means; error bars represent SD. Modified from (Talbot *et al.* 2014) under Creative Commons license (CC BY 3.0).

The work described so far was conducted under laboratory conditions and utilised isocapnic normobaric hypoxia. Other experiments have exploited the hypobaric hypoxia of high altitude to investigate the effects of iron status on HPV. There may be differences in physiological responses to hypobaric versus normobaric hypoxia (Millet *et al.* 2012), and the conditions are poikilocapnic in this setting. A study in Peru that took healthy individuals from sea level to 4,340 m and measured PASP over a week found the expected significant rise in PASP induced by hypobaric hypoxia shortly after ascent. After three days at this elevation, individuals were randomised in a double-blind fashion to receive either IV iron

(200 mg iron-sucrose) or saline. Those receiving iron showed a significant fall in PASP after four hours; overall approximately 40% of the pulmonary hypertensive response to hypobaric hypoxia was reversed by iron infusion (Smith *et al.* 2009). This effect was evident not solely during the phase of grossly elevated serum iron levels (Figure 2-11), suggesting that an increase in tissue iron availability was important, as would be expected if iron were acting on oxygen-regulated gene transcription, rather than, say, ion channels.

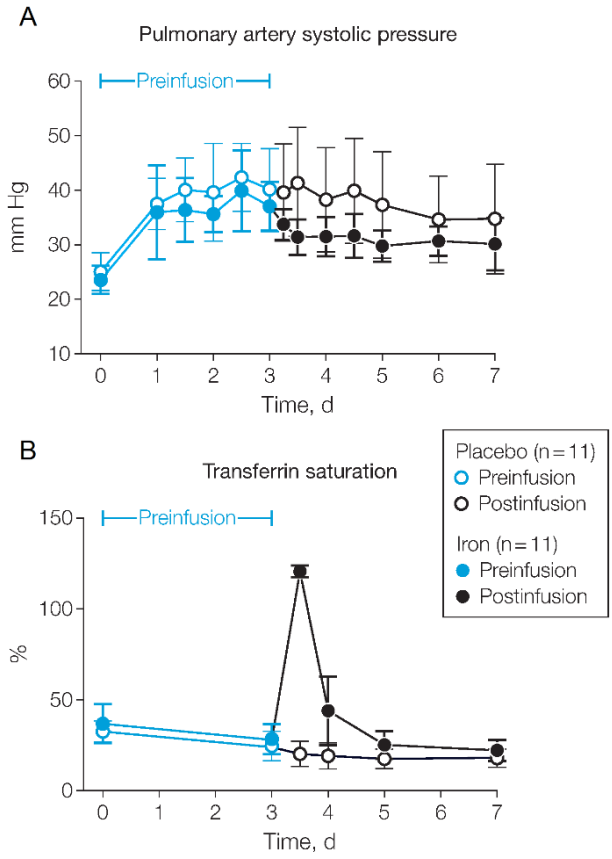

Figure 2-11. Effect of acute iron loading on elevated PASP after several days at high altitude

(A) PASP and (B) TSat in two groups of healthy male sea-level inhabitants during a week spent at an altitude of 4,340 m. Iron-sucrose (200 mg) or placebo was infused on day 3. RAP was assumed to be 5 mmHg. Values are means; error bars represent SD. Adapted with permission from (Smith et al. 2009). Copyright© (2009) American Medical Association. All rights reserved.

2.2. Aims

> *"…it has been suggested that iron infusions may attenuate hypoxic vasoconstriction, and trials are underway to evaluate the effect…in patients with primary pulmonary arterial hypertension. These initiatives are being performed despite a paucity of mechanistic evidence concerning the pathobiologic relationship between pulmonary hypertension and iron deficiency." (Robinson et al. 2014)*

The aim of *Study A* was to determine whether iron deficiency influences normal human responses to hypoxia. The specific focus of this component of the study was the pulmonary circulation. In view of the work already described, the hypothesis was that iron deficiency would exaggerate HPV, reflected by a greater rise in PASP at the end of a sustained hypoxic exposure in otherwise healthy iron-deficient adults, versus iron-replete controls. Additionally, it was hypothesised that iron-deficient individuals would show a more marked response to IV iron, that is, a greater attenuation, by prior administration of iron, of the rise in PASP during hypoxia. Other outcomes of interest were ventilatory and CO responses to hypoxia, and the manner in which these were influenced by IV iron infusion.

2.3. Methods

The contributions of this author and other individuals to the design and execution of *Study A* are described in detail in Appendix A. This was a prospective, non-randomised, controlled, clinical physiology study. Otherwise healthy adults with absolute iron deficiency were recruited, with iron-replete age- and sex-matched volunteers serving as controls. Participants were studied on two occasions, a week or more apart, during a sustained hypoxic exposure. An infusion of IV iron was administered to all participants at the beginning of the second study day.

A flow diagram illustrating the study protocol is given in Figure 2-12.

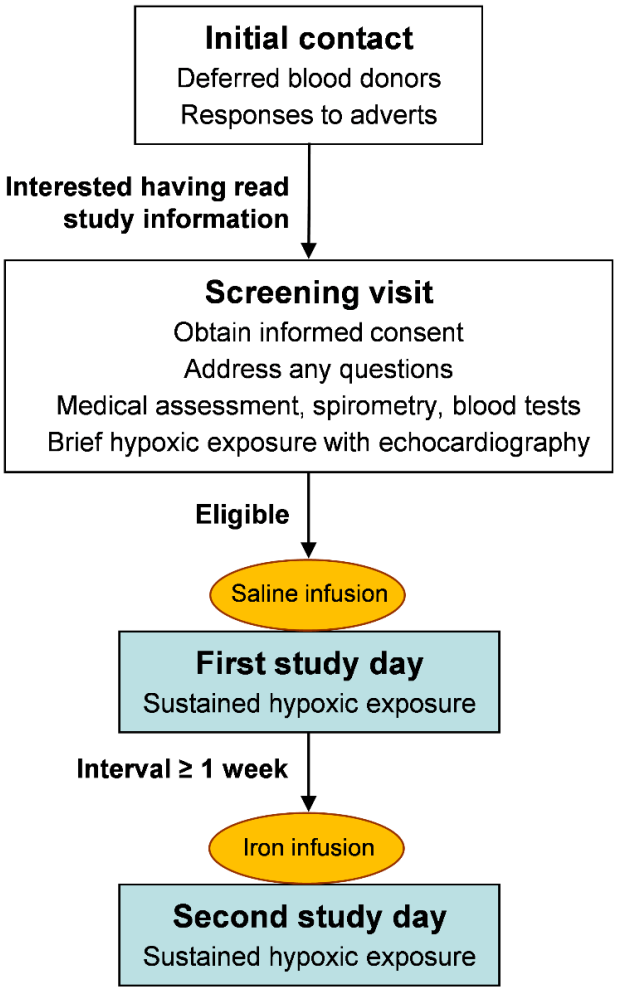

Figure 2-12. Overview of protocol for *Study A*

A detailed description of the recruitment methods and experimental techniques employed may be found in sections 2.3.3 to 2.3.5. Note that the order of infusions was not randomised.

2.3.1. Eligibility criteria

Inclusion criteria were: ability to give informed consent; aged at least 18 years; and presence of detectable tricuspid regurgitation (TR) on transthoracic Doppler echocardiography enabling measurement of PASP. For recruitment to the iron-deficient

(ID) group, both a serum ferritin ≤15 μg/L and a TSat < 16% were required. For iron-replete (IR) volunteers, these values were ≥ 20 μg/L and ≥ 20%, respectively. Exclusion criteria were: haemoglobin < 8 g/dl; haemoglobinopathy; serum ferritin > 300 μg/L; euoxic S_pO_2 < 94%; iron supplementation or blood transfusion within six weeks; pregnancy or breastfeeding; and any significant comorbidity potentially affecting haematinics, pulmonary vascular responses to hypoxia, or ventilation. Volunteers were also excluded if recently exposed to altitude > 2,500 m or air travel longer than four hours, since even these types of exposure to hypobaric hypoxia may induce a degree of pulmonary vascular and ventilatory acclimatisation (Fatemian *et al.* 2001, Smith *et al.* 2012).

The challenge of defining iron deficiency has already been discussed at length in Chapter 1. The values adopted to define the ID and IR groups in the present study were not intended to reflect a universally accepted definition of iron deficiency, since no such definition exists (Zimmermann and Hurrell 2007). Instead, the primary function of the laboratory indices employed was to generate two groups differing significantly in iron status, whilst avoiding recruitment of individuals of what might be considered 'indeterminate' iron status. The coincidence of serum ferritin ≤ 15 μg/L and TSat < 16% was taken to be indicative of significantly diminished iron stores *and* an imbalance between iron supply and demand; both parameters were required to be abnormally low for enrolment to the ID group. Combining indices in this way has been shown to be powerful as it avoids inadvertently misclassifying individuals as ID on the basis of a single abnormal parameter (Cook *et al.* 1976). The IR group definition used an essentially arbitrary cut-off of serum ferritin ≥ 20 μg/L coupled with TSat > 20%. This later value is that which reliably indicates sufficiency of iron supply for the demands of the tissue (Bainton and Finch 1964, Cook *et al.* 1974), albeit only at the point the blood is sampled.

2.3.2. Statistical analysis

The pre-specified primary outcome measure was the rise in PASP over the initial six-hour hypoxic exposure in ID compared with IR participants. The study was designed to have 80% power to detect a difference in the rise in PASP between groups of 4 mmHg with a two-sided significance level of 0.05; it was estimated that 12-14 pairs of participants would be required based on these calculations.

Data were analysed and graphs generated using SigmaPlot for Windows (version 13.0, Systat Software). Repeated measures analysis of variance (RM-ANOVA) and mixed-effects modelling (MEM) were performed with SPSS (version 20, IBM). Group characteristics were compared using a two-tailed unpaired Student's t-test (t-test), or the Mann Whitney U (MWU) test if data were not normally distributed. RM-ANOVA was used for comparisons within and between groups when measures were repeated in a given participant. If the assumptions underlying ANOVA were violated (including those of normally distributed data, equal variances, and uncorrelated residuals), and this compromised the security of conclusions based on such analysis, then MEM was used instead (Gueorguieva and Krystal 2004, Ma *et al.* 2012, Detry and Ma 2016). In this case, candidate covariance structures were identified based on exploration of graphical representations of the data. The most parsimonious converging model and covariance structure were selected. In all cases, $P < 0.05$ was taken as an arbitrary level of statistical significance.

2.3.3. Participant recruitment and matching

During the period of recruitment between February 2013 and April 2014, blood donors in Oxfordshire, UK were offered information about the study if below the haemoglobin threshold to donate, since such individuals frequently have iron deficiency as a

consequence of repeated venesection (Smith *et al.* 2014). Advertisements were placed concurrently for controls. Volunteers attended a screening visit conducted by a physician including medical history, examination, spirometry (MicroLab™, CareFusion, UK), transthoracic Doppler echocardiography (Vivid-q™, GE Healthcare), venous blood sampling, and a brief hypoxic exposure in a normobaric chamber, to establish eligibility and familiarise participants with the study procedures.

During the course of recruitment, in view of the relatively small sample size, ID and IR participants were nominally matched in pairs according to sex and age. The aim of this approach was to ensure that by the end of the study two groups broadly similar in all but iron status had been recruited. Since both age and sex may affect pulmonary vascular physiology (McQuillan *et al.* 2001, Balanos *et al.* 2015, Turner *et al.* 2015, Berendsen *et al.* 2016, Fatemian *et al.* 2016), imbalance in these variables would have been undesirable.

2.3.4. Exposure to hypoxia

Volunteers found to be eligible following a screening visit were invited to attend two subsequent full study days. Each study day entailed a six-hour eucapnic hypoxic exposure (P_{ETO_2} = 55 mmHg) in a normobaric chamber. This apparatus includes a computerised system for continuously monitoring end-tidal gases via a nasal cannula; continuous electrocardiography (ECG) and pulse oximetry are performed, and ventilation monitored by computerised analysis of gas entrained from the nasal cannula (Howard *et al.* 1995).

Nitrogen, CO_2 and oxygen are introduced via a rapid fan-mixing system, and CO_2 removed by passing ambient gas through a soda-lime filtration system, permitting inspired gas concentrations to be controlled tightly. This ongoing process is computer controlled, though the system is constantly monitored and human interventions made to optimise gas control

as necessary, and ensure safety of the participant. A schematic representation is given in Figure 2-13, and a photographic image in Figure 2-14.

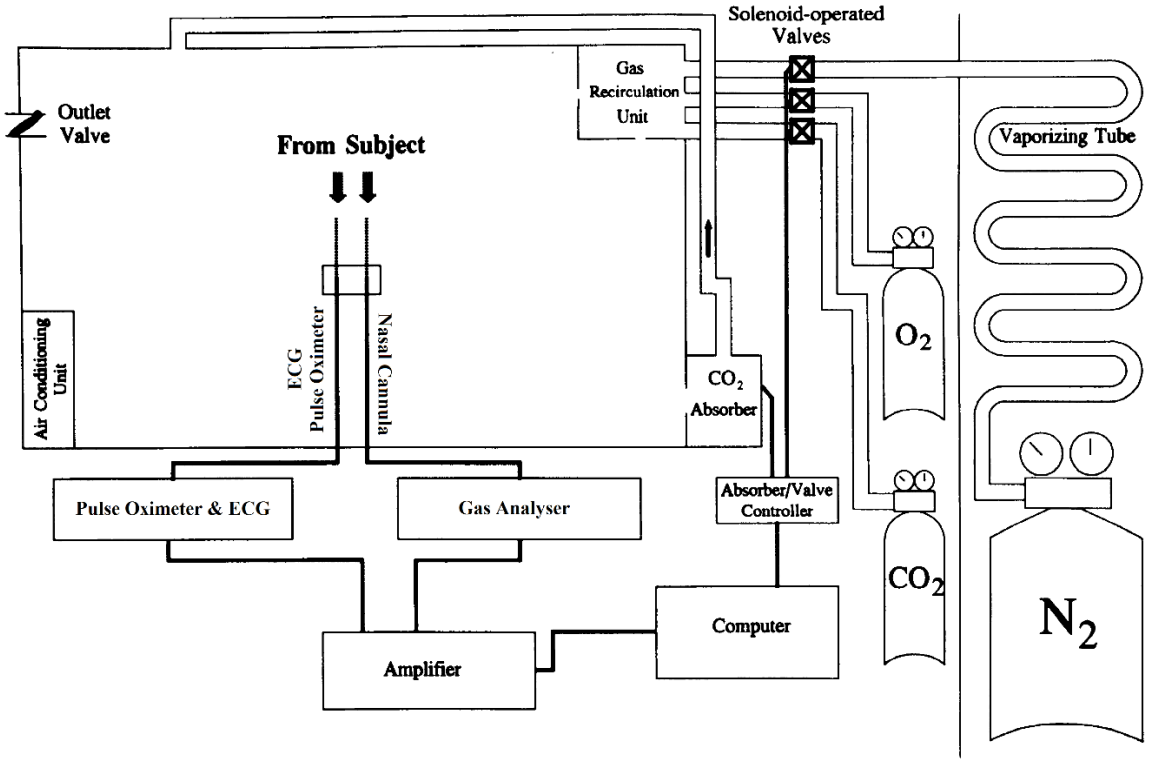

Figure 2-13. Schematic representation of normobaric chamber and associated apparatus

Participants were monitored continuously by 3-lead ECG, earlobe pulse-oximetry, and analysis of inspired and expired gas sampled by nasal cannula. Automatic adjustments were made according to a computer algorithm, to maintain the desired $P_{ET}O_2$ and $P_{ET}CO_2$ levels throughout. This was achieved by automatic control of three solenoid valves allowing entry of oxygen, carbon dioxide and nitrogen into the chamber via a recirculation unit. The operator could make manual adjustments to gas control as necessary. A separate hypoxia alarm (not illustrated here) constantly entrained gas from the chamber, and an alarm sounded if the F_IO_2 fell below a level predetermined by the operator. Adapted from the original description by (Howard *et al.* 1995).

Figure 2-14. Chamber for end-tidal gas control in *Study A*

The investigator was able directly to observe the participant throughout, with ECG, S_pO_2 and ventilatory data displayed continuously. A blind could be drawn over the exterior window and the lights dimmed to facilitate image acquisition during echocardiography. 1, gas recirculation unit; 2, participant wearing nasal cannula (the ECG leads and oximetry probe are not visible in this view); 3, solenoid-operated valves; 4, CO_2 absorber; 5, monitoring stack (pulse oximeter, gas analyser and ECG monitor); 6, computer monitor displaying physiological data in real time; 7, hypoxia alarm; 8, controller for absorber and solenoid valves; 9, centrifuge; 10, investigator.

Participants were asked to report to the Sherrington Building between 8 and 9 o'clock in the morning, having enjoyed a light breakfast but avoided caffeine and exercise earlier in the day. After a period of quiet rest, $P_{ET}CO_2$ was determined whilst seated during spontaneous breathing over a period of 5-10 minutes, or longer if judged necessary by the

investigator. The value thus obtained was used for the maintenance of isocapnia for the remainder of that day. Initial euoxic measurements were made at P_{ETO_2} = 100 mmHg; these same conditions were used after cessation of hypoxia at 6 hours. Participants were provided with light refreshment *ad libitum*, and were able to move around, enjoy audio-visual entertainment and leave the chamber briefly to use the lavatory if required.

2.3.5. Blood sampling and infusions

On each study day a 20-gauge venous cannula (Venflon™, Becton Dickinson) was inserted aseptically into a forearm vein and 20 ml blood withdrawn slowly into a 20 ml plastic syringe, with great care taken to avoid haemolysis. The venous blood was immediately decanted into blood collection tubes (Vacutainer®, Becton Dickinson), including those for plasma (ethylenediaminetetraacetic acid (EDTA) and lithium heparin) and serum (SST™ II Advance). Routine assays for full blood count (FBC), ferritin, serum iron, transferrin and TSat were performed by the John Radcliffe Hospital clinical pathology laboratory. Samples were refrigerated at 4°C prior to transportation to the laboratory; delivery was by hand, directly to the laboratory processing area, in order to minimise delays in processing, physical trauma to the samples, and risk of haemolysis.

In addition to the samples sent for analysis in this manner, aliquots of serum and EDTA plasma were obtained by centrifugation and immediately frozen and stored at -80°C until the conclusion of the final study visit by the final participant, following which they were analysed by enzyme-linked immunosorbent assay (ELISA). Serum erythropoietin and plasma sTfR were analysed by sandwich ELISA (both Quantikine®, R&D Systems, Abingdon, UK). Plasma hepcidin was analysed using a competitive ELISA (Hepcidin-25 EIA kit, Bachem, Peninsula Laboratories, San Carlos, USA). In all cases samples were assayed in triplicate, and in accordance with manufacturer's instructions.

On the first study day, immediately prior to commencing the hypoxic exposure, 0.9% saline was administered via the cannula. On the second, ferric carboxymaltose (Ferinject®, Vifor Pharma, Switzerland) 15 mg/kg (maximum 1 g) was added to an appropriate volume of 0.9% saline and infused. The IV infusions on each day were 250 ml in total volume and given over 15 minutes. Though it was not possible to randomise the order of infusions, participants were blindfolded during administration and not told that the infusions would follow a consistent order.

2.3.6. Doppler echocardiography

Throughout each hypoxic exposure, PASP and CO were measured echocardiographically (Yock and Popp 1984, Berger *et al.* 1985, Currie *et al.* 1985, Chan *et al.* 1987, Yoshida *et al.* 1988, Stevenson 1989, Jobic *et al.* 1993, Allemann *et al.* 2000, McQuillan *et al.* 2001). Initial measurements were made under euoxia (P_{ETO_2} = 100 mmHg), when 30 minutes of exposure to hypoxia (P_{ETO_2} = 55 mmHg) had passed, after an hour of hypoxia, and thereafter at hourly intervals. At the end of the six-hour hypoxic exposure, participants were returned to isocapnic euoxia (P_{ETO_2} = 100 mmHg) and one final set of measurements made after 30 minutes. All measurements were made with the same device, which was that used at the screening visit.

Participants rested comfortably on a customised couch (with a section removed to facilitate optimal positioning for image acquisition) in the left lateral position, facing the operator whilst ΔP_{MAX} was determined from an apical four chamber view of the heart using continuous-wave Doppler (Figure 2-15).

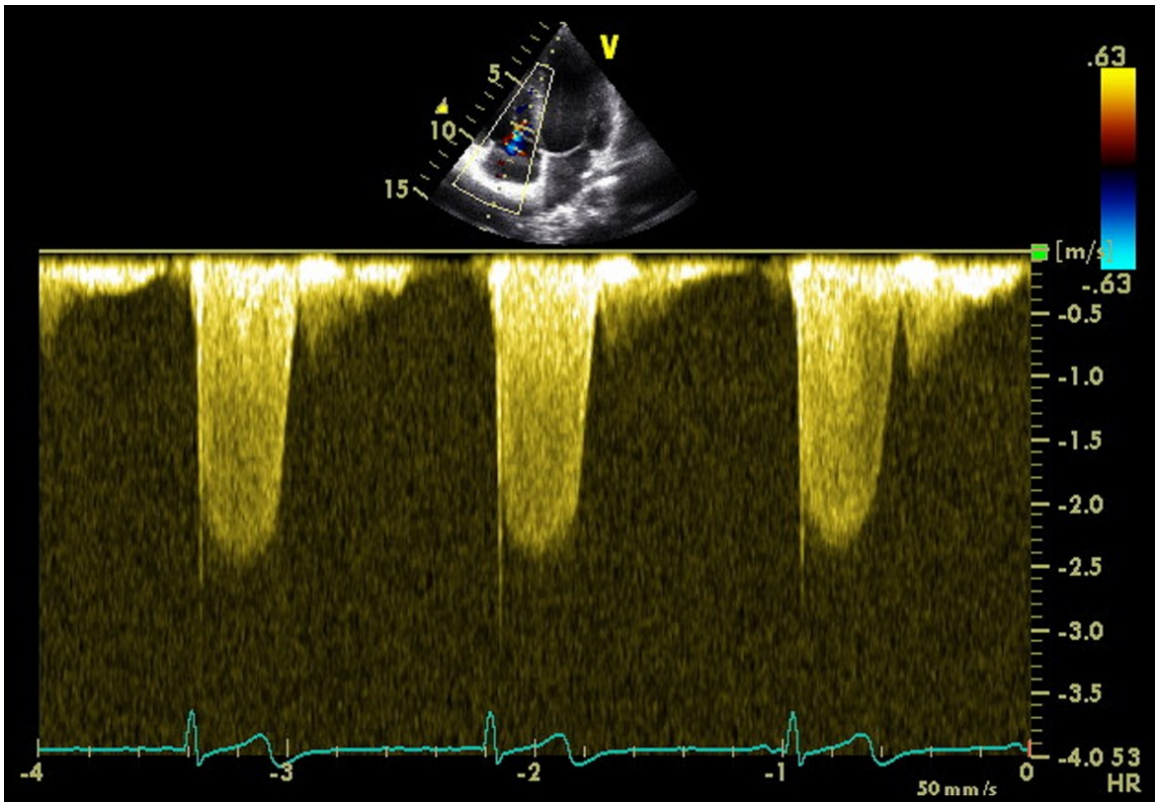

Figure 2-15. ΔPMAX estimation using Doppler echocardiography

An apical four chamber view of the heart is visible at the top of the image, the position having been optimised to align the direction of the probe (dotted line) with the trans-tricuspid regurgitant jet (blue). On the spectral Doppler image below, three envelopes are visible, with the corresponding ECG trace in turquoise at the bottom. This image was acquired at the 2-hour time point during the first hypoxic exposure in an iron-replete female participant. The maximum velocity of the TR jet in this case of 2.4 m/s equates to a ΔPMAX of 23 mmHg, or a PASP of 28 mmHg with an assumed RAP of 5 mmHg.

Stroke volume (SV) was measured from the velocity-time integral of left ventricular outflow tract (LVOT) blood flow using pulsed-wave Doppler in an apical five chamber view, having determined LVOT diameter from a parasternal long axis view at the beginning of the first study visit. To determine CO, SV (taken from the mean of five separate envelopes) was multiplied by heart rate as recorded simultaneously by ECG. Pulmonary

artery systolic pressure was calculated by adding 5 mmHg, as an estimate of RAP, to ΔP_{MAX} (Smith *et al.* 2008a, Smith *et al.* 2009, Talbot *et al.* 2014), the latter having been determined from the mean of ten separate envelopes. Analysis of echocardiographic data in this manner was performed offline following the final visit of the final study participant.

2.3.7. Study approval

The study was conducted in accordance with the Declaration of Helsinki and received ethical approval from the National Research Ethics Service NHS South Central Portsmouth Research Ethics Committee (reference: 12/SC/0710). Though not a clinical trial of an investigative medicinal product (CTIMP), the study was nevertheless registered prospectively with ClinicalTrials.gov (reference: NCT01847352). The study sponsor was the University of Oxford. All participants provided written informed consent.

2.4. Results

2.4.1. Baseline characteristics of participants in *Study A*

Thirteen pairs of ID and IR healthy individuals were identified as illustrated in Figure 2-16. Characteristics of these participants are given in Table 2-1. There were no significant differences in body mass index (BMI) or spirometric parameters between groups. Mean ferritin in the ID group was 6.4 μg/L and TSat 8.4%, consistent with absolute iron deficiency. Corresponding values for the IR group were 69.6 μg/L and 29.2%, respectively. Plasma sTfR was significantly higher in the ID group; the mean exceeded the upper limit of normal of 28.1 nmol/L in the healthy population for the assay used (Allen *et al.* 1998). None of the IR group had an elevated sTfR. Plasma hepcidin was very heavily suppressed in the ID group.

Characteristic	ID Group (n=13)	IR Group (n=13)	*P*-value
Female sex – no. (%)	9 (69)	9 (69)	
Age – years	37.5 ± 11	36.7 ± 13	0.86
Body mass index – kg/m^2	24.2 ± 3.7	23.5 ± 3.2	0.65
Smoking status – current:ex:never	1:3:9	0:4:9	0.56†
Euoxic S_pO_2 – %	97.8 ± 1.0	97.7 ± 1.0	0.70
Systolic blood pressure – mmHg	126 ± 16	122 ± 16	0.59
Diastolic blood pressure – mmHg	80 ± 12	76 ± 11	0.38
FEV_1 – % predicted	105 ± 11	106 ± 15	0.90
Serum ferritin – µg/L	6.4 ± 3.9	66.9 ± 52.1	
Serum TSat – %	8.4 ± 3.6	29.2 ± 5.7	
Serum iron – µmol/L	6.9 ± 3.0	18.7 ± 4.6	**< 0.001**
Serum transferrin – g/L	3.8 ± 0.6	2.9 ± 0.5	**< 0.001**
Plasma sTfR – nmol/L	29.3 ± 8.3	19.5 ± 4.2	**< 0.001**
Plasma hepcidin – µg/L	1.8 (0.8 - 5.0)	22.1 (18.5 - 29.1)	**< 0.001**
Haemoglobin concentration – g/dl	12.1 ± 1.5	14.1 ± 0.9	**< 0.001**
Haematocrit – %	38.0 ± 3.3	42.6 ± 2.9	**< 0.001**
Mean cell volume – fl	82.0 ± 8.2	90.8 ± 4.7	**0.003**
Serum vitamin B12 – ng/L	364 ± 109	355 ± 104	0.82
Serum folate – µg/L	8.2 (5.4 - 10.8)	9.6 (8.0 - 12.8)	0.12

Table 2-1. Participant characteristics on enrolment to *Study A*

Body mass index (BMI) is the weight divided by the square of the height. Values are means ± SD, for normally distributed data, and comparisons by two-tailed unpaired Student's t-test; or medians (IQR), if not normally distributed, in which case comparisons are by Mann Whitney U test. †, Chi-squared test; FEV_1, forced expiratory volume in one second. *P*-values for comparisons where statistically significant differences are present appear in bold.

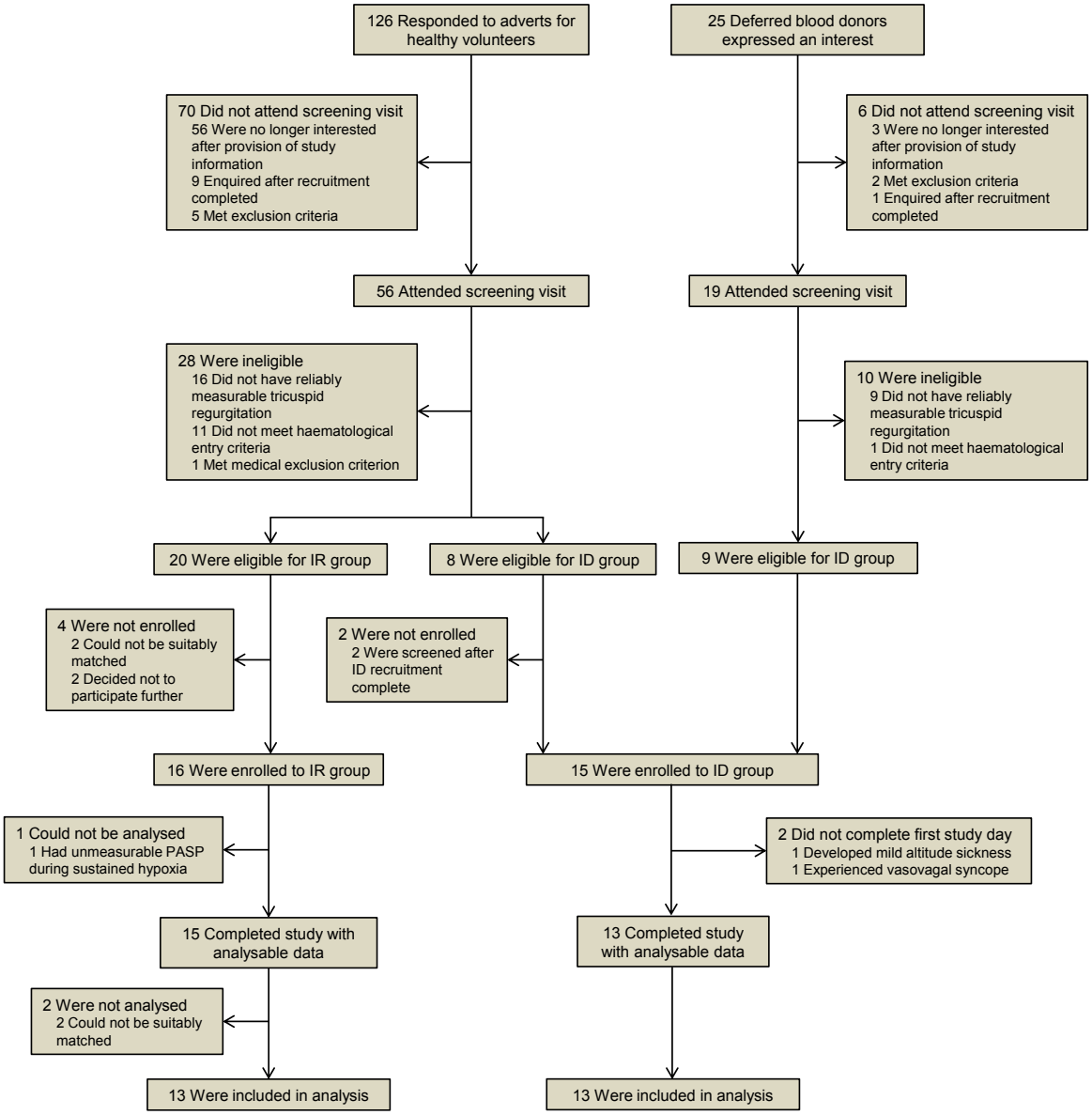

Figure 2-16. Study recruitment flow diagram for *Study A*

There were 25 expressions of interest from deferred blood donors and 126 responses to advertisements. Sixteen participants were enrolled to the IR group and fifteen to the ID group. interestingly, of the latter, a significant proportion were identified having responded to advertisements. Two ID females were withdrawn during the first hypoxic exposure; one developed headache and nausea (effectively altitude sickness); the other experienced vasovagal syncope. Both recovered promptly without sequelae once returned to air. One participant in the IR group had echocardiographic data during hypoxia precluding determination of ΔP_{MAX}, despite success at the screening visit. No ID participants could be recruited as suitable matches for two young men enrolled to the IR group early in the course of the study; their data were not analysed.

2.4.2. Haematological parameters over the course of *Study A*

All of the screening visit laboratory measurements given in Table 2-1, with the exception of serum B12 and folate, were repeated at the start of each experimental day, and are given in Table 2-2.

	ID Group (n = 13)			IR Group (n = 13)			*P*-value (RM-ANOVA)		
	Screen	**Day 1**	**Day 2**	**Screen**	**Day 1**	**Day 2**	**Group**	**Day**	**Inter-action**
Hb – g/dl	12.2 ± 1.5	12.1 ± 1.3	11.9 ± 1.2	14.1 ± 0.9	14.4 ± 1.1	14.3 ± 1.2	**< 0.001**	0.39	0.23
Platelets – 10^9/L	310 ± 72	323 ± 74	310 ± 76	235 ± 47	229 ± 58	233 ± 59	**0.003**	0.76	0.30
MCV – fl	82.0 ± 8	82.9 ± 5.2	83.6 ± 6.1	90.8 ± 4.5	89.7 ± 4.5	90.5 ± 5.1	**0.002**	0.44	0.28
Ferritin – µg/L	6.4 ± 3.9	6.9 ± 4.1	6.7 ± 3.7	66.9 ± 52.1	61.6 ± 55.2	62.4 ± 52.2	**< 0.001**	0.43	0.30
TSat – %	8.4 ± 3.6	14.6† ± 7.2	14.9 ± 8.8	29.2 ± 5.7	27.6† ± 10.1	30.3† ± 12.6	**< 0.001**	0.18	0.15
Iron – µmol/L	6.9 ± 3.0	11.1† ± 4.6	11.5 ± 6.0	18.7 ± 4.6	17.0† ± 6.2	19.7† ± 7.4	**< 0.001**	0.13	0.084
Transferrin – g/L	3.78 ± 0.57	3.70 ± 0.64	3.67 ± 0.61	2.93 ± 0.46	2.85 ± 0.36	2.82 ± 0.31	**< 0.001**	**0.042**	0.99
sTfR – nmol/L	29.3 ± 8.3	28.8 ± 10.6	27.7 ± 9.0	19.5 ± 4.2	19.4 ± 4.3	19.4 ± 3.6	**0.003**	0.28	0.40
Hepcidin – µg/L	2.8 ± 2.7	4.5 ± 5.8	3.2 ± 4.5	24.0 ± 11.4	20.1 ± 17.1	18.9 ± 15.4	**< 0.001**	0.56	0.35

Table 2-2. Haematological and iron parameters over the course of the study

Values are means ± SD. On the first study day, one sample in each group was reported to show evidence of haemolysis, so values for serum iron and TSat for these individuals were excluded from the analysis. A similar issue affected a single sample in the IR group on the second study day, which was also excluded. †, n = 12 at this time point, for this variable. *P*-values for comparisons where statistically significant differences are present appear in bold.

At each time point, for every variable listed in Table 2-2, values differed significantly between the ID and IR groups (post-hoc testing using the Holm-Sidak method for pairwise multiple comparison procedures; $P < 0.01$ in all cases). Specifically, in the ID group, [Hb], MCV, serum ferritin, TSat, serum iron and plasma hepcidin were lower; platelet count, serum transferrin and plasma sTfR were higher.

In the case of transferrin, where a statistically significant effect of time was suggested, post-hoc testing indicated a significant within-group difference only for the ID group, with respect to the screening visit versus study day 2 ($P = 0.043$). In the case of TSat in the ID group, both the day 1 and day 2 values were significantly higher than the screening visit value ($P = 0.048$ and $P = 0.049$, respectively) though the values on the two study days were not significantly different. This observation was largely explained by a significantly higher serum iron on both study day 1 and study day 2 than at the screening visit ($P = 0.041$ and $P = 0.035$, respectively).

2.4.3. PASP during hypoxia

PASP responses were analysed using mixed-effects modelling. Euoxic PASP prior to the hypoxic exposure did not differ across group or study day (ID vs IR: mean 27.3 vs 26.3 mmHg, on first day; 27.2 vs 26.6 mmHg, on second day; $P = 0.59$ for group; $P = 0.79$ for study day; $P = 0.75$ for interaction). At the end of the 6-hour hypoxic exposure on the first day, the ID group reached a mean PASP of 44.2 mmHg compared with 37.0 mmHg in the IR group (Figure 2-17). The rise in PASP was therefore 6.2 mmHg greater in the ID group (95% confidence interval (CI) for difference, 2.7 to 9.7 mmHg, $P = 0.001$).

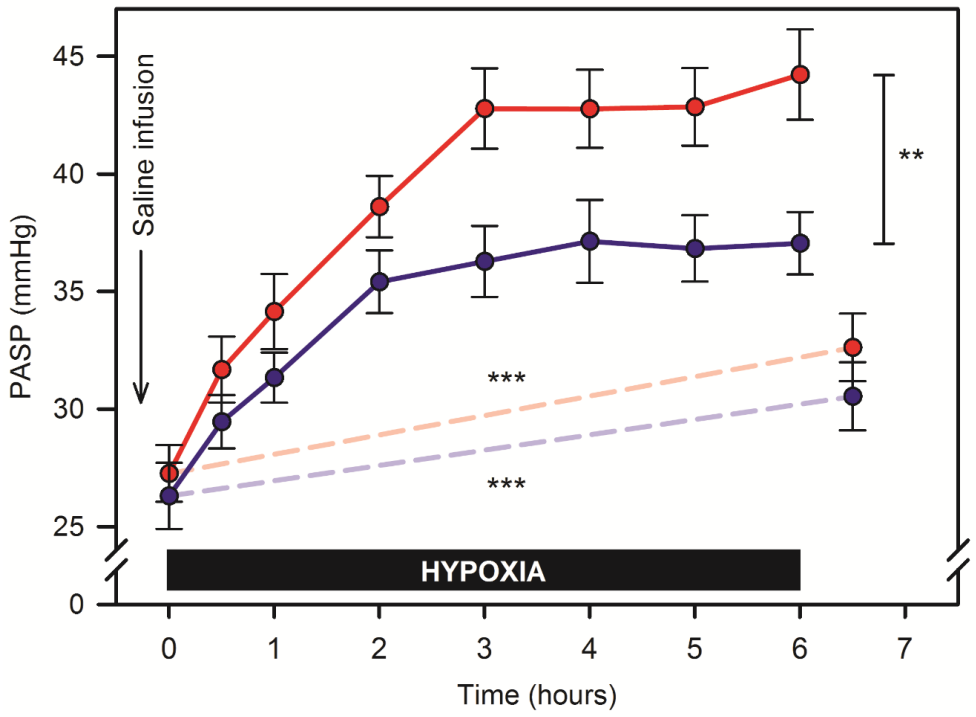

Figure 2-17. PASP responses to hypoxia on the first study day

Intravenous 0.9% saline was infused prior to the first echocardiographic measurements. Values are means ± SEM; the ID group are shown in red; the IR group in blue. The black bar indicates the period of isocapnic hypoxia. Continuous lines represent responses during hypoxia; broken lines the change in air-breathing PASP induced by hypoxia (acclimatisation). Asterisks indicate the significance of comparisons between or within groups: **, $P < 0.01$; ***, $P < 0.001$.

On the second day, following IV iron, the rise in PASP diminished in both groups (Figure 2-18). At six hours, the mean in the ID group was 33.1 mmHg and in the IR group 30.5 mmHg, representing absolute reductions in the rise in PASP during hypoxia of 11.1 and 6.8 mmHg, respectively (Figure 2-19). The magnitude of the absolute reduction seen in the ID group was significantly greater than that in the IR group (-4.3 mmHg, 95% CI for difference, -8.3 to -0.3 mmHg, $P = 0.035$).

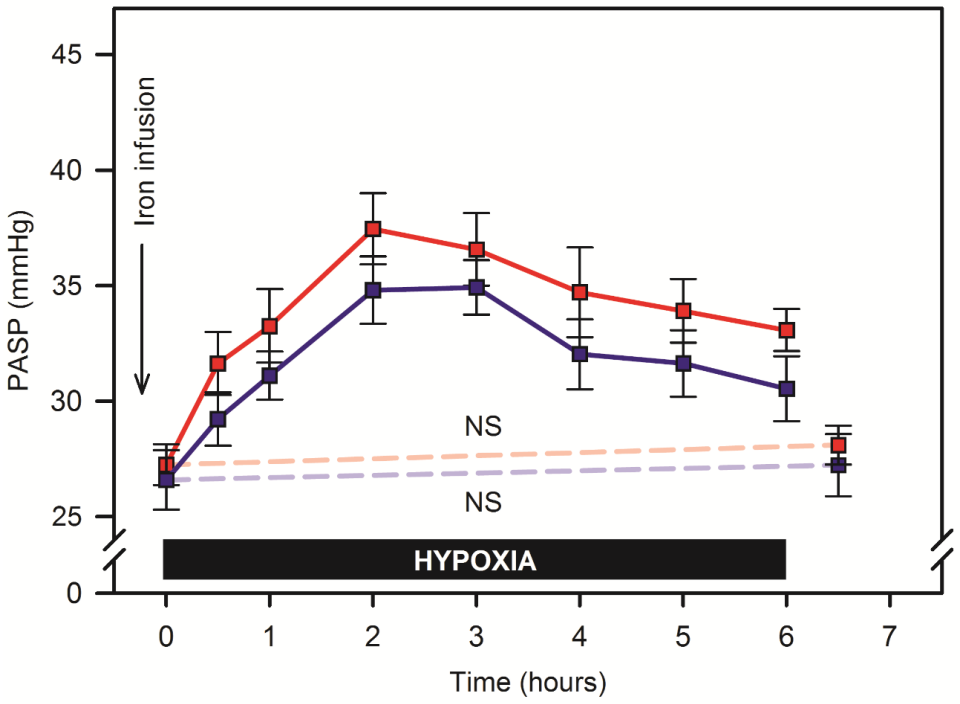

Figure 2-18. PASP responses to hypoxia on the second study day

Intravenous iron was infused prior to the first echocardiographic measurements. Values are means ± SEM; the ID group are shown in red; the IR group in blue. The black bar indicates the period of isocapnic hypoxia. Continuous lines represent responses during hypoxia; broken lines acclimatisation. The latter was not significant (NS) on this occasion.

2.4.4. Pulmonary vascular acclimatisation

After 30 minutes of euoxia following cessation of hypoxia on the first day, euoxic PASP remained elevated in both groups compared with values prior to hypoxia (mean increase in euoxic PASP: ID, 5.4 mmHg; IR, 4.2 mmHg; $P < 0.001$ for both groups) reflecting acclimatisation of the pulmonary vasculature (Figure 2-17). On the second day, with intravenous iron, an increase in euoxic PASP at 30 minutes post-hypoxia was no longer evident (Figure 2-18). The difference between study days was significant (reduction in

euoxic PASP at 30 minutes post-hypoxia on the second day, compared with the first: ID, -4.5 mmHg; IR, -3.6 mmHg; $P < 0.001$ for both groups).

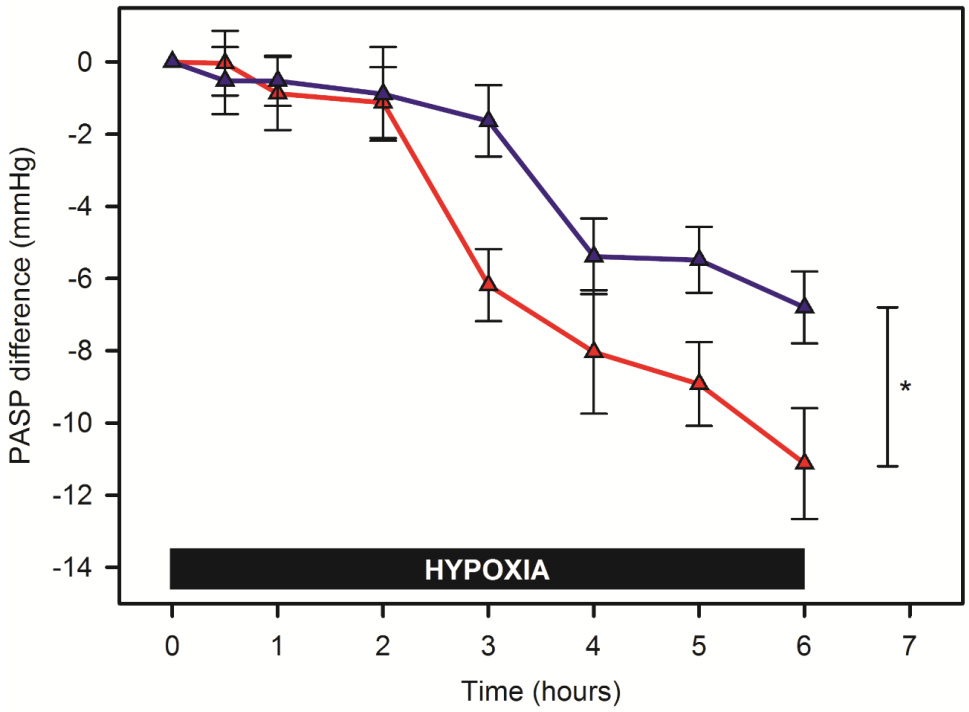

Figure 2-19. Difference in PASP response to hypoxia between study days

The PASP difference was calculated by subtracting the rise in PASP that had occurred on day one, from that occurring on day two, for each participant, at each time point. Thus, values for both groups are zero at time zero. Values are means ± SEM; the ID group are shown in red; the IR group in blue. The black bar indicates the period of isocapnic hypoxia. Asterisks indicate the significance of comparisons between groups: *, P < 0.05.

2.4.5. Cardiac output responses to hypoxia

Cardiac output responses were analysed using mixed-effects modelling. Initial euoxic CO did not differ between groups on either study day, nor on the different days within groups (ID vs IR: mean 5.6 vs 5.3 L/min, respectively, on first study day; 5.6 vs 5.1 L/min,

respectively, on second study day; $P = 0.45$ for group; $P = 0.48$ for study day; $P = 0.57$ for interaction). Cardiac output increased during hypoxia in both groups on both days (ID and IR: first study day 1.1 and 0.8 L/min, respectively; second study day 0.8 and 1.0 L/min, respectively; $P < 0.001$ for each group, each day) (Figure 2-20). In contrast to PASP, the CO response at the end of the six-hour hypoxic exposure did not differ between groups or study days, and there was no differential effect of IV iron between groups ($P = 0.29$ for group; $P = 0.91$ for study day; $P = 0.25$ for interaction).

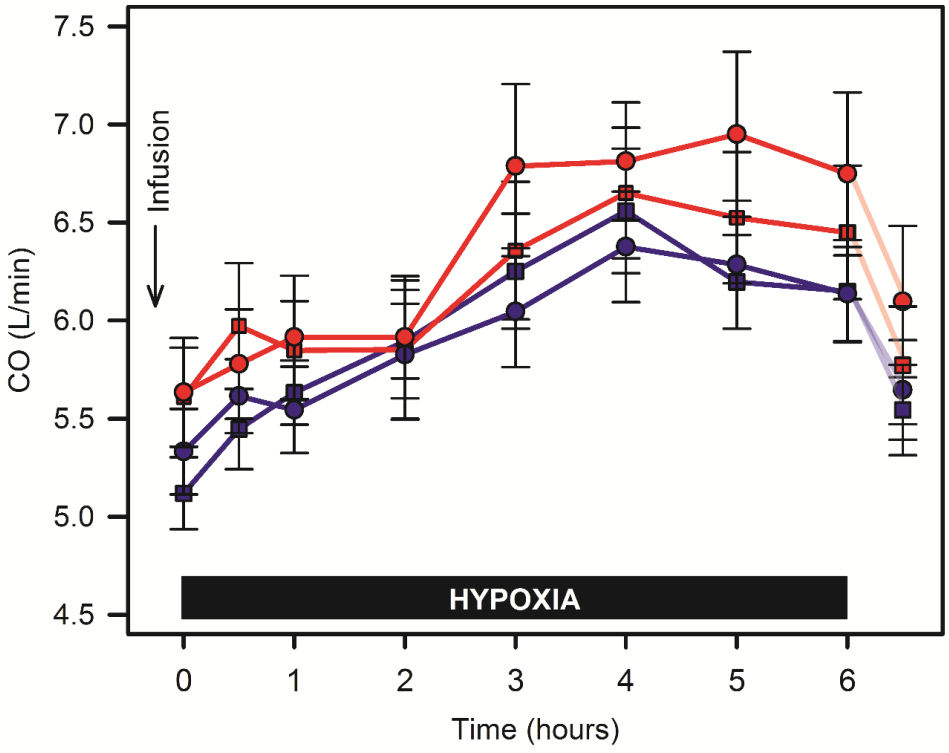

Figure 2-20. Cardiac output responses on both study days

Aside from the within-group rise in CO during hypoxia in each group on each day, no significant interactions between iron status, time or study day were detected. Circles, first study day; squares, second study day. Translucent lines indicate the fall in CO after cessation of hypoxia. Values are means ± SEM; the ID group are shown in red; the IR group in blue. The black bar indicates the period of isocapnic hypoxia.

2.4.6. Ventilation and peripheral oxyhaemoglobin saturation

Figure 2-21 shows the end-tidal and inspired partial pressures of gases and S_pO_2 in both groups on each study day. Euoxic $P_{ET}CO_2$ did not differ between groups on either study day, nor on the different days within groups (ID vs IR: mean 35.9 vs 35.4 mmHg, respectively, on the first day; 35.5 vs 34.9 mmHg, respectively, on the second day; $P = 0.45$ for group; $P = 0.052$ for study day; $P = 0.77$ for interaction).

The mean $P_{ET}O_2$ during the hypoxic exposures did not vary between groups or study days ($P = 0.96$ for group; $P = 0.22$ for study day; $P = 0.74$ for interaction) confirming that uniform hypoxic stimuli were delivered. Similarly, the mean S_pO_2 during the hypoxic exposures did not vary between groups or study days ($P = 0.78$ for group; $P = 0.33$ for study day; $P = 0.83$ for interaction).

The P_ICO_2, which is an index of ventilation under the experimental conditions in the present study when $P_{ET}CO_2$ is clamped to ensure isocapnia, did not differ between groups or study days at 30 minutes ($P = 0.94$ for group; $P = 0.61$ for study day; $P = 0.50$ for interaction) or at six hours ($P = 0.67$ for group; $P = 0.08$ for study day; $P = 0.51$ for interaction).

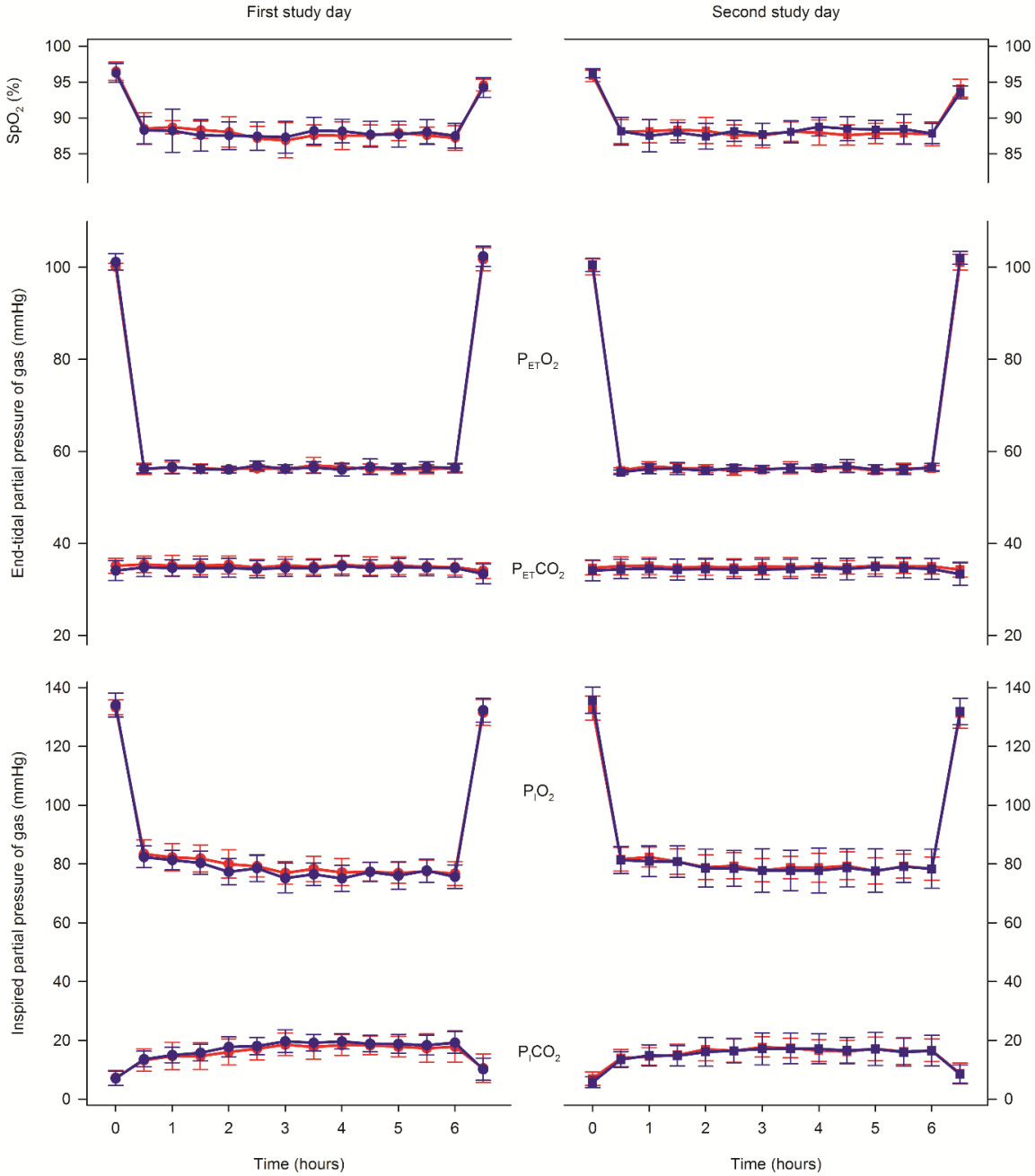

Figure 2-21. S_pO_2 and gas control on each study day

Left: First study day, when saline was infused at the outset (circles). *Right:* Second study day, when iron was infused at the outset (squares). Top panel, S_pO_2; middle panel, end-tidal gas partial pressures; bottom panel, inspired gas partial pressures. Data for the ID group are shown in red and for the IR group in blue; data are means based on 30-minute averages of continuous recordings; error bars represent SD.

2.5. Discussion

2.5.1. Main findings

The main finding of this study is of greatly exaggerated HPV in healthy individuals with iron deficiency. After only six hours of moderate alveolar hypoxia, the ID group showed a mean rise in PASP that was in excess of fifty percent greater than IR controls, despite a similar euoxic PASP to that of the IR group at the outset. Cardiac output rose during hypoxia to a similar extent in both groups on both study days, by approximately 1 L/min at six hours. The extent to which changes in CO contribute directly to the change in PASP that occurs during hypoxia is estimated to be only 0.61-0.66 mmHg for every 1 L/min rise in CO (Balanos *et al.* 2005, Croft *et al.* 2013). Thus the overwhelming majority of the rise in PASP during hypoxia on both experimental days must be due to HPV, and neither the greater PASP rise in the ID group on the first experimental day, nor the greater response to iron infusion in the ID group on the second, can be explained by differences in CO responses during hypoxia.

2.5.2. Advances over previous work

A major difficulty in securely extrapolating the findings of work using iron chelation (Balanos *et al.* 2002, Smith *et al.* 2008a) is that acute infusion of DFO is very different indeed from the insidious development of iron deficiency that is seen in clinical practice. Desferrioxamine permeates cell membranes slowly, preferentially depletes hepatic and reticuloendothelial iron, and cannot effectively chelate iron bound to circulating transferrin (Breuer *et al.* 2001). Thus the pattern of tissue iron depletion that results from an acute infusion of DFO is likely to differ considerably from that seen in naturally occurring iron deficiency. Moreover, DFO has actions aside from iron chelation: it participates in

oxidation and reduction reactions, and has free-radical scavenging properties (Zhu *et al.* 1998). As already discussed, ROS are implicated in hypoxia-sensing and signalling pathways in a number of ways (Weir *et al.* 2005), so DFO may interfere directly with human responses to hypoxia independently of any effect on iron bioavailability. Indeed, fatal pulmonary toxicity has been reported from DFO infusion used to treat ingestion of high doses of iron, possibly meditated by the production of ROS (Tenenbein *et al.* 1992).

Similarly, acute delivery of a large dose of iron intravenously cannot be assumed to be similar to a physiological state of greater iron repletion. A concern with previous studies (Smith *et al.* 2008a, Smith *et al.* 2009, Talbot *et al.* 2014) might be that the action is in some way dependent upon supraphysiological levels of serum iron, bringing about an effect that does not ordinarily occur when total body iron stores are higher but appropriately partitioned. The present study overcomes these problems and moves from short-term experimental manipulation of iron bioavailability to the demonstration that the predicted effects of absolute iron deficiency on responses to hypoxia are significant for human health.

2.5.3. Limitations

2.5.3.1. Haemoglobin concentration

Haemoglobin concentration in the ID group was somewhat lower than that in the IR group, though this is not surprising considering the severity of iron deficiency in those recruited to the present study. Indeed, a mean [Hb] of ~ 9 g/dl has previously been reported in a group of almost one hundred women and men (in a similar ratio to that of the present study) with nearly identical serum ferritin and TSat values, who had absent stainable iron on bone marrow examination (Krause and Stolc 1979). Though not unexpected, it must be considered whether this difference could have confounded the findings; specifically, could the greater hypoxic rise in PASP in the ID group have been explained by the lower [Hb]?

Evidence from a range of settings indicates that, rather than acting to augment HPV, a reduction in [Hb] has an attenuating effect. First, detailed animal experiments using perfused rabbit lungs (Deem *et al.* 1998) and intact dogs (Kerbaul *et al.* 2004) indicated that a lower haematocrit directly impaired HPV. Secondly, in high-altitude residents with PH secondary to chronic hypobaric hypoxia, isovolaemic haemodilution quickly brought about a fall in PASP and PVR (Winslow *et al.* 1985, Manier *et al.* 1988). Thirdly, in patients with severe chronic hypoxaemic lung disease, gradual reduction of [Hb] by repeated small volume venesection resulted in a significant fall in mPAP and PVR (Weisse *et al.* 1975).

Taking these observations together, it is very difficult indeed to see how a lower [Hb] could account for the much greater hypoxic PASP rise seen in the ID group since, if there is any influence, it would be anticipated to act in entirely the opposite direction. Indeed, some investigators argue that when examining the behaviour of the pulmonary circulation during hypoxia, a correction should be applied for this attenuating effect on HPV of a lower [Hb] (Naeije and Dedobbeleer 2013). Before moving on from this point, it is important to acknowledge the suggestion made by Smith *et al.,* that in conditions characterised by PH and polycythaemia, where venesection is used therapeutically, the well-recognised tendency of the benefit provided to diminish with time may well be explained by the concomitant induction of iron deficiency (Smith *et al.* 2008a).

Having argued that the causative nature of the relationship between the iron deficiency itself and exaggerated HPV is further attested by the significantly greater attenuation, by IV iron, of hypoxic pulmonary hypertension in the ID group, it should equally be considered whether the lower [Hb] contributed in some way to this finding. For instance, if the IV iron infused were somehow to be more bioavailable in the ID group, or taken up

more avidly by the pulmonary endothelium and PASMCs, then this might explain the greater 'sensitivity' to IV iron in the ID group.

Again, it appears to be the case that the direction of action of any such effect may reasonably be expected to be the opposite to that which would affect the main conclusions of the study. Radioactive isotope studies indicate that when infused into profoundly iron-deficient individuals, iron is directed rapidly towards erythropoiesis (Geisser and Burckhardt 2011); the bone marrow seems to take priority in this setting. Thus the expected effect is to constrain iron availability for the pulmonary vasculature in the ID group, not increase it.

It might be argued that this difference in [Hb] could have been avoided altogether if any of several possible strategies had been employed. For instance, some combination of allogenic blood transfusion in the ID group and isovolaemic haemodilution in the IR group could have been used, as in the studies in animals and patients discussed above. This would seem to be problematic ethically, particularly the transfusion of allogeneic blood with its attendant risks to individuals with a [Hb] within or close to the normal range. Also, venesecting the IR group would have been likely to induce by the time of the second study day a state of functional iron deficiency in many previously IR participants, hardly desirable given the aim of the study. Perhaps, though, exclusively ID individuals with a [Hb] well into the normal range could have been recruited? Though at first glance attractive, this strategy would arguably have been more likely to risk compromising the study than enhancing it, since it would have selected a biologically atypical group. With this approach, the ID group with have consisted exclusively of individuals able to preserve their [Hb] in the face of very marked iron deficiency, perhaps explained by physiological differences in iron and oxygen homeostasis, or the regulation of erythropoiesis itself.

2.5.3.2. Echocardiography for assessment of PASP

The utility of PASP as an index of HPV has already been discussed. The technique used to determine PASP in the present study relied on the presence of a systolic tricuspid regurgitant jet. With progressive technological advances in echocardiography it has become apparent that most healthy individuals have physiological TR, which is not considered a reflection of underlying pathology (Yoshida *et al.* 1988, Jobic *et al.* 1993, McQuillan *et al.* 2001). Invasive measurements of PAP correlate very well in a wide range of clinical settings with those obtained using echocardiography (Yock and Popp 1984, Berger *et al.* 1985, Currie *et al.* 1985, Chan *et al.* 1987, Stevenson 1989), and this holds true for healthy individuals rendered hypoxic (Allemann *et al.* 2000).

It is of interest, though, whether healthy individuals without readily demonstrable TR are biologically different in some way, since this might limit the generalisability of the findings of *Study A*. The prevalence of detectable TR has risen markedly as echocardiographic instruments have improved, without any accompanying change in the mean measured PASP (McQuillan *et al.* 2001, Okura *et al.* 2011). This implies that the absence of TR does not simply reflect lower pressures in the pulmonary circulation. Furthermore, mortality is no different in individuals with and without TR sufficient to determine PASP echocardiographically (Lam *et al.* 2009) so, if there are biological differences, their clinical significance is questionable. Associations between echocardiographic TR and characteristics of the left heart have been reported, including left atrial size, left ventricular end-diastolic diameter, and ejection fraction, though the size of the differences is small, and the direction of reported effects is not consistent (McQuillan *et al.* 2001, Okura *et al.* 2011).

Taking all this together, the possibility cannot completely be discounted that pulmonary vascular behaviour during hypoxia might differ, in some small way, in the minority of

healthy individuals who do not have TR sufficient to measure PASP echocardiographically, though it is difficult to see how this could have any bearing on the findings of *Study A*.

A final point in relation to echocardiography concerns the small difference in the density of blood that might be expected between ID and IR participants as a consequence of the marginally lower [Hb]. The modified Bernoulli equation used to determine ΔP_{MAX} will have assumed a value of 1.06 kg/L. That of plasma devoid of red cells is ~ 1.025 kg/L. With ~ 15% lower [Hb] in the ID group, the density would therefore be ~ 1.055 kg/L. Thus, the effect of the difference in [Hb] will be for Doppler echocardiography consistently to overestimate PASP in the ID group by around half of one percent, which clearly cannot compromise the findings of the present work.

2.5.3.3. Interval between study days

The hypoxic exposure on the first day induced a degree of acclimatisation. Experiments using similar sustained hypoxic exposures indicate that after return to euoxia for 3 hours, it is possible to demonstrate some residual PASP elevation and augmented HPV in response to a further acute hypoxic challenge (Dorrington *et al.* 1997). If a week of euoxia is allowed to pass, however, no elevation in euoxic PASP remains, and the magnitude of HPV returns completely to normal (Smith *et al.* 2008a, Herigstad and Robbins 2009). Thus, in the present study, an interval of a week or more was imposed between experimental days to ensure that the hypoxic exposure on the first day would not affect findings during the second. Acute IV iron loading does not alter the first phase of HPV (Smith *et al.* 2008a, Talbot *et al.* 2014), so within each group, the near identical euoxic PASPs and very similar acute PASP responses to hypoxia, on the second experimental day compared with the first, provide further evidence that the interval was sufficient for any acclimatisation to resolve.

2.5.3.4. Haematological parameters over the course of the study

The recruitment strategy and definitions of ID and IR adopted performed very well insofar as two groups were generated that, upon enrolment and throughout the course of the study, differed very significantly with respect to iron status, despite the absence of marked anaemia in the ID group. One thing this study cannot do, however, is determine the level of iron stores at which there ceases to be an effect on HPV. The addition of a group with higher physiological iron stores – a serum ferritin > 100 μg/L for instance – would be informative in this regard, though it might be challenging to recruit premenopausal females with such values.

As discussed, the study was designed in such a way as to ensure that any effects from the hypoxic exposure on the first study day had resolved by the time each participant's second study day was undertaken. The only other significant intervention – iron infusion – was given following venous blood sampling at the beginning of the second study day. Accordingly, sizeable changes over the course of the study in the euoxic laboratory parameters measured were not anticipated. As Table 2-2 makes clear, this was almost invariably the case. However, it was notable that in the ID group, serum iron – and therefore TSat – was significantly higher on both study days than it had been at the screening visit. A similar effect was absent in the IR group. It is possible that, since participants were told their iron status prior to enrolment to enable them to make an informed decision about participation, some altered their behaviour in the intervening period prior to the first study day to increase their dietary iron intake. Against this suggestion is that ferritin, [Hb], sTfR and hepcidin did not show any significant changes over the course of the study, which would have been expected if the iron balance of the group had altered to any extent.

A more likely explanation, in view of the great intra-individual variability in serum iron levels already discussed (Dale *et al.* 2002), is that the observed 'change' is nothing of the sort but is instead simply regression to the mean. Though the ID group had a mean TSat of 8.4% when enrolled – a rather extreme value – measuring the TSat a second and third time at the study visits has yielded less extreme results. That values on both study days were similar supports this view. Even if this apparent change is real, it does not affect the conclusions of the study in any way.

One additional finding that is noteworthy is of a persistently higher platelet count in the ID group, though values remain within what is considered the physiological range of 150-450 x 10^9/L. Significant IDA has long been recognised to be associated with thrombocytosis (Schloesser *et al.* 1965) and more recently some of the mechanisms by which iron deficiency influences megakaryopoiesis have been elucidated (Evstatiev *et al.* 2014). Importantly, though a single report suggests a possible influence of platelets on the potency of HPV in a rat lung preparation (Hauge and Melmon 1968), a collection of other studies that have examined this issue, including several using intact animals, together indicate that there exists no significant effect (Sylvester *et al.* 2012).

2.5.3.5. Duration of study

It would perhaps have been desirable to examine the behaviour of the pulmonary circulation in our participants a third or even a fourth time, to investigate the duration of the effects of iron loading on HPV, and the influence of a gradually climbing [Hb] in the ID group. Some previous related physiology studies have recruited volunteers willing to return repeatedly in such a manner. However, in the present work such a strategy might have risked dissuading ID participants from taking part. It must be remembered that many individuals who volunteered did so having been informed about *Study A* when deferred at a blood

donation session, rather than actively seeking out an opportunity to take part in a clinical physiology study involving two days confined to a normobaric chamber.

2.5.4. Comparison with previous studies

In the present study, euoxic PASP and the time course and magnitude of PASP responses to hypoxia in IR individuals, were very similar to those reported previously (Balanos *et al.* 2002, Balanos *et al.* 2005, Petousi *et al.* 2014, Talbot *et al.* 2014). However, an unexpected finding was the time course of the PASP response on the second study day. Following IV iron, both groups exhibited a peak in PASP after two hours of hypoxia, after which PASP declined. In a previous study using a similar duration of hypoxia, this secondary decline was not observed (Talbot *et al.* 2014). Instead, PASP was stable from one hour onwards. In that study, IV iron was administered as 200 mg iron-sucrose over 105 minutes, prior to hypoxia. This contrasts with the present study, in which up to 1000 mg ferric carboxymaltose was administered over 15 minutes prior to the exposure.

Iron-sucrose and ferric carboxymaltose differ quite significantly in the way they are handled after infusion. With iron-sucrose, the iron administered appears to be able rapidly to bind circulating transferrin (Geisser 2009), and thus can be taken up quickly by any tissue expressing TfR1. Ferric carboxymaltose, on the other hand, does not tend to liberate iron, instead requiring endocytosis by macrophages, which process and subsequently export iron into the circulation via ferroportin (Lyseng-Williamson and Keating 2009, Geisser and Burckhardt 2011), a process that will tend to delay the delivery of iron to circulating transferrin. Accordingly, the likely explanation for the difference between our findings and those of the previous study, is that the iron within ferric carboxymaltose, even at the higher dose, had less time to downregulate the mechanisms underlying the second phase of HPV,

before the hypoxia was introduced. Thus, in *Study A*, the second phase of HPV is evident in both groups on the second experimental day, before the effect of IV iron supervenes.

No differences were seen in ventilation between the groups, nor was a discernible acute effect of IV iron supplementation on ventilation evident. The method used to measure ventilation was rather indirect and certainly inferior to pneumotachography. Having said this, these findings are in agreement with those of a previous study in which acute iron chelation did not affect ventilation (Ren *et al.* 2000). As discussed, the PHD2-HIF-2α axis appears to be dominant in both the pulmonary vasculature and carotid body, so the apparent insensitivity of the latter to chronic differences in iron bioavailability and acute iron loading is puzzling. This may perhaps be explained by differences between the two related to the IRP-IRE system, peculiarities of mitochondrial biology in type I cells, or perhaps differences in iron-transport mechanisms.

2.5.5. Underlying mechanisms

This clinical physiology study was not designed to determine with certainty the precise mechanisms underlying the observed effects of iron status. Rather it was motivated ultimately by a knowledge of the molecular biology of iron- and oxygen-sensitive pathways, and a desire to determine whether *in vitro* phenomena translated into a meaningful effect for human physiology. This does not mean, though, that no attempt can be made to draw mechanistic inferences from some aspects of the present work.

As already discussed, a collection of animal and human studies have confirmed the centrality of the HIF pathway in coordinating pulmonary vascular responses to hypoxia. Though the initial, rapid phase of HPV that becomes maximal within minutes is too brisk to be mediated by a transcription factor pathway such as HIF, the second phase, which is

associated with acclimatisation, is a very different matter, and HIF plays a crucial role in the hypoxic responses of the pulmonary vasculature from this point onwards (Shimoda and Laurie 2014, Veith *et al.* 2016). With this in mind, that the effect of IV iron to attenuate PASP elevation during hypoxia was evident in *Study A* during the second phase of HPV, but not the first, is consistent with iron acting on hypoxia-regulated gene expression but not on those processes underlying acute HPV. A similar conclusion might be drawn from the behaviour observed in the previous study by Talbot and colleagues illustrated in Figure 2-10 (Talbot *et al.* 2014).

However, *Study A* cannot prove that iron deficiency and supplementation are acting through the HIF-hydroxylases, or for that matter the IRP-IRE system, to influence HIF-regulated gene transcription. Further evidence would come from direct examination of pulmonary arterial tissue, which is clearly not possible in healthy humans. It has been suggested that pulmonary arterial endothelial cells can be found in small numbers in the bloodstream, and that it might be possible to isolate these from the systemic circulation in humans (Bull *et al.* 2003). Determining with certainty their origin would be problematic, and it would also be difficult to know whether such cells were representative of those *in situ* within the pulmonary vasculature. Direct evidence of this sort from examination of animal tissue is, though, provided by the observation that air-breathing rats fed a very iron-restricted diet rapidly develop PH and RV hypertrophy, in association with increased lung expression of HIF-1α, HIF-2α, and genes of relevance to the pathogenesis of PH in humans known to be under transcriptional regulation by HIF (Cotroneo *et al.* 2015).

If iron is acting through HIF, then we would expect to find that expression of genes known to be of importance in regulating HPV, and indeed in the pathogenesis of PH in humans, is modulated by the HIF pathway. This does indeed appear to be the case. For example, the

gene encoding endothelin-1, *EDN1*, is directly transcriptionally regulated by HIF (Hu *et al.* 1998, Minchenko and Caro 2000), and endothelin-1 itself appears to play an important role in regulating the expression of voltage-gated K^+ channels in response to sustained hypoxia (Whitman *et al.* 2008). Transient receptor potential calcium channels, encoded by *TRPC1* and *TRPC6*, are also HIF-regulated (Wang *et al.* 2006), apparently via BMP4, the promoter region for which contains HIF binding sites (Wang *et al.* 2015).

It is worth briefly considering at this point the human disease idiopathic pulmonary arterial hypertension (IPAH), previously referred to as primary pulmonary hypertension; both names indicating that it does not arise secondary to any sort of primary disease of the lung parenchyma or airways. IPAH is a rare but devastating disease that predominantly afflicts young women. Mutations in BMPR2 underlie a significant proportion of cases of IPAH; a heritable form of the condition is recognised, and mutations may also contribute to PH arising in the setting of chronic lung disease (McLaughlin *et al.* 2009). IPAH will be considered further in Chapter 5, but it is relevant to the present discussion that, as mentioned earlier, BMP signalling plays an important role in human iron homeostasis by regulating hepatic hepcidin expression (Figure 1-12), and iron deficiency with elevated circulating hepcidin appears to be an significant pathophysiological feature in the condition (Rhodes *et al.* 2011, Ruiter *et al.* 2011, Soon *et al.* 2011). Importantly, human and animal studies also implicate activation of the HIF pathway in the pathogenesis of IPAH (Bonnet *et al.* 2006, Fijalkowska *et al.* 2010), though the relationship between disordered iron homeostasis and HIF activation in the condition is yet to be characterised.

2.5.6. Clinical implications of findings

The significance of the findings in the present study could be viewed from two angles. On the one hand, they might be taken to reveal ‘unexpected’ physiological consequences of

iron deficiency in otherwise healthy adults. This raises the possibly that some of the symptoms reported in clinical studies of individuals with NAID – typically premenopausal women – could be explained by an effect on oxygen-sensing and signalling pathways. Such symptoms include depression, fatigue and impaired concentration (Murray-Kolb 2011).

On the other hand, they demonstrate a fundamental aspect of the regulation of physiological responses to hypoxia in humans, raising the possibility that some of the clinical features in diseases in which hypoxia plays a role may be influenced by iron status. Of special interest in this regard, considerable evidence has emerged in recent years of the harm associated with iron deficiency in cardiopulmonary diseases, and of a benefit from administering IV iron in these conditions. Iron deficiency – independent of anaemia – appears to be particularly deleterious in pulmonary vascular disease (Rhodes *et al.* 2011, Ruiter *et al.* 2011, Soon *et al.* 2011, Ruiter *et al.* 2014, Viethen *et al.* 2014) and has also been linked to poorer outcomes in chronic heart failure (CHF) (Anker *et al.* 2009, Klip *et al.* 2013, Ponikowski *et al.* 2015) and acute heart failure (Jankowska *et al.* 2014). Whether iron deficiency might similarly play a role in COPD is a question that the work described in Chapter 5 addresses.

In each of these disease states, pathological upregulation by iron deficiency of HPV – and other HIF-regulated processes – offers a plausible mechanism by which these associations might be explained. Of note, severity of PH, which drives progressive RV hypertrophy and failure, predicts mortality in both IPAH (D'Alonzo *et al.* 1991) and PH secondary to a wide range of chronic lung diseases (Seeger *et al.* 2013). With respect to CHF, PH is an independent predictor of mortality whether function of the left ventricle (LV) is impaired or preserved (Kjaergaard *et al.* 2007). In animal models, cardiac-specific PHD inactivation was found to phenocopy ischaemic cardiomyopathy (Moslehi *et al.* 2010), and drastically

restricting iron supply to murine cardiomyocytes using targeted inactivation of TfR1 led to death in the second week of life with a similar cardiac phenotype (Xu *et al.* 2015). Additionally, a single small human study has suggested an improvement in LV function after correction of iron deficiency in humans with CHF and chronic renal impairment (Toblli *et al.* 2015).

2.6. Conclusions

This component of *Study A* provides the first evidence of a clinically meaningful effect of absolute iron deficiency on pulmonary vascular biology. It implies that iron status modulates the HIF pathway *in vivo* in a significant way, and it confirms the potential of manipulation of iron homeostasis as a tool to treat diseases in which hypoxia plays a role.

Chapter 3. Interactions of iron- and oxygen-signalling pathways

3.1. Introduction

In addition to the work described in the previous chapter, during the course of *Study A* measurements were made of mediators of importance for iron and oxygen homeostasis: erythropoietin, hepcidin and IL-6. It is these that are the subject of this chapter. Before describing the methodologies used it will be useful to revisit some of the studies considered previously, as well as others that are of relevance, particularly those in which the interaction of iron- and hypoxia-regulated pathways was a specific focus.

3.1.1. Erythropoietin

3.1.1.1. Historical aspects

> *"During the past century, few proteins have matched erythropoietin in capturing the imagination of physiologists, molecular biologists, and, more recently, physicians and patients. Its appeal rests on its commanding role as the premier erythroid cytokine, the elegant mechanism underlying the regulation of its gene, and its remarkable impact as a therapeutic agent..." (Bunn 2013)*

Denis Jourdanet, who spent many years during the middle of the 19th Century practising high-altitude medicine in Mexico, was probably the first to recognise the polycythaemia that occurs at high altitude, manifest as an increase in the viscosity of the blood (Jourdanet 1863). The much better known French physiologist Paul Bert, with whom Jourdanet worked, then correctly surmised that this was explained by an increase in the concentration of circulating red cells (Bert 1878).

Credit for the first clear description of the erythropoietic response to hypobaric hypoxia in humans is, though, generally given to another Frenchman, Francois-Gilbert Viault, who observed a rise in his own red cell count from ~ 5 to over 7 million/mm^3 during a two-week sojourn at an altitude in excess of 4,200 m in a mountainous region of Peru (Viault 1890). Subsequently, a clear relationship in health between haematocrit and altitude of residence (Figure 3-1) was demonstrated by Mabel FitzGerald, who made careful measurements in individuals residing over a range of elevations as part of the Pike's Peak expedition (FitzGerald 1913), but who receives almost no recognition for her painstaking work. Later studies confirmed the relationship she described (Talbott 1936, Hurtado *et al.* 1945).

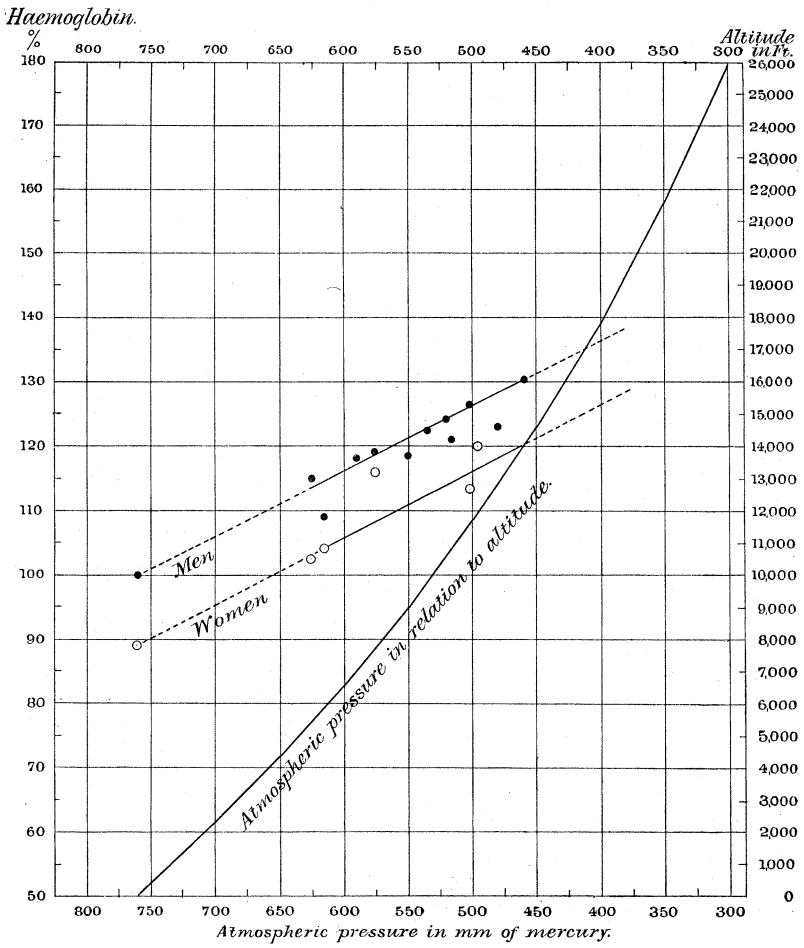

Figure 3-1. Relationship of haematocrit to atmospheric pressure in the Pike's Peak Expedition

Values are based on 128 separate blood samples taken from individuals who had been resident for at least a year at high altitude. Mean haematocrit (left hand scale) is expressed as a percentage of that determined for males at sea level in Oxford. Filled circles represent values for males; open circles, values for females. The right hand scale relates to the curved line describing the relationship between altitude and atmospheric pressure. Reproduced from (FitzGerald 1913).

The first proposed mechanism for the hypoxic stimulation of erythropoiesis that resembles the physiology as we understand it today came with the observation that the red cell count in healthy rabbits could be increased by infusion of serum taken from anaemic animals (Carnot and Deflandre 1906). This suggested regulation by a humoral factor in the plasma, rather than a direct action of hypoxia on the bone marrow. Much later, fundamentally

similar experiments that incorporated determination of reticulocyte counts confirmed the induction of new red cell production in animals by injection of anaemic serum (Krumdieck 1943, Bonsdorff and Jalavisto 1948, Oliva *et al.* 1949, Erslev 1953).

Around the same time, experiments using parabiotic pairs of rats – animals whose circulations had been connected by means of overlapping flaps of skin – showed that when only one rat was rendered hypoxic, both animals exhibited a marked increase in new red cell production (Reissmann 1950, Ruhenstroth-Bauer 1950). Collectively, these experiments provided strong evidence for the existence of a circulating factor, which came to be known as erythropoietin. Subsequently, the kidney was demonstrated to be the major, but not sole, site of production of erythropoietin in adult animals and humans (Jacobson *et al.* 1957, Nathan *et al.* 1964), and some time later, with considerable technical difficulty, the protein itself was purified (Miyake *et al.* 1977).

3.1.1.2. Role of the HIF pathway in regulating erythropoietin expression

The cells responsible for renal erythropoietin synthesis were identified using transgenic mice as being fibroblast-like cells located in the interstitium of the cortex and outer medulla (Maxwell *et al.* 1993a). By experimentally inducing renal ischaemia in rats it was further possible to demonstrate within these cells nuclear accumulation of HIF-2α under hypoxic conditions, whereas HIF-1α was stabilised elsewhere within the organ (Rosenberger *et al.* 2002), implying a selective role for the former in regulation of renal erythropoietin production. Subsequently, using conditional postnatal ablation of HIF-2 in mice, it was confirmed that this paralogue was necessary for normal adult erythropoiesis, since acute deletion led to anaemia (Gruber *et al.* 2007). Furthermore, somatic PHD2 inactivation was shown to promote erythropoietin overexpression, leading to profound polycythaemia (Minamishima *et al.* 2008), which again implied that, as in the pulmonary vasculature and

carotid body, the PHD2-HIF-2α axis was central. However, since HIF-2α had also been shown to regulate hepatic production of erythropoietin (Rankin *et al.* 2007), experiments using specific deletion of HIF-2α in the murine kidney were necessary to demonstrate that renal erythropoietin was exclusively HIF-2α regulated (Kapitsinou *et al.* 2010).

Just as rare genetic diseases affecting the HIF pathway disturb human pulmonary vascular physiology, so too do they dysregulate erythropoiesis. Percy *et al.* have described a family with (inappropriately) normal circulating erythropoietin levels despite polycythaemia, in whom a mutation in PHD2 was identified (Percy *et al.* 2006). Experiments *in vitro* suggested impaired HIF-α binding by, and greatly diminished HIF-hydroxylase activity of, the mutant PHD2, effectively causing near-haploinsufficiency of PHD2. Subsequently, a mutation in HIF-2α was reported to cause a similar picture (Percy *et al.* 2008), the molecular basis being that the altered residue was close to one of the two proline residues targeted for hydroxylation by the PHDs.

These experiments of nature further confirm the centrality of the PHD2-HIF-2α axis in regulating erythropoietin synthesis in adult humans. With increased recognition that HIF-pathway mutations may underlie familial erythrocytosis syndromes (McMullin 2010, Lee and Percy 2011), further reports have emerged of other mutations with variable phenotypic characteristics (Percy *et al.* 2012, Lorenzo *et al.* 2013, Perrotta *et al.* 2013, Yang *et al.* 2013). It has been pointed out that such mutations do not solely affect renal erythropoietin production, but appear in some cases to alter the sensitivity of erythrocyte precursors themselves to the hormone (Lanikova *et al.* 2013), such that there is a dual mechanism of action operating. We have seen this complication arise via a different mechanism when considering the origin of excessive erythropoiesis in patients with CP (Russell *et al.* 2011).

3.1.1.3. The kidney as a haemoglobin sensor

Why should the kidney be the site of erythropoietin synthesis? This question was elegantly posed in 1985 by Erslev and colleagues, who observed that:

> *"Severe erythrocytosis is associated with increased whole blood viscosity and impaired blood flow. Since a reduced blood flow will cause tissue hypoxia and since tissue hypoxia is associated with increased synthesis of erythropoietin, erythrocytosis per se should cause an increase in the rate of red cell production. This, however, does not occur and severe erythrocytosis in patients with polycythaemia vera does not lead to increased synthesis of erythropoietin." (Erslev et al. 1985)*

Erslev *et al.* hypothesised that a unique aspect of renal physiology explained the main location for erythropoietin production being the kidney. Specifically, decreased blood flow to the organ does not, in fact, cause renal tissue hypoxia. They argued that renal oxygen tension was largely determined by the oxygen consumption requirement for sodium reabsorption; since sodium reabsorption could be expected to be proportional to glomerular filtration, a decreased flow of blood to the kidney should be matched by a decrease in oxygen consumption. Thus, renal tissue oxygen tension could be expected to be effectively independent of renal perfusion, and instead mainly determined by blood oxygen content, C_aO_2 (Erslev *et al.* 1985).

Outside a hyperbaric chamber C_aO_2 is, of course, primarily a reflection of [Hb]. So it can be seen that the kidney uses an oxygen-sensing pathway to sense [Hb]. As an unavoidable consequence, a fall in blood oxygen content due to hypoxaemia without any change in [Hb] acts to stimulate erythropoietin production. An interesting upshot of this arrangement is that the fall in renal erythropoietin output in the setting of chronic renal disease, which contributes very significantly to the anaemia seen in the condition, might be viewed in part

as resulting from a state of relative renal hyperoxia. In the situation where the kidney remains perfused but no longer consumes as much oxygen doing the work of tubular reabsorption, the stimulus to produce erythropoietin is impaired (Eckardt *et al.* 1989b, Priyadarshi *et al.* 2002). In this setting, stabilising HIF-2α and reintroducing the hypoxic signal using small molecule inhibitors of the PHDs seems to be effective in correcting this blunted erythropoietin response to renal anaemia (Bernhardt *et al.* 2010, Flamme *et al.* 2014), though systemic changes in iron metabolism also appear to contribute significantly (Provenzano *et al.* 2016).

3.1.1.4. Hypoxia-driven erythropoietin synthesis

Textbooks of high-altitude medicine and physiology, as well as reviews on the subject, not infrequently give the impression that hypoxic stimulation of erythropoiesis has been subject to selection pressure because it is a mechanism that is of benefit on ascent to high altitude. This idea is attractive, since expansion of the red cell mass is an effective way to improve oxygen delivery to tissues in the face of hypobaric hypoxia. However, there are multiple difficulties with this argument, not least that there has been no sustained selection pressure of this sort on the majority of the world's population. Moreover, as already discussed, in those regions where such a pressure has existed, such as the Tibetan plateau, the effect seems to be in the opposite direction; a genetic make-up restraining marked erythrocytosis appears advantageous. Also, if we examine the haematological adaptations that have occurred in high-altitude vertebrates, an increased haematocrit does not appear to be an advantageous strategy to overcome the lower P_{IO_2}. Instead, sequence changes that alter the affinity of haemoglobin for oxygen have undergone positive selection; these act to optimise tissue oxygen delivery despite hypobaric hypoxia (Storz and Moriyama 2008).

Although erythropoietin synthesis in response to the renal tissue hypoxia brought about by a fall in ambient oxygen tension, rather than a fall [Hb], might teleologically be described as unintentional, studies at altitude – both simulated and actual – nevertheless offer an excellent opportunity to investigate the underlying mechanisms regulating erythropoietin synthesis, erythropoiesis, and iron metabolism. Initial studies clearly demonstrated a very marked rise in circulating erythropoietin on exposure to hypobaric hypoxia (Siri *et al.* 1966, Faura *et al.* 1969, Abbrecht and Littell 1972, Milledge and Cotes 1985), as well as the tendency for levels to fall back to near-normal with time despite absent or only very small increases in [Hb]. Improved temporal resolution with respect to the early phase of erythropoietin production during acute exposure to hypoxia was provided by a study using simulated altitudes of 3,000 m and 4,000 m in a decompression chamber, coupled with a sensitive radioimmunoassay. With this approach, Eckardt and colleagues were able to detect a rise in serum erythropoietin after 114 and 84 minutes, respectively (Eckardt *et al.* 1989a) (Figure 3-2), much earlier than the figure of around 12 hours reported previously.

Subsequently, in a study involving ascent by a small number of individuals to, and prolonged residence at, extreme altitude (6,542 m), Richalet *et al*. reported a close correlation between early changes in C_aO_2 and erythropoietin production (Richalet *et al.* 1994). This study also examined the influence of iron on erythropoietin and erythropoiesis, but the authors did not feel able to conclude more than that iron stores seemed to be one of several factors of importance. Though the liver is also able to synthesise erythropoietin, a study using glycoform heterogeneity analysis supports the view that the kidney is the predominant source in adults for the increase in plasma erythropoietin seen during hypoxia (Lundby *et al.* 2014).

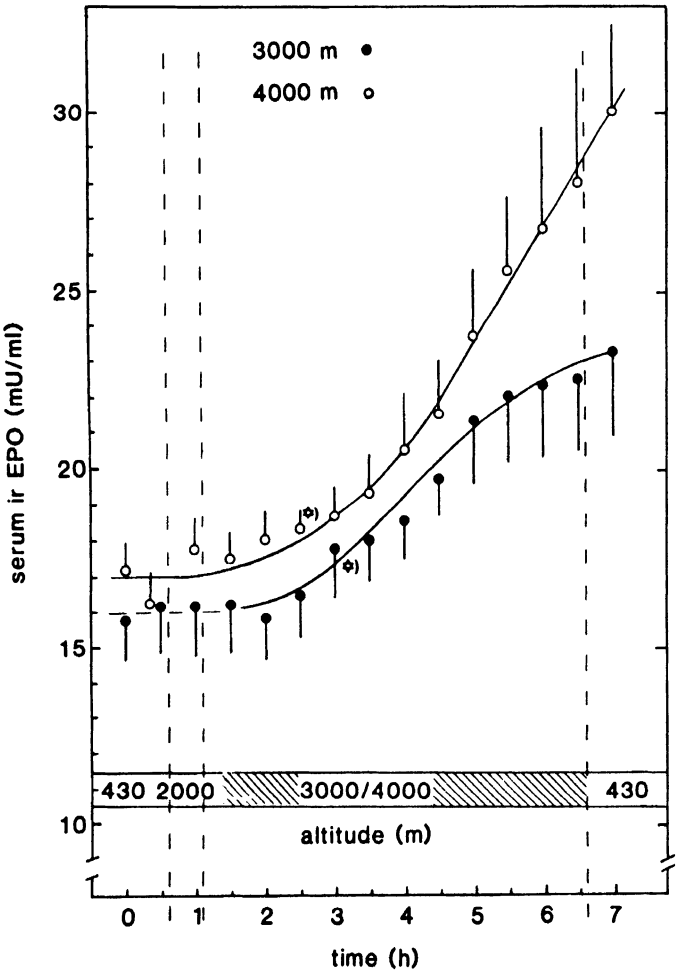

Figure 3-2. Time course of serum erythropoietin in response to hypobaric hypoxia

Data are for six healthy young men exposed to simulated altitude in a decompression chamber for five-and-a-half hours. Each subject was exposed to 3,000 m and 4,000 m, with exposures separated by an interval of over three weeks. Atmospheric pressure was gradually reduced from an initial value of 720 Torr (430 m above sea level) to 520 Torr and 460 Torr, respectively, within 30 minutes, with a 15-minute interruption at 600 Torr (2,000 m). Dashed vertical lines indicate points at which pressure within the chamber was altered. *, $P < 0.05$ for within-group comparison with time zero; ir EPO, immunoreactive erythropoietin. Reproduced from (Eckardt *et al.* 1989a).

3.1.1.5. Iron bioavailability and erythropoietin synthesis

The central part played by erythropoietin in the history of the field of iron and hypoxia sensing, including early studies with cobalt and iron chelation, was described in Chapter 1.

Of particular interest is the repeated observation that erythropoietin levels appear to be higher, at any given [Hb], in patients with IDA than in those with anaemias of other common aetiologies, including pernicious anaemia (Ward *et al.* 1971, Sheridan and Gatenby 1975, de Klerk *et al.* 1981, Camacho *et al.* 1991). This phenomenon generally seems to be taken to indicate an inappropriately low synthesis of erythropoietin in anaemias not due to iron deficiency. Indeed, a contemporary study examining anaemia in patients aged 60 years or older found that erythropoietin levels in chronic kidney disease (CKD), anaemia of chronic disease (ACD) and anaemia of uncertain aetiology, were lower by 48%, 46% and 27%, respectively, compared with IDA, even after adjusting for [Hb], estimated glomerular filtration rate (eGFR), and comorbidities (Gowanlock *et al.* 2016). This finding was interpreted as indicative of inappropriately low erythropoietin in anaemia of unknown aetiology. It might be argued instead that erythropoietin is peculiarly high in IDA.

Though these observations from anaemic patients provide some insight, studies using iron chelation are more informative. Following the demonstration that DFO could induce *EPO* expression in both cell culture and the intact mouse (Wang and Semenza 1993), Kling and colleagues showed using an anti-TfR antibody that blockade of transferrin-mediated iron uptake stimulated erythropoietin production *in vitro* (Kling *et al.* 1996). The induction of erythropoietin either by this antibody or by DFO could be suppressed by adding iron to the culture medium. These investigators also treated healthy volunteers with DFO linked to hydroxyethyl starch (HES-DFO) and found a significant rise in serum erythropoietin, but only after several days had elapsed. They also administered the anti-TfR antibody to patients with advanced malignancy and demonstrated a similar effect.

Ren and colleagues also explored the increased circulating erythropoietin levels brought about in healthy humans by iron chelation. An increase in serum erythropoietin was seen

by the end of an 8-hour DFO infusion, much sooner than with HES-DFO, and this persisted for four hours after cessation of the infusion, and in some cases until the next day (Ren *et al.* 2000). Though no significant correlation between the erythropoietin response to DFO and iron status was evident, these authors did observe the greatest erythropoietin responses in the two subjects with the lowest levels of serum ferritin.

Plasma erythropoietin concentrations were also determined in the study by Smith *et al.* investigating the effects on the rapid phase of HPV of both DFO and IV iron supplementation. In the DFO protocol, erythropoietin levels were initially similar on the two experimental days (mean 10.4 mIU/ml with saline vs 8.3 mIU/ml with DFO), and DFO again induced a significant rise, in percentage terms, in plasma erythropoietin (mean increase 2% with saline vs 794% with DFO). Levels prior to the infusions in the iron protocol were also initially similar on the two experimental days (mean 13.2 mIU/ml with saline vs 13.3 mIU/ml with iron), but rose to similar degrees during the 8-hour hypoxic exposure, whether saline or iron was administered (mean increase 67% with saline vs 61% with iron) (Smith *et al.* 2008a). Thus, acute iron loading with 200 mg iron-sucrose did not appear significantly to attenuate the plasma erythropoietin response to sustained moderate alveolar hypoxia.

It would be of interest to know whether iron chelation exaggerates the erythropoietin response to hypoxia, as a synergistic effect might perhaps be expected via a common action on HIF-hydroxylase activity. Such an effect was not seen *in vitro* (Kling *et al.* 1996), nor in a human experiment addressing this question that has been reported in abstract form (Talbot *et al.* 2007).

3.1.2. Hepcidin

Early ferrokinetic studies suggested that iron absorption from the gut was regulated not only by body iron stores but also the prevailing rate of erythropoiesis (Bothwell *et al.* 1958). From these observations came the concept of both a "store regulator" and an "erythroid regulator" of iron homeostasis (Finch 1994). Though we now understand that hepcidin integrates both of these signals, many aspects are still poorly understood. One question of particular interest relates to the nature of the pathway that signals enhanced erythropoietic activity, and thus iron demand, to suppress hepcidin and promote increased iron availability for red cell production.

It is clear that suppression of hepcidin in response to anaemia requires erythropoietic activity, rather than simply being a direct action of erythropoietin, from experiments in mice using venesection and myelotoxic agents (Pak *et al.* 2006), and somewhat more complex studies in genetically modified animals in which erythropoietin synthesis is uncoupled from HIF activation (Liu *et al.* 2012). Based on a study in healthy humans ascending to high altitude, Piperno *et al.* argued that iron itself (or the kinetics of iron use in response to hypoxia) was the signal responsible for hepcidin downregulation (Piperno *et al.* 2011). This suggestion was subsequently refuted by a study of similar design but offering higher temporal resolution, which demonstrated that the suppression by hypobaric hypoxia of hepcidin was too rapid to be driven by a reduction in iron availability (Talbot *et al.* 2012). By iron loading some of the volunteers prior to ascent in the latter study, it was possible to show that during the period for which TSat remained elevated, but not during the entire period for which serum ferritin remained elevated, the suppression of hepcidin by hypoxia could be overcome. Talbot and colleagues noted that this observation was consistent with the notion of TSat as the major signal linking iron availability to hepcidin upregulation (Lin *et al.* 2007, Schmidt *et al.* 2008).

As for the identity of the putative agent suppressing hepcidin, this has been given the name erythroferrone, and reported to be produced by erythroblasts in response to erythropoietin during stress erythropoiesis in mice (Kautz *et al.* 2014). Animals deficient in erythroferrone were observed to fail to suppress hepcidin rapidly after haemorrhage, exhibiting a delayed recovery from blood loss, but a recovery none the less. This area is somewhat controversial at the time of writing, since the gene encoding erythroferrone in the mouse appears to be homologous to that in humans encoding the adipokine myonectin, reported previously to have a somewhat different role in skeletal muscle and lipid metabolism (Seldin *et al.* 2012). Furthermore, when myonectin was measured in historical samples from participants in a high-altitude study, no clear role for circulating myonectin could be determined in linking erythropoietic drive to hepcidin suppression (Talbot *et al.* 2016).

3.2. Aims

This component of *Study A* aimed to determine the effect of iron status on erythropoietin and hepcidin responses to sustained moderate hypoxia. It was hypothesised that iron deficiency would exaggerate the synthesis of erythropoietin, and the suppression of hepcidin, in response to hypoxia.

3.3. Methods

The bulk of the experimental protocol and details of statistical analyses for *Study A* have already been described in section 2.3. At all time points where venous blood was sampled, aliquots of serum and plasma were immediately frozen and stored at -80°C until the conclusion of the final study visit of the final participant, following which they were analysed by ELISA. The blood sample drawn at the end of each study day was taken with

the participant still in the normobaric chamber whilst the P_{ETO_2} remained at 55 mmHg. Serum erythropoietin, plasma sTfR, and serum IL-6 were analysed by sandwich ELISA (all Quantikine®, R&D Systems, Abingdon, UK). Plasma hepcidin was analysed using a competitive ELISA (Hepcidin-25 EIA kit, Bachem, Peninsula Laboratories, San Carlos, USA).

The main outcome of interest was the change in a given blood parameter over the six-hour hypoxic exposure on the first study day in ID compared with IR participants. Additionally, the manner in which behaviour on the second study day, when iron was infused at the outset, compared with the first, was also assessed. The statistical approach was as described in section 2.3.2, except that in addition, Spearman's rank correlation coefficient (ρ) was calculated to investigate correlations between non-normally-distributed variables, and multiple linear regression analysis was used to explore factors that might determine the change in a variable during hypoxia.

3.4. Results

Table 3-1 gives values and statistical comparisons for erythropoietin, hepcidin and IL-6 in each group on each study day. In all three cases, there were no significant within-group differences in mean starting values between the first and second study days.

3.4.1. Erythropoietin

Serum erythropoietin was higher prior to the hypoxic exposure in the ID than the IR group at the start of both study days but within-group values on the two days were similar (ID vs IR: mean 25.4 vs 7.5 mIU/ml, on the first day; 20.1 vs 6.8 mIU/ml, on the second day; $P = 0.018$ for group; $P = 0.08$ for day; $P = 0.16$ for interaction). Erythropoietin increased

significantly during the hypoxic exposure on the first study day irrespective of iron status ($P < 0.001$ for effect of time); however, the effect was greater in the ID group (absolute rise, ID vs IR: mean 12.1 vs 6.2 mIU/ml; 95% CI for difference: 0.06 – 11.6 mIU/ml; $P = 0.048$). Erythropoietin rises during the hypoxic exposure on the second experimental day were not significantly different from those observed on the first ($P = 0.13$ for interaction between time and day; $P = 0.47$ for interaction between group, time and day). These findings are illustrated in Figure 3-3.

		ID Group (n=13)		IR Group (n=13)		*P*-value for fixed effects (MEM)			
								Interactions	
		Start	End	Start	End	Time	Group	Time & group	Day, time & group
Erythropoietin (mIU/ml)	**Day 1**	25.4 ± 7.0	37.4 ± 9.5	7.5 ± 0.6	13.7 ± 1.2	**< 0.001**	**0.018**	**0.048**	0.47
	Day 2	20.1 ± 4.4	36.4 ± 9.9	6.8 ± 0.8	14.6 ± 1.6				
Hepcidin (µg/L)	**Day 1**	4.5 ± 1.6	6.0 ± 2.8	20.1 ± 4.8	36.5 ± 8.8	**0.015**	**0.005**	**0.039**	**< 0.001**
	Day 2	3.2 ± 1.2	9.5 ± 4.6	18.9 ± 4.3	78.1 ± 10.5				
IL-6 (ng/L)	**Day 1**	0.6 ± 0.2	2.0 ± 0.4	1.1 ± 0.3	2.6 ± 0.7	**0.001**	0.21	0.76	0.58
	Day 2	0.7 ± 0.1	2.1 ± 0.4	0.9 ± 0.2	2.0 ± 0.5				

Table 3-1. Behaviour of erythropoietin, hepcidin and IL-6 on both days in *Study A*

Values are means ± SEM. *P*-values for comparisons where statistically significant differences are present appear in bold.

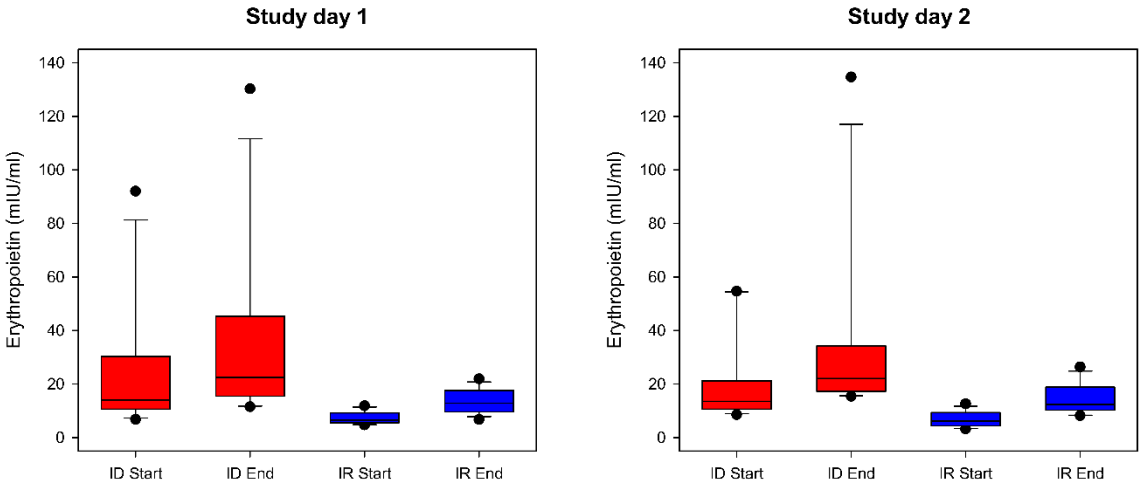

Figure 3-3. Erythropoietin responses during hypoxia

Box plots show the distribution of serum erythropoietin levels in the ID (red) and IR (blue) groups prior to (start) and at the end of each hypoxic exposure. Boxes show IQR; the median is indicated by the horizontal line; whiskers are 10th and 90th centiles; circles are values falling above the 90th or below the 10th centile. Details of statistical comparisons are given in the main text.

3.4.2. Hepcidin

Plasma hepcidin was lower prior to the hypoxic exposure in the ID than the IR group at the start of both study days but within-group values on the two days were similar (ID vs IR: mean 4.5 vs 20.1 µg/L, on the first day; 3.2 vs 18.9 µg/L, on the second day; $P = 0.005$ for group; $P = 0.63$ for day; $P = 0.97$ for interaction). Unexpectedly, hepcidin increased significantly during the hypoxic exposure on the first study day irrespective of iron status ($P = 0.015$ for effect of time). However, the effect was greatly attenuated in the ID group (absolute rise, ID vs IR: mean 1.5 vs 16.4 µg/L; 95% CI for difference: 0.78 – 29.1 µg/L; $P = 0.039$).

Hepcidin again rose in both groups during the hypoxic exposure on the second experimental day but the magnitude was significantly increased following IV iron ($P < 0.001$ for

interaction between time and day). However, the size of this effect differed between groups ($P < 0.001$ for interaction between group, time and day); the increase in rise was attenuated in the ID group (absolute increase in rise compared with first study day, ID vs IR: mean 4.8 vs 42.8 µg/L; 95% CI for difference: 20.1 – 55.8 µg/L; $P < 0.001$). These findings are illustrated in Figure 3-4.

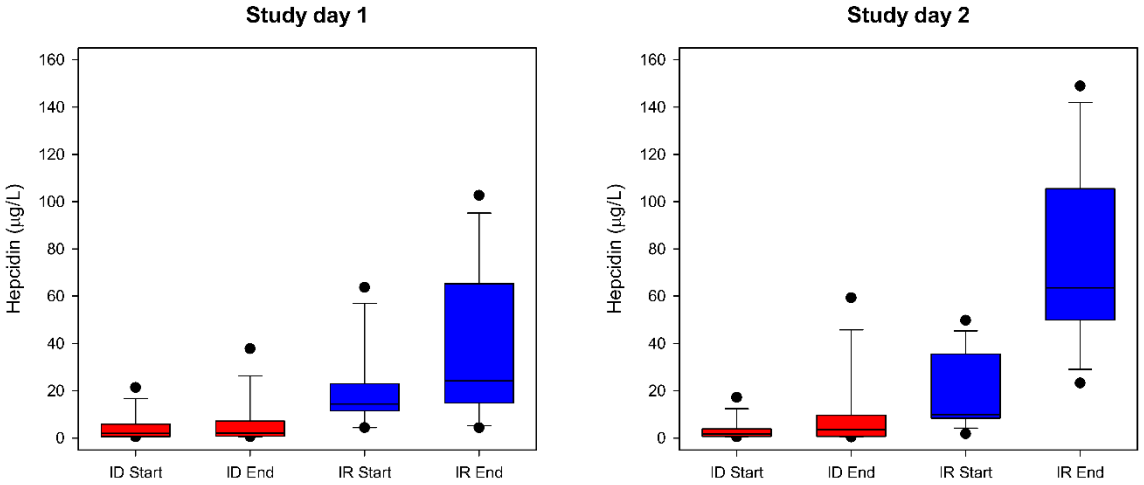

Figure 3-4. Hepcidin responses during hypoxia

Box plots show the distribution of plasma hepcidin levels in the ID (red) and IR (blue) groups, in the manner described for Figure 3-3. Details of statistical comparisons are given in the main text.

3.4.3. Interleukin-6

Serum IL-6 prior to each hypoxic exposure did not differ by group or study day (ID vs IR: mean 0.6 vs 1.1 ng/L, on the first day; 0.7 vs 0.9 ng/L, on the second day; $P = 0.21$ for group; $P = 0.81$ for day; $P = 0.53$ for interaction). IL-6 increased significantly during the hypoxic exposure on the first study day irrespective of iron status but there was no difference in the rise between groups (absolute rise, ID vs IR: mean 1.3 vs 1.6 ng/L; 95%

CI for difference: -1.8 – 1.4 ng/L; $P = 0.76$). IL-6 rises during the hypoxic exposure on the second study day were not significantly different to those observed on the first ($P = 0.60$ for interaction between time and day; $P = 0.58$ for interaction between group, time and day). These findings are illustrated in Figure 3-5.

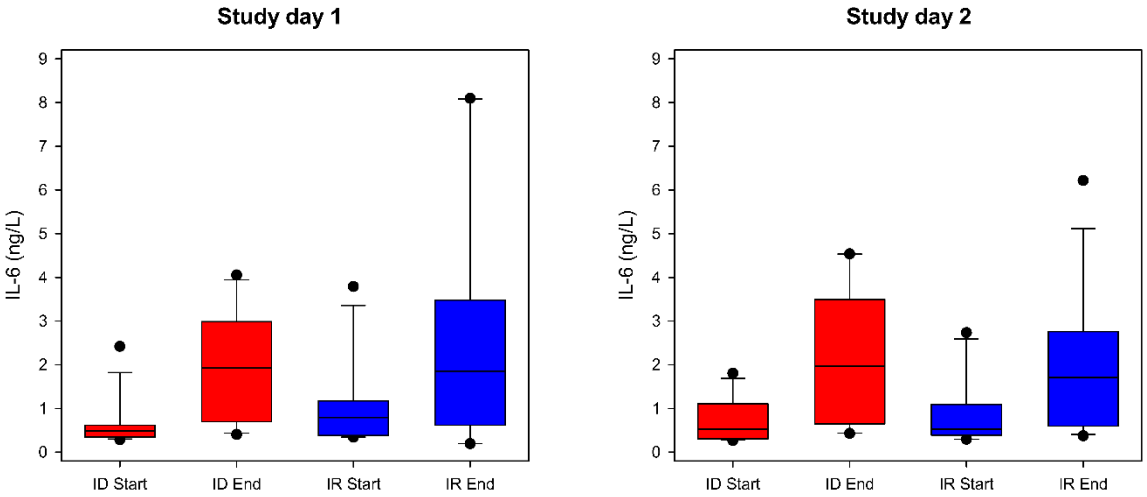

Figure 3-5. IL-6 responses during hypoxia

Box plots show distribution of serum IL-6 levels in the ID (red) and IR (blue) groups in the manner described for Figure 3-3. Details of statistical comparisons are given in the main text.

3.4.4. Relationships between haematological variables

In the ID group, euoxic serum erythropoietin on the first study day correlated equally strongly with both serum ferritin and [Hb] ($\sigma = -0.69$, $P = 0.009$, for both relationships). Serum ferritin and [Hb] also correlated with one another ($\sigma = 0.65$, $P = 0.016$). Thus, multiple collinearity was identified for these variables in the ID group. In contrast, euoxic serum erythropoietin in the IR group on the first study day did not correlate with either serum ferritin ($\sigma = 0.02$, $P = 0.92$) or [Hb] ($\sigma = -0.165$, $P = 0.58$). Serum ferritin and [Hb]

did, though, correlate with one another ($\sigma = 0.64$, $P = 0.017$), as in the ID group. The relationship between euoxic serum erythropoietin and [Hb] in each group is illustrated in Figure 3-6.

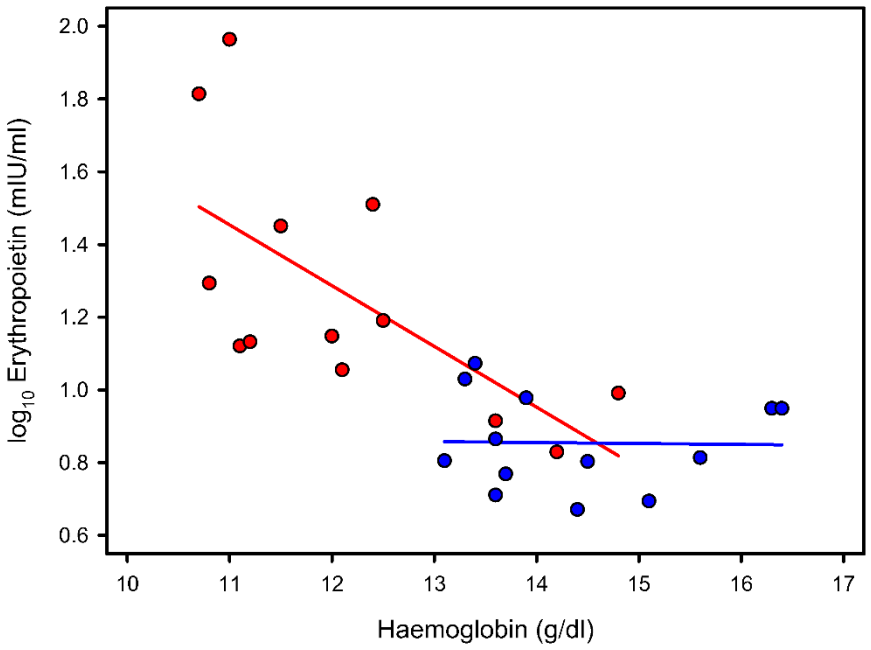

Figure 3-6. Relationship between euoxic serum erythropoietin and haemoglobin concentration

Data are from venous blood samples drawn at the start of the first study day. Red circles represent ID participants; blue circles, IR participants. A linear line of best fit is shown for each group over their respective ranges. There were insufficient data to determine with appropriate power whether iron status moderated the relationship between serum erythropoietin and [Hb].

Multiple linear regression analysis indicated that in both groups, erythropoietin levels at the end of the hypoxic exposure on the first study day could be predicted by erythropoietin levels at the start (adjusted $R^2 = 0.98$, $P < 0.001$, for the ID group; adjusted $R^2 = 0.53$, $P = 0.003$, for IR group). In both cases, inclusion of neither serum ferritin nor [Hb] improved the model. The same was true of the erythropoietin responses on the second study

day. In the case of hepcidin, similar analyses indicated that in both groups, levels at the end of the hypoxic exposure on the first study day could be predicted by hepcidin levels at the start (adjusted $R^2 = 0.89$, $P < 0.001$, for ID group; adjusted $R^2 = 0.38$, $P = 0.014$, for IR group). In both cases, inclusion of neither serum ferritin nor IL-6 values improved the model. The same was true of the hepcidin responses on the second study day. With respect to IL-6 levels at the end of each hypoxic exposure, no variables were identified with significant predictive power in either group, on either day.

3.5. Discussion

3.5.1. Erythropoietin

As expected, serum erythropoietin levels rose during hypoxia in both groups on the first study day. In accordance with the study hypothesis, the rise was greater in the ID group. However, euoxic levels were also greater in the ID group, and because ID participants had a lower [Hb] the question arises as to whether this accounts for these findings, either alone or in combination with the iron deficiency. Unfortunately, the multiple collinearity of ferritin, [Hb] and euoxic erythropoietin evident in the present study makes it difficult to draw firm conclusions. That is, the more severely iron deficient participants were, the lower their [Hb] tended to be, which is not surprising.

The analysis represented in Figure 3-6 suggests, but cannot prove, that iron deficiency alters the relationship in euoxia between [Hb] and erythropoietin, which would provide support for a direct action of iron on erythropoietin expression *in vivo*. However, it is conceded that this arguably adds little to the studies in frankly anaemic patients already described. Since the only variable found to have predictive value for the rise in erythropoietin during hypoxia

was the euoxic erythropoietin level, it is also difficult to draw any inferences about the effect of iron deficiency in this regard.

Though it would be possible to estimate for each participant the change in renal oxygen delivery as a consequence of the fall in C_aO_2 during hypoxia, which is the ultimate stimulus for renal erythropoietin synthesis (Wenger and Hoogewijs 2010), in the absence of data regarding the half-life of circulating erythropoietin and the kinetics of synthesis for a given change in C_aO_2, both of which are likely to vary considerably between individuals, this would be unlikely to provide much further insight.

Acute IV iron loading had no detectable effect on the erythropoietin responses during hypoxia in either group, mirroring the findings of the one previous study to examine this issue (Smith *et al.* 2008a). This was despite the present study using a dose up to five times higher. It may be that there simply was not sufficient time for the ferric carboxymaltose, even at this higher dose, significantly to increase the intracellular iron concentration within renal interstitial fibroblasts, especially given the delay in onset of action seen in the pulmonary vasculature, the reasons for which have been considered in Chapter 2. With this in mind, it is of note that in the study by Smith and colleagues at high altitude in Peru, the group of participants given IV iron after three days at altitude did show a trend towards a more rapid fall in circulating erythropoietin levels (Smith *et al.* 2009), suggesting a direct effect of iron to supress erythropoietin synthesis.

Alternatively, renal interstitial fibroblasts may be peculiar with respect to the manner in which they acquire iron, being somehow resistant in the face of acute elevations in circulating levels compared with the pulmonary endothelium and smooth muscle cells. Renal iron biology is increasingly the focus for research efforts, though studies tend to

centre on tubular iron handling and the role of the kidney in whole-body iron homeostasis (Smith and Thevenod 2009). Data relating to the regulation of intracellular iron levels within renal interstitial fibroblasts appear to be absent. Notwithstanding this gap in our knowledge, many authorities appear to imply that the IRP-IRE system is certain to be a physiologically important regulator of HIF-2α translation, and thus erythropoietin expression, in humans, with intracellular iron deficiency taken to be a factor restraining erythropoietin production and contributing to the reduction in [Hb] seen in IDA (Sanchez *et al.* 2007, Wilkinson and Pantopoulos 2014, Zhang *et al.* 2014a). Whilst this seems very reasonable in view of the abnormalities demonstrated in mice deficient in IRP1 (Anderson *et al.* 2013), in whom polycythaemia is paradoxically stimulated by iron deficiency (Ghosh *et al.* 2013), direct evidence regarding the relationship between whole body iron status and intracellular concentrations of iron in the cells responsible for erythropoietin production would be extremely helpful.

As one final observation, a direct action of iron status on erythropoietin secretion of the sort postulated above, would provide an explanation for a puzzling observation from historical studies in animals:

> *"The response of polycythaemic laboratory animals to hypoxia is more difficult to fit within the concept of an oxygen sensor responsive both to anaemic and hypoxic hypoxia. If the polycythaemia was induced by hypertransfusion, erythropoietin production in response to hypoxia was, as predicted, less than that observed in normal animals. If, however the polycythaemia was induced by previous exposure to hypoxia, the animals responded to hypoxia as though they were not polycythaemic. An explanation for this challenging observation may provide a clue as to the operation of the oxygen sensor." (Erslev and Caro 1987)*

A rise in [Hb] brought about by exposure to hypoxia will inevitably deplete tissue iron stores to meet the needs of erythropoiesis, whereas the polycythaemia induced by transfusion will not have this effect, indeed it will significantly increase total body iron.

3.5.2. Hepcidin and IL-6

As already discussed, hepcidin expression is suppressed indirectly by hypoxia via stimulation of erythropoiesis (Volke *et al.* 2009, Liu *et al.* 2012, Gammella *et al.* 2015), an effect putatively mediated by the hormone erythroferrone (Kautz *et al.* 2014). Unexpectedly, we found that plasma hepcidin rose in both groups on both study days during the six-hour hypoxic exposure. Reliable data regarding the half-life of circulating hepcidin in humans are lacking (Ruchala and Nemeth 2014), but studies in Cynomolgus Monkeys yielded a value of under two and a half minutes (Xiao *et al.* 2010). Thus, even a very small early effect of hypoxia on hepcidin expression would be expected to be detectable as a fall in levels within the period of hypoxia in the present study.

One of the challenges in interpreting data from the current experiments is the absence of a control group in whom no hypoxic exposure was given. The primary outcome of interest in *Study A* was the behaviour of the pulmonary vasculature during hypoxia. It is firmly established that resting euoxic PASP does not vary over time (Balanos *et al.* 2002, Balanos *et al.* 2003, Balanos *et al.* 2005, Talbot *et al.* 2005, Smith *et al.* 2008a, Herigstad and Robbins 2009, Dorrington *et al.* 2010, Balanos *et al.* 2015), so a euoxic control group was not justified. However, when examining these secondary outcomes, because care was taken to begin experiments at a similar time in the morning for all participants, the possibility that changes in the measured variables during the period of hypoxia may to a degree reflect diurnal variation must be considered. Additionally, measurement of hepcidin has not yet entered routine clinical practice and remains an experimental tool, with a variety of

techniques used by different groups. The various commercially available ELISAs also vary considerably in their performance.

With these caveats in mind, diurnal variation in hepcidin levels was initially reported based on measurements made using time-of-flight mass spectrometry (Kroot *et al.* 2009). More recently, Schaap and colleagues found hepcidin rose by a factor of ~ 1.8 in iron-replete men and women between a fasted blood sample taken at 0800 h and an unfasted sample at 1600 h (Schaap *et al.* 2013). In that study individuals had been fed an iron-deficient diet the preceding day, and most of the rise in hepcidin was seen early in the day on which samples were taken. It is possible, therefore, that all of the increase in plasma hepcidin in *Study A* on the first day was due to diurnal variation.

However, our participants ate normally the day before experiments, were not fasted for the first blood sample, and had the initial blood test somewhat later than 0800 h. All of these factors will tend to diminish the effect reported by Schaap and colleagues. Even if we were to assume this degree of diurnal variation to be present in our subjects, and to account for all of the rise on the first day, then we would still be permitted to conclude that no or very little suppression of hepcidin expression by hypoxia is operational during this acute period. This is an interesting observation in itself given how quickly the stimulation by erythropoietin of the bone marrow can be expected to have occurred.

Given that IL-6 directly regulates hepcidin transcription via the STAT3 pathway (Wrighting and Andrews 2006, Verga Falzacappa *et al.* 2007), and IL-6 rose during hypoxia, to a similar extent in both groups on both days, another possibility is that acute hypoxia generated an inflammatory signal that drove hepcidin expression over hours, before the suppressive effect of erythropoietic drive could supervene. Nemeth and

colleagues infused recombinant IL-6 into heathy humans and found a marked increase in urinary hepcidin excretion within five hours (Nemeth *et al.* 2004a), suggesting a time course compatible with this possibility. However, the circulating IL-6 levels achieved were several orders of magnitude higher than those in the current study. Furthermore, in the present experiments no relationship between the IL-6 rise during hypoxia and that simultaneously observed in hepcidin was evident.

Just as with hepcidin, it is possible that the rise in IL-6 during hypoxia is simply a reflection of diurnal variation. IL-6 has been found to rise in the afternoon in some settings (Bonda *et al.* 2010) but in others a fall has been observed (Miles *et al.* 2008). It has also been argued that some of the reported patterns are artefactual, and arise due to technical issues such as the use of an indwelling cannula to take samples (Haack *et al.* 2002).

In favour of a true effect of the hypoxic exposure on IL-6, several previous studies have suggested an increase in response to acute hypoxia of a similar order of magnitude to that observed in *Study A*. Goetze *et al.* reported very similar euoxic values before, and hypoxic values the day after, rapid ascent to the *Capanna Regina Margherita* at 4559 m (Goetze *et al.* 2013), though some participants in that study had received dexamethasone. Comparable IL-6 starting values and elevations after 72 hours at the same altitude were also reported by another group (Ravasi *et al.* 2014). Piperno and colleagues, however, reported values ten-fold higher under euoxia during an expedition to Nepal, and a doubling after several days at high altitude (Piperno *et al.* 2011). This latter study used the same brand of ELISA kit to measure IL-6 as the work presented in this thesis, as apparently did those performed in Italy. These high-altitude hypoxia studies also measured hepcidin, which had very clearly fallen after a day or more at high altitude, though not with sufficient temporal resolution to

exclude an initial rise. None of these studies found a significant correlation between the IL-6 response to hypoxia and that seen in hepcidin.

One study that did examine the early change in hepcidin levels with hypoxia suggested a significant fall at 6 hours, with levels approximately halving (Sonnweber *et al.* 2014). Though the duration of hypoxia was the same as that used in the present work, several aspects of that study were quite different. First, poikilocapnic normobaric hypoxia (F_{IO_2} = 0.11, P_{ETO_2} and P_{ETCO_2} not clamped) was used throughout. Secondly, volunteers did not rest throughout the hypoxic exposure but were required instead to exercise for two 30-minute periods after 2 and 5 hours of hypoxia, thus the blood drawn after six hours was effectively a 30-minute post-hypoxic-exercise sample. Thirdly, volunteers had considerably higher starting values for ferritin and hepcidin than in the present study, despite similar demographics. Fourthly, is not clear at what time of day these experiments were performed, and whether this was the same for all participants. The authors highlighted associations between baseline iron indices and the absolute change in hepcidin with hypoxia, but it was in fact euoxic IL-6 that was the strongest predictor of the fall in hepcidin with hypoxia. Interestingly, Sonnweber *et al.* also reported a five-fold rise in IL-6 after six hours of hypoxia, much higher than any of the studies already discussed. Exercise, whether in euoxia or hypoxia, has been reported to induce a significant rise in IL-6, though this was associated with a rise, not a fall, in hepcidin (Govus *et al.* 2014). Thus, the explanation for the discordant IL-6 and hepcidin changes in the study by Sonnweber *et al.* is not immediately clear.

Finally, it is possible that an early transient rise in hepcidin during hypoxia was present in the Peru expedition already discussed (Talbot *et al.* 2012). After an 8-hour journey by road to 4,340 m, hepcidin (and indeed ferritin) showed a trend towards an increase before

subsequently falling. Again, though, this might simply reflect the tendency of hepcidin to increase over the course of a day. If the rise in hepcidin on the first study day is causally related to hypoxia, rather than something peculiar to the experimental conditions in the present work or simply diurnal variation, then there might be implications for diseases characterised by recurrent short periods of hypoxia, as opposed to chronic hypoxia. One might speculate that in these conditions intermittent hypoxia could contribute to inflammation and elevated hepcidin. Of note, the latter has recently been suggested by two small studies in patients with obstructive sleep apnoea (OSA) (Abakay *et al.* 2015, Liu *et al.* 2015).

Considering now the observed changes in hepcidin on the second study day, the greater increase in both groups is entirely anticipated. Changes in serum iron, signalled via a mechanism involving TfR2 on the surface of hepatocytes (Zhao *et al.* 2013), very rapidly bring about a change in hepcidin concentration. Even a single 240 mg dose of iron administered orally, of which only a fraction is absorbed, produces a five- to ten-fold rise in circulating hepcidin after 9 hours (Moretti *et al.* 2015). In terms of an interaction with hypoxia, when iron was given prior to ascent to high altitude (Talbot *et al.* 2012), hepcidin rose four-fold within 10 hours of infusion despite ascent to 4,340 m, and the suppression, by hypoxia, of circulating hepcidin levels was not seen until several days later.

The behaviour of IL-6 was no different on the second study day. If IL-6 did contribute to the hepcidin rise on the first study day, then the implication is that the difference in hepcidin behaviour on the second was accounted for by the action of IV iron. It is also of interest that acute infusion of 15 mg/kg iron did not generate acute inflammation in our healthy volunteers, at least as measured by IL-6. Concern has previously been expressed that large doses of IV iron could be harmful in this respect, by promoting free-radical generation. In

patients with chronic renal failure given a single IV dose of 100 mg iron-sucrose, for example, evidence of a rapid increase in oxidative stress and transient proteinuria were seen in one study (Agarwal *et al.* 2004). Conversely, a study giving repeated IV doses of 200 mg iron-sucrose weekly for five weeks to patients with CHF, renal impairment and IDA, found that iron loading in fact resulted in a fall in inflammatory markers and improved renal function (Toblli *et al.* 2015). The effect of IV iron in this regard is likely to vary according to the clinical setting. Ferric carboxymaltose may additionally be less likely to generate oxidative stress than older IV iron preparations (Geisser and Burckhardt 2011).

The magnitude of the rise in hepcidin on the first study day was much more substantial in the IR group, presumably due to a potent suppressive effect of low serum and hepatocyte iron on hepcidin secretion in the ID participants, whatever the stimulus for the increase may have been. When IV iron was given, hepcidin levels rose more markedly, as expected, in response to the acute elevation in serum iron. The rise was again constrained in the ID group, suggesting that existing tissue iron depletion still acts as a very strong negative regulatory signal even when serum iron levels are acutely elevated.

With this in mind, it is interesting to note that in Ethiopian highlanders with elevated [Hb], hepcidin is not heavily suppressed despite exposure to chronic steady-state hypoxia; iron demand and body iron stores instead appear to be the primary regulators of circulating hepcidin in this setting (Lundgrin *et al.* 2013). Equally, venesection of Peruvian high-altitude residents suffering from chronic mountain sickness – a condition in which polycythaemia, hypoxaemia and PH are features (Naeije and Vanderpool 2013) – brings about a very rapid fall in circulating hepcidin levels, consistent with an erythroid regulator signalling the tension between erythropoietic drive and iron supply (Talbot *et al.* 2016).

3.6. Conclusions

This aspect of *Study A* has demonstrated that both euoxic serum erythropoietin, and the rise in response to hypoxia, are abnormally high in iron-deficient individuals, though the relative contributions from [Hb] and iron status cannot be determined with certainty. A slightly longer interval following iron supplementation would help address this issue, and this will be explored further in Chapter 4.

These experiments also suggest that hypoxia does not act to suppress circulating hepcidin levels for some hours, and may even initially promote an increase in hepcidin. They indicate a rapid increase in plasma hepcidin in response to IV iron loading in iron-replete individuals, and very marked attenuation of this effect by iron deficiency, consistent with signals from the *store regulator* and *erythroid regulator* that cannot be immediately overridden in this situation. Finally, a small increase in IL-6 in response to hypoxia is suggested, and a significant acute effect of iron-loading using ferric carboxymaltose to promote inflammation in healthy individuals, refuted.

Chapter 4. Iron status and skeletal muscle metabolism

4.1. Introduction

So far this thesis has considered the effects of iron deficiency on the pulmonary vasculature and, to a lesser extent, the kidney; two sites where hypoxia is sensed and responses engendered to optimise the systemic supply of oxygen. Though the oxygen-sensing cells at these sites of course themselves consume oxygen, their major role is in triggering integrated physiological processes central to organismal oxygen homeostasis. In moving now to consider skeletal muscle, it will become clear that a very different situation exists; this tissue is a major consumer – frequently *the* major consumer – of oxygen, given the requirement to oxidise lipid and carbohydrate in order to meet the very considerable energy demands of muscular work.

4.1.1. Energetics and oxygen cost of skeletal muscle contraction

The immediate energy source for muscular contraction is ATP, which can be generated from a variety of fuels (Table 4-1). The most energy-dense and therefore efficient means for the body to *store* energy is as fat within adipose tissue (~ 9.3 kcal/g); in comparison, only a very small amount of energy is stored as carbohydrate, which is less than half as energy dense (~ 4.1 kcal/g). In fact, carbohydrate is made even less favourable in this respect owing to the intracellular accumulation of water that inevitably accompanies glycogen synthesis (Cahill 1970). However, in terms of the liberation of energy that may usefully be employed in the resynthesis of ATP from ADP, oxidation of carbohydrate offers a considerable advantage in that, as can be seen from Table 4-1, oxidation of lipid does not allow as rapid a production of ATP as is the case if muscle carbohydrate is used.

Fuel source	Maximal ATP production rate (mmol/s)	Total P-equivalent available (mol)
Muscle ATP		0.2
Muscle PCr	73.3	0.45
Muscle glycogen – glycolysis	39.1	6.7
Muscle glycogen – oxidation	16.7	84
Liver glycogen – oxidation	6.2	19
Adipose tissue fatty acids – oxidation	6.7	4000

Table 4-1. Sources of fuel for human skeletal muscle contraction

The P-equivalent is the amount of ATP that may be resynthesized from ADP and inorganic phosphate (Pi) using a given fuel source. Note that although ATP for skeletal muscle contraction may be synthesised most rapidly by hydrolysis of phosphocreatine (PCr), if used exclusively this energy source would be exhausted in only 6 seconds. Data taken from (Berg *et al.* 2012) page 835.

Additionally, textbooks of exercise physiology often state that oxidation of fat is more costly with respect to oxygen than is oxidation of carbohydrate. For the complete oxidation of a single molecule of glucose we have:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

This reaction is generally stated to yield 36-38 molecules of ATP. Whereas for lipid, in this case palmitate, we find that the following reaction yields 130 molecules of ATP:

$$CH_3(CH_2)_{14}COOH + 23O_2 \rightarrow 16CO_2 + 16H_2O$$

On the face of it, therefore, oxidation of carbohydrate requires less oxygen per ATP molecule resynthesized than does fat. However, whereas the anaerobic metabolism of glucose to pyruvate predictably yields 2 molecules of ATP, owing to peculiarities of mitochondrial oxidative phosphorylation the stoichiometry of the metabolism of pyruvate to carbon dioxide and water is not fixed, and the true yield may in fact be closer to 30 molecules of ATP (Hinkle *et al.* 1991). Thus, the factor of most importance when considering fuel selection for aerobic muscular work is likely to be the rate of ATP resynthesis possible, rather than the oxygen cost of a given fuel.

That the major available fuels for muscular contraction vary both in their abundance, and the speed at which they can be harnessed to regenerate ATP, has important consequences for exercise physiology. These can be illustrated by considering different intensities of muscular work. At one extreme, studies of human locomotion are remarkable for reporting consistently that fasted adult humans walk at a characteristic speed, termed the preferred walking speed (PWS). In one study, which investigated oxygen consumption and fuel

selection using indirect calorimetry during human walking, Willis and colleagues found this to be ~ 4.7 km/hour:

> *"At speeds less than or equal to PWS, muscle carbohydrate oxidation rates were quite low, in the range that could be matched by gluconeogenesis. Above 4.8 km/hour carbohydrate oxidation rate increased abruptly...We conclude that PWS is just below a threshold speed, above which carbohydrate oxidation abruptly increases." (Willis et al. 2005).*

Thus, as would be expected, the metabolic preference appears to be to burn the most abundant fuel at low workloads. For endurance activities in which exercise significantly above walking pace is undertaken, we find the body forced to utilise carbohydrate. At the outset of a marathon, for example, carbohydrate oxidation dominates. Though it is certainly true that over the course of such an event fat is increasingly utilised, exhaustion of muscle glycogen has disastrous consequences for athletic performance in this situation (Costill 1972). Endurance athletes recognise the point at which this occurs as 'hitting the wall', though the integrated physiology of this phenomenon is probably somewhat more complex simply than running out of carbohydrate (Rapoport 2010).

At the other extreme from walking, during short periods of extremely intense exercise such as maximal sprinting over a distance of 100 m, oxidative processes are essentially non-contributory. In this situation, PCr degradation accounts for about half of total anaerobic ATP production with the remainder provided by anaerobic glycolysis (Gaitanos *et al.* 1993). Though during this sort of intense exercise ATP resynthesis proceeds in the absence of oxygen, the process cannot continue indefinitely; eventually the lactate derived from pyruvate that would otherwise have entered the tricarboxylic acid cycle will have to be

metabolised. To put it another way, the 'oxygen debt' that has been built up during this period of intense activity will have to be paid:

> *"The lactacid debt amounts at most to 0.22 kcal/kg or about 3 litres 0_2 for man, on the average…the lactacid debt process is completed in about 40 seconds in the most strenuous exercise. Therefore, at the highest workloads, the only energy source available after 40 seconds resides in oxidative processes." (Margaria et al. 1964)*

However, it sometimes seems to be implied that for human cells generally a 'switch' to anaerobic glycolysis is an excellent strategy for times when oxygen is scarce. For example:

> *"Activation of glycolytic genes by HIF-1 is considered critical for metabolic adaptation to hypoxia through increased conversion of glucose…to lactate…HIF-1 also actively suppresses metabolism through the TCA cycle by directly trans-activating the gene encoding pyruvate dehydrogenase kinase 1…a hypoxia-induced metabolic switch that shunts glucose metabolites from the mitochondria to glycolysis to maintain ATP production…" (Kim et al. 2006)*

Similarly:

> *"A key feature of metabolic pathways is their plasticity. Changes in nutrient availability or oxygen levels are the best-characterised drivers of metabolic reprogramming. For example, hypoxia is a well-known driver of glycolysis, as an oxygen deficit results in limited oxidative phosphorylation. Under these circumstances cells must rely on glycolysis to generate ATP. HIF-1α is critical for this process, as it induces the expression of glycolytic enzymes such as hexokinase and phosphofructokinase, thereby allowing for sustained ATP production." (Corcoran and O'Neill 2016)*

Though such statements have an inherent verisimilitude, there is a real danger in assuming that the manner in which cells – often immortalised cancer cells – behave in culture is a good reflection of the situation that is to be found when they are organised neatly into a whole human organism. The suggestion that skeletal muscle ATP resynthesis might be 'maintained' or 'sustained' by anaerobic glycolysis is clearly refuted by the brief discussion of bioenergetics above. Leverve puts it rather well:

> *"In prokaryotes…complete oxidation involving a respiratory chain, and thus requiring oxygen, is not mandatory because these organisms can release the excess of reducing equivalents in the medium (by releasing reduced compounds such as lactate, ethanol, glycerol, etc.). By contrast, in mammals, as in all superior eukaryotes, full oxidation of energetic substrates is mandatory, leading to CO_2 and H_2O formation."*
> *(Leverve 2007)*

Thus, whilst it is true that oxidation of muscle carbohydrate rather than fatty acids offers an improvement in the rate of ATP resynthesis achievable, it is evident that metabolism of pyruvate to lactate, to escape altogether the requirement of skeletal muscle for oxygen, is not tractable – it is simply a temporising measure. This is essential to bear in mind when considering the role of the HIF pathway in skeletal muscle.

At this point it will be helpful to consider the concept of thresholds, a variety of which are ubiquitous in the exercise literature. It might mistakenly be assumed that there exists a point during exercise at which work intensity becomes so high that a 'switch' to anaerobic respiration is necessary in order to continue any further, with entirely aerobic metabolism up until this point. This would be a gross misrepresentation; at *any* workload there will be some skeletal muscle units respiring anaerobically and some aerobically; the balance between these will determine the extent to which circulating blood lactate levels rise (Myers

and Ashley 1997), if indeed they do so at all. This point is nicely illustrated by the finding that energy provision for *gracilis* muscle contraction in the walking dog is met entirely through anaerobic glycolysis despite abundant oxygen (Connett *et al.* 1984); this also highlights that fuel selection and metabolic strategy are not simply determined by oxygen supply. In fact, skeletal muscle is not solely important in this regard; other tissues are capable of disposing of lactate:

> *"An erythrocyte is a completely anaerobic cell, since it lacks mitochondria, therefore the only way to produce ATP is the non-oxidative glycolytic pathway, resulting in obligatory lactate release. On the contrary, the liver is a full aerobic organ producing ATP from the aerobic fatty acid (beta)-oxidation, and using this energy to synthesise glucose from lactate. Hence, by recycling the carbons between lactate and glucose, the liver actually 'respires' for the erythrocyte mass."*
> *(Leverve 1999)*

Thus, the lactate generated by skeletal muscle contraction may be metabolised elsewhere, and the kinetics of blood lactate accumulation during muscular exercise will reflect the integrated metabolic behaviour of the body's tissues as a whole; if levels are rising or falling there must be net lactate production or disposal, respectively, at that point in time. It is certainly the case, though, that in a given individual there exists a point at which the rate of change with increasing work of blood [lactate] noticeably accelerates. This inflection point on the line describing the relationship between work and [lactate] is described as the lactate threshold, and is generally seen to coincide with a similar ventilatory threshold. Whatever the exact physiological basis for such thresholds, they do offer a convenient index of aerobic exercise metabolism that may be exploited in a range of settings, including clinical physiology studies of skeletal muscle metabolism.

4.1.2. HIF and skeletal muscle

If we consider certain aspects of the physiology of the pulmonary vasculature and interstitial fibroblasts of the kidney, it is not terribly difficult to bring to mind likely roles of an oxygen-sensing pathway at these sites. Given that it has just been argued that muscular contraction cannot escape the requirement for oxygen, and rather a lot of it at that, it might be a struggle to generate hypotheses surrounding the role of HIF in skeletal muscle. We could hypothesise, from what has gone before, that the HIF pathway functions here to 'fine-tune' metabolic pathways in some way, according to prevailing fuel availability or the type of muscular work to which an individual is habituated – repeated short bursts of anaerobic exercise versus long periods of sustained submaximal exertion, for example. HIF might perhaps also play a role in regulating the abundance of the organelle responsive for the bulk of skeletal muscle oxygen consumption – the mitochondrion.

Evidence regarding the role of HIF in skeletal muscle comes from two main sources, which not infrequently overlap: (i) studies of humans or animals with genetic abnormalities of, or variation within, HIF-pathway components; and (ii) studies where athletic performance is of primary interest to the investigators. In the case of the former, Mason and colleagues found that tissue-specific deletion of murine skeletal muscle HIF-1α blocked exercise-induced changes in muscle gene expression and significantly decreased amounts of lactate in the serum of exercising mice (Mason *et al.* 2004). Interestingly, exercise times in the knockout animals were at first prolonged, but repeated exercise bouts caused extensive muscle damage, similar to that seen in human diseases caused by deficiencies of enzymes important for glycogenolysis and glycolysis. The same group later reported upregulated oxidative capacity in these knockout animals in the absence of training, in tandem with decreased expression of pyruvate dehydrogenase kinase 1 (PDK1) (Mason *et al.* 2007),

which, as alluded to above, is encoded by a HIF-target gene. In effect, they argued, loss of HIF-1α induced "endurance adaption" in skeletal muscle.

On the other hand, it was reported that in mice, stimulating mitochondrial biogenesis (using overexpression of peroxisome proliferator-activated receptor-coactivator-1α), led to HIF-1α protein stabilisation; this phenomenon apparently resulted from an increase in cellular oxygen consumption (O'Hagan *et al.* 2009). It was therefore suggested that HIF might play a role in matching oxygen supply to demand, both by regulating angiogenesis in response to training-induced mitochondrial biogenesis, and by negatively regulating mitochondrial biogenesis itself. A further study showed that, as would be expected based on these previous investigations, loss of PHD1 altered murine skeletal muscle glucose metabolism, promoting glycolysis to generate ATP, and impairing oxidative muscle performance (Aragones *et al.* 2008). One study has reported the opposite effects, specifically that loss of PHD2 in mice and consequent stabilisation of HIF enhanced the effects of endurance running training (Nunomiya *et al.* 2016). However, this study did not use skeletal-muscle-specific silencing of PHD2 expression, so these animals had a [Hb] fifty-percent higher than controls, which one would anticipate to be a very significant confounding factor.

In humans, Formenti *et al*. reported that, in addition to the abnormalities already described, CP patients had impaired skeletal muscle oxidative phosphorylation (Formenti *et al.* 2010). Using ^{31}P-magnetic resonance spectroscopy (MRS) they showed more marked PCr depletion and intracellular acidosis during small muscle mass exercise (Figure 4-1). Given the nature of this exercise challenge, it appeared very unlikely that differences in [Hb] or local blood flow regulation explained the abnormalities demonstrated.

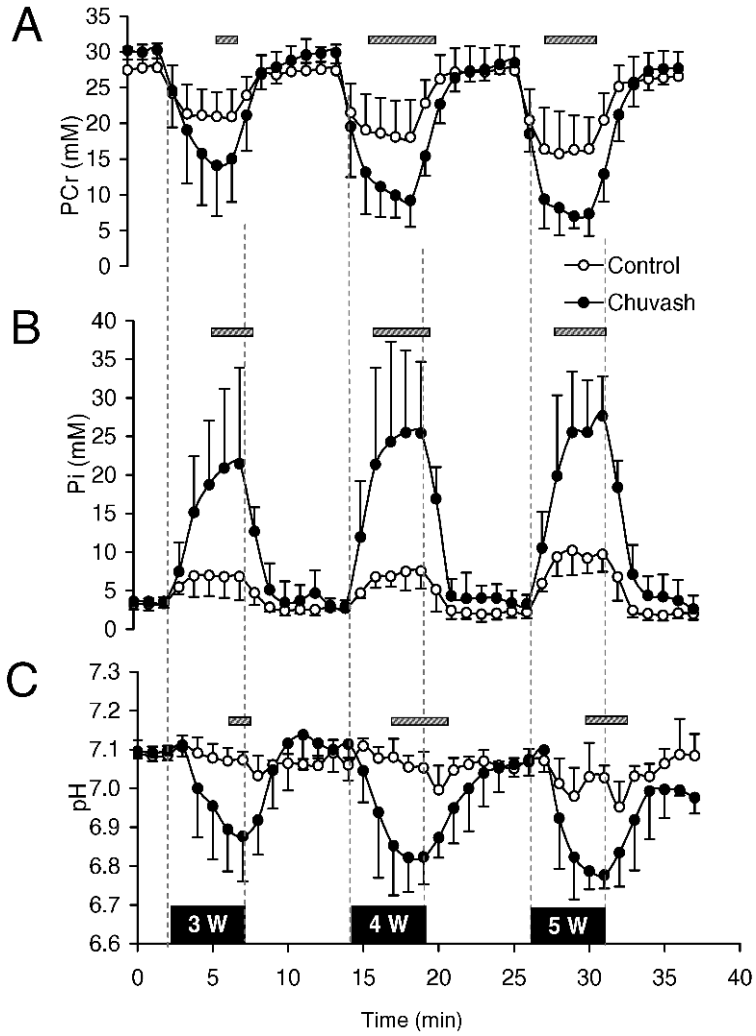

Figure 4-1. Phosphocreatine metabolism during small muscle mass exercise in patients with CP

(A) PCr concentration; (B) inorganic phosphate (Pi) concentration; and (C) pH, over time. Data were obtained using ^{31}P-MRS of the right calf muscle. Three periods of plantarflexion exercise were undertaken against a spring-loaded footplate, at increasing power outputs (indicated by black bars). Vertical dashed lines indicate the onset and offset of these 5-minute exercise bouts; empty circles show results for the control group (n=5); filled circles those for the CP group (n=5). Values are minute averages ± SD. Hatched horizontal bars indicate periods of significant difference in values between groups ($P < 0.05$). Reproduced from (Formenti *et al.* 2010).

Additionally, patients with CP showed an earlier rise in blood [lactate] during cardiopulmonary exercise testing (CPET) using a bicycle ergometer, and analysis of *vastus*

lateralis biopsies suggested upregulation of HIF-target genes, including those encoding several PDK isoforms, favouring glycolysis. However, this study was rather small, involving as it did only five patients. Additionally, at least three of these individuals were likely to have been profoundly iron deficient as a consequence of therapeutic venesection. Other work in CP patients, and mice harbouring the Chuvash mutation, has suggested greater skeletal muscle glucose uptake as a result of increased expression of the glucose transporter GLUT4 (McClain *et al.* 2013), with lower blood glucose levels as a consequence.

Studies in Tibetans also provide some interesting observations. For example, a study at an altitude of 4,700 m involving 17 lifelong Tibetan residents and 14 acclimatised Han Chinese newcomers, reported that mean anaerobic threshold values in the Tibetan and Han groups were 84.1 and 61.6% $\dot{V}O_2MAX$ (maximal oxygen uptake), respectively, with Tibetans also showing lower blood lactate levels before and at the end of exercise (Ge *et al.* 1994). These observations imply that, just as in the pulmonary vasculature, a downregulated HIF system has the expected effect of attenuating some of the physiological responses to hypobaric hypoxia. The Han Chinese displayed a lower lactate threshold and higher end-exercise lactate, consistent with the expected promotion of glycolysis by hypoxia – the higher eukaryotic equivalent of the Pasteur effect (Racker 1974).

In the case of studies of HIF that have focused on athletic performance, Lindholm and colleagues studied elite athletes, and moderately active individuals who undertook an exercise programme. They reported HIF to be very strongly negatively regulated in the skeletal muscle of elite athletes, which appeared to be largely the result of PHD2 upregulation (Lindholm *et al.* 2014). These investigators suggested that this phenomenon might attenuate PDK1 expression leading to activation of the pyruvate dehydrogenase

(PDH) complex, which is the rate-limiting step in aerobic ATP synthesis. An exercise programme in the initially moderately active individuals induced some aspects of the changes seen in elite athletes within a matter of weeks. Conversely, high-intensity interval exercise has been reported to increase HIF activity and promote glycolysis, at least in an animal study (Abe *et al.* 2015).

4.1.3. Skeletal muscle and systemic iron homeostasis

Despite containing a significant proportion of the body's iron, much less is known about the role of skeletal muscle in systemic iron homeostasis than is the case for the liver. It is apparent, though, that many genes of importance for hepatic regulation of iron homeostasis are also expressed in skeletal muscle, including those encoding TfR1, haemojuvelin, HFE, DMT1 and ferroportin; whereas hepcidin and TfR2 are near-absent (Polonifi *et al.* 2010). Haemojuvelin is very highly expressed, so it is surprising to find that skeletal-muscle-specific loss of haemojuvelin has no detectable consequences for iron homeostasis in mice (Chen *et al.* 2011), whereas hepatic loss of haemojuvelin causes haemochromatosis.

Much of the iron in skeletal muscle is within the haem moiety of myoglobin. The importance of the ferrous ion in haem for the function of the tetrameric oxygen-transport protein haemoglobin was considered at the outset of this thesis. The relationship between the partial pressure of oxygen and its binding to myoglobin is quite different from that of haemoglobin, and its role in human skeletal muscle has been surmised to relate to optimisation of oxygen diffusion (Duteil *et al.* 2004). Having said this, there is still uncertainty despite 75 years of debate on the issue (Millikan 1939), and it is notable that myoglobin-knockout mice exhibit preserved exercise capacity, intact ventilatory responses to hypoxia, and grossly normal skeletal muscle metabolism (Garry *et al.* 1998). In terms of whether iron is lost from skeletal muscle when a state of negative iron balance exists, it was

reported that, in rats fed diets lacking in iron, the degree of depletion of muscle cytochrome c mirrored the fall in [Hb] as a result of IDE; myoglobin content seemed to be more resistant, but did ultimately fall (Siimes *et al.* 1980) (Figure 4-2).

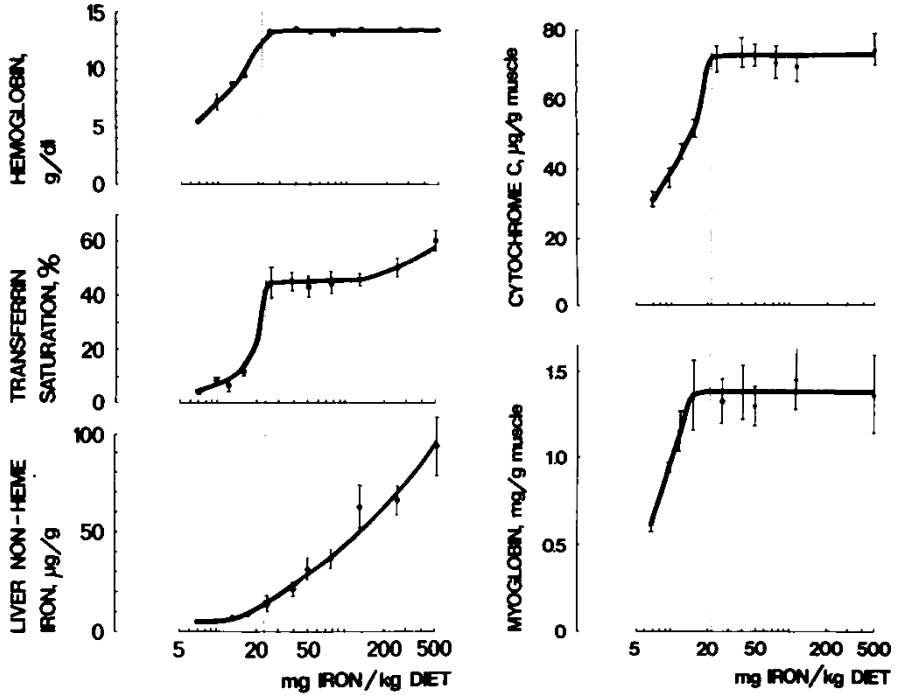

Figure 4-2. Effect of dietary iron on systemic iron homeostasis in rats

Animals (n=6 for each diet) were fed a controlled diet for three weeks, from three weeks of age, before measurements were made. Note that [Hb] and skeletal muscle cytochrome c content show a threshold effect, being more or less stable above a dietary intake of around 25 mg/kg, but falling steadily below this level; muscle myoglobin content shows a similar phenomenon but is preserved until dietary iron is further restricted. Liver non-haem iron content rises progressively as dietary iron intake increases, as would be expected for the major site of iron storage. TSat is stable over the middle of the range of dietary iron intake, but falls rapidly at the point at which erythropoiesis becomes restricted by iron availability, as is seen in human IDE. It is possible to overload hepatic iron storage capacity, such that TSat rises, as in human haemochromatosis. Values are means; error bars represent SEM. Note the log scale on the x-axis. Adapted from (Siimes *et al.* 1980) with permission.

Other animal studies have similarly reported a fall in skeletal muscle oxidative enzyme content using this experimental approach (Finch *et al.* 1979, McLane *et al.* 1981, McKay *et al.* 1983). On the other hand, during ascent to altitude in humans, it was reported that the stimulation of erythropoiesis by hypobaric hypoxia was sufficient to bring about a significant lowering of skeletal muscle myoglobin expression and down-regulation of iron-related proteins including ferritin and TfR1 (Robach *et al.* 2007). This was accompanied by a fall in iron content of the muscle to around two-thirds of initial values. These changes were noted to be concurrent with increased ferroportin mRNA levels, suggesting possible enhanced iron export, though it is difficult from this study alone to determine whether the observed changes represent regulated redistribution of iron from skeletal muscle. Levels of skeletal muscle oxidative enzymes were not reported in this study.

4.1.4. Functional consequences of skeletal muscle iron deficiency

4.1.4.1. Animal studies

Despite being a haematologist, Finch was very interested in the extra-haematological consequences of iron deficiency, and with his colleagues undertook several animal studies designed to identify direct effects on skeletal muscle function. Notably, it was demonstrated that work performance on a treadmill was considerably impaired in iron-deficient rats, despite removal of anaemia as a confounding factor using isovolaemic transfusion to match [Hb] with iron-replete control animals (Finch *et al.* 1976). When administered to iron-deficient animals, intraperitoneal iron dextran therapy corrected the observed abnormalities within 4 days. Finch thought it likely that a specific underlying defect of mitochondrial function was responsible.

This work was extended to demonstrate that in iron-deficient rats, muscular work was associated with a higher blood [lactate] than in iron-replete animals (Finch *et al.* 1979).

Importantly, in this second study it was demonstrated using infusion of ^{14}C-labelled lactate that hyperlactataemia was the result of excessive lactate production rather than impaired clearance from the blood. Additionally, it was shown that energy substrates – blood glucose, free fatty acids, and muscle glycogen – were adequate in iron-deficient animals. Further disambiguation of the effects of [Hb] and iron status was provided by work undertaken by Dallman's group, which suggested that whilst anaemia limits oxygen delivery to exercising muscle, tissue iron deficiency limits oxidative metabolism (Figure 4-3). Like the work of Finch, these studies involved iron repletion and exchange transfusion in rats (Davies *et al.* 1982, Davies *et al.* 1984).

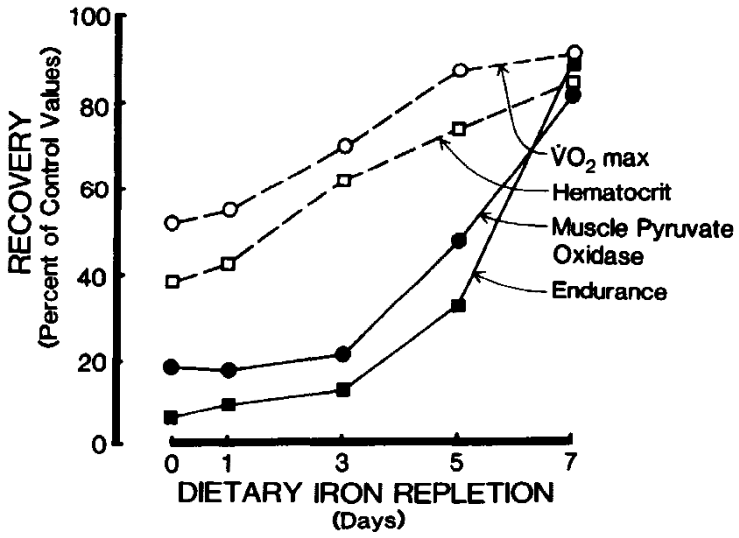

Figure 4-3. Recovery of various parameters during dietary iron repletion in iron-deficient rats

The "recovery" values given are expressed as a percentage of those achieved by a control group on corresponding days. A strong correlation of $\dot{V}O_{2MAX}$ with haematocrit is evident, as is a relationship between endurance and skeletal muscle oxidative enzyme activity. Pyruvate oxidase is synonymous with PDH. Reproduced from (Davies *et al.* 1982).

Subsequently, using ^{31}P-MRS during electrical stimulation of the gastrocnemius muscle in rats (Figure 4-4), iron deficiency was shown to be associated with increased PCr breakdown and acid production in contracting skeletal muscle (Thompson *et al.* 1993a), very similar to the findings in patients with CP already discussed.

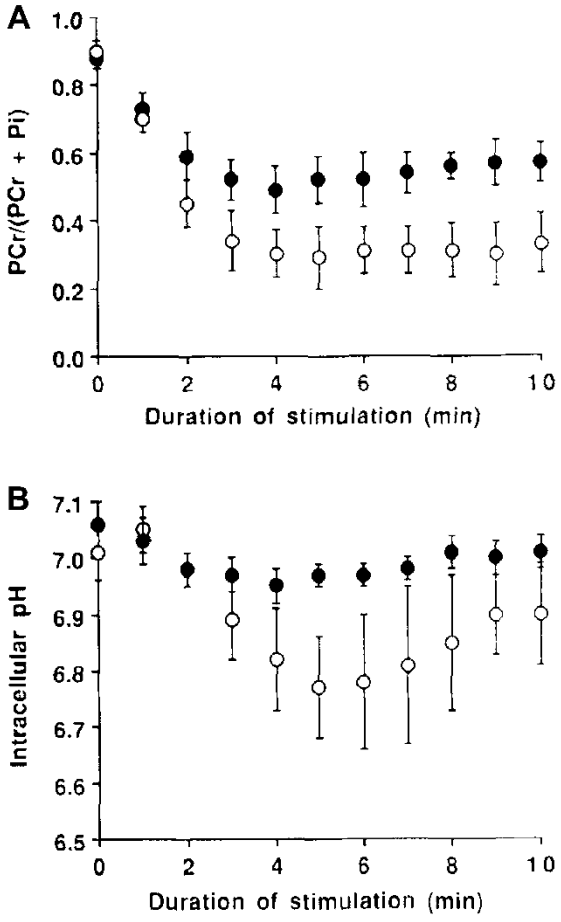

Figure 4-4. ^{31}P-MRS data obtained using sciatic nerve stimulation in rats

Changes in (A) PCr, and (B) pH, during stimulation at 2 Hz. Filled circles represent animals fed a normal diet; open circles, those fed an iron-deficient diet. Values are means; error bars represent SD. PCr breakdown and intracellular acidosis were both significantly greater in the iron-deficient animals ($P < 0.001$, both comparisons). Adapted from (Thompson *et al.* 1993a) with permission.

Finally, perhaps the most dramatic illustration of the importance of iron for skeletal muscle function comes from a study in which TfR1 was conditionally knocked out in the skeletal muscle of mice (Barrientos *et al.* 2015). These animals developed severe skeletal muscle metabolic derangement and growth arrest, and died around two weeks after birth. Clearly, depriving any large tissue mass of iron is a rather blunt tool, and it might be argued that it is not surprising that this sort of insult was, to use the authors' own description of the phenotype, "catastrophic". However, associated dramatic changes occurred in other tissues; gluconeogenesis was stimulated in the liver, which itself became iron deficient with suppressed hepcidin production, and hypoglycaemia ensued. Thus, the complexity of interactions between iron homeostasis and metabolism was further highlighted.

4.1.4.2. Human studies

> *"One must be cautious about extrapolating these data to human iron deficiency because of the possible influences of species difference, aetiology of the deficiency and differing severity of the iron deficiency. In animals, iron deficiency is usually induced by severe dietary iron restriction to the growing rat…In this model, myoglobin concentrations are also reduced as are iron dependent enzymes important in mitochondrial oxidative phosphorylation…In man, the situation appears different. In adults the aetiology is usually chronic blood loss. Muscle myoglobin and enzyme concentrations may be preserved despite a reduction in haemoglobin…In severe iron deficiency in the rat, we have confirmed the presence of a mitochondrial defect in vivo. More moderate iron deficiency in man may not show these metabolic differences." (Thompson et al. 1993a)*

Though work in rodents strongly suggests a defect in mitochondrial oxidative phosphorylation attributable to iron deficiency, considerable caution is required when drawing inferences for humans from such experiments, as the authors of the study from

which Figure 4-4 is taken articulate so well in the above quote. Indeed, using repeated venesection to induce severe iron deficiency for four weeks in healthy men, followed by transfusion to correct the ensuing anaemia, one group had previously reported no evidence of any effect on maximal oxidative enzyme activity in human skeletal muscle (Celsing *et al.* 1986). Subsequently, these investigators also found that an [Hb] of 8-10 g/dl, as a result of naturally occurring iron deficiency, was not associated with the sort of impaired skeletal muscle biochemical function seen in rodents (Celsing *et al.* 1988), though this study was performed in Indonesian subjects who the authors noted appeared – somewhat unexpectedly – to differ rather substantially from Caucasian individuals with respect to skeletal muscle enzyme function.

On the other hand, that iron deficiency might directly influence work capacity and lactate kinetics during exercise has been suggested by a number of studies over several decades. For example, a study of the effect of 3 months of oral iron supplementation in the "elderly" (defined as men and women aged 58-71 years) reported improvements in physical work capacity in those receiving iron, despite no concomitant increase in [Hb] (Ericsson 1970). Also, in subjects with moderately severe anaemia (mean [Hb] 6.6 g/dl), maximal exercise time increased significantly within four days of IV iron dextran therapy, and post-exercise venous blood lactate was similar with successive experiments even though subjects reached higher workloads (Ohira *et al.* 1979). However, with the degree of anaemia in this latter study, [Hb] was seen to rise within days, confounding the findings somewhat, though the authors commented that the elevation of Hb concentration did not seem to be entirely responsible for their findings.

In a small study of trained female athletes with and without iron deficiency, two weeks of oral iron therapy had no effect on performance during incremental exercise to exhaustion

on a bicycle ergometer, but blood lactate levels at maximum exercise in the iron-deficient group decreased significantly from 10.3 ± 0.6 mmol/L before therapy to 8.42 ± 0.7 mmol/L after therapy (Schoene et al. 1983). The authors took this to imply that iron played a role in oxidative metabolism; unfortunately, but predictably, [Hb] in the iron-deficient group rose significantly with therapy, confounding the findings.

A recent systematic review and meta-analysis of the iron and exercise literature found fifteen eligible studies using oral iron and two using intramuscular iron; based on these it was concluded that iron improved the aerobic capacity of endurance athletes with NAID (Burden *et al.* 2015a). The group who conducted this review subsequently reported the findings of a study wherein they administered 500 mg IV ferric carboxymaltose to iron-deficient elite athletes (Burden *et al.* 2015b). Surprisingly, in view of the results of their meta-analysis, no difference was demonstrated in $\dot{V}O_2MAX$, submaximal blood lactate, running economy, RPE, or time to exhaustion during incremental exercise tests to volitional fatigue.

4.2. Aims

Fundamentally, *Study B*, sought to determine whether iron deficiency in otherwise healthy individuals has consequences in any way similar to those seen in animals, and, based on the premise that iron deficiency upregulates the HIF pathway, the observed abnormalities of metabolism in patients with CP. The hypothesis was that iron deficiency would act significantly to alter human skeletal muscle metabolism, leading to impaired oxidative phosphorylation, as assessed using ^{31}P-MRS, and augmented lactate accumulation during exercise, and that these effects would be independent of [Hb].

4.3. Methods

The contributions of this author and other individuals to the design and execution of *Study B* are described in detail in Appendix A. The design of *Study B* had much in common with *Study A*, with the notable difference that in this instance not all participants were given IV iron. This was instead a prospective, randomised controlled clinical study, with participants and investigators blinded to intervention. We recruited otherwise healthy adults with absolute iron deficiency. Iron-replete age- and sex-matched volunteers served as controls. Participants were studied on two occasions, approximately a week apart. A flow diagram illustrating the study protocol is given in Figure 4-5.

4.3.1. Eligibility criteria

The iron parameters defining ID and IR groups were as for *Study A*. Eligibility criteria were also similar, with the notable addition of the requirement for participants to be MRI compatible. Thus, participants with ferromagnetic implants or any other characteristics precluding safe performance of ^{31}P-MRS were excluded.

4.3.2. Participant recruitment

During the period of recruitment between October 2014 and September 2016, blood donors in Oxfordshire, UK were offered information about the study if below the haemoglobin threshold to donate. Advertisements were placed concurrently for controls; in contrast to *Study A*, on this occasion it was also made explicit that individuals known to be iron deficient but otherwise healthy would be welcome to volunteer. This strategy was adopted in an attempt to facilitate recruitment to the ID group.

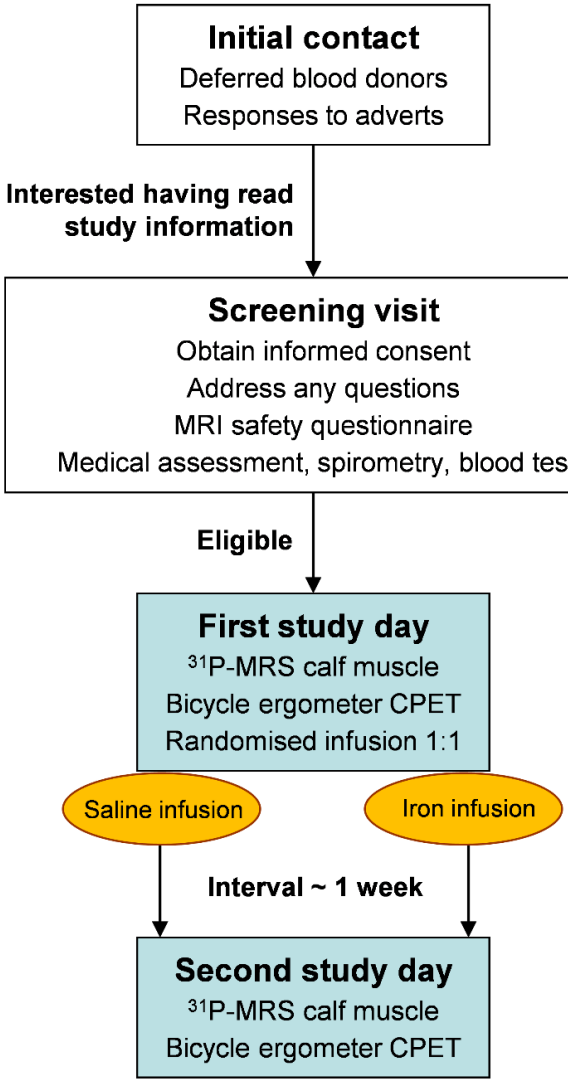

Figure 4-5. Overview of protocol for *Study B*

A detailed description of the experimental techniques employed can be found in section 4.3.3. Compare with the somewhat different protocol for *Study A* (Figure 2-12), in which infusions were given at the beginning of each study day, with all participants receiving an iron infusion on the second. MRS, magnetic resonance spectroscopy; CPET, cardiopulmonary exercise testing.

Volunteers attended a screening visit conducted by a physician including medical history, questioning regarding exercise habits, examination, spirometry (MicroLab™, CareFusion,

UK), venous blood sampling and familiarisation with study procedures. The latter included an explanation of the requirements of the plantarflexion exercise protocol within the MRI scanner and subsequent CPET.

4.3.3. Study visits

Participants were asked to have a normal dinner on the evening prior to each experimental day but avoid alcohol. The next morning, having fasted and drunk only water after waking, they were collected by taxi and brought to the John Radcliffe Hospital, where they were met and escorted to the University of Oxford Centre for Clinical Magnetic Resonance Research (OCMR). This strategy was adopted to ensure that participants arrived on time, relaxed, and without having exercised in any way. ^{31}P-MRS was performed in OCMR, typically from 0700-0800 h, before moving to the University of Oxford Cardiovascular Clinical Research Facility (CCRF) for CPET.

4.3.3.1. ^{31}P Magnetic resonance spectroscopy

Skeletal muscle ^{31}P-MRS was performed in the supine position with a dual-tuned ^{31}P and ^{1}H 6-cm-diameter surface coil secured under the right calf. The right foot was fastened to a custom-built plantarflexion exercise apparatus (Figure 4-6) with the leg straightened, and the calf exercised at 1 Hz in time to a digital metronome played via the integral MRI scanner headphones. Three 5-minute periods of exercise, at 3 W, 4 W and finally 5 W, were alternated with 7-minute recovery periods. Participants were given handgrips to hold, which were connected by means of a narrow band of inelastic polypropylene webbing that ran around the distal aspect of the plantarflexion apparatus; this was to ensure isolation of the desired exercise muscle group and minimise the extent to which the participant was thrust away upon plantarflexion.

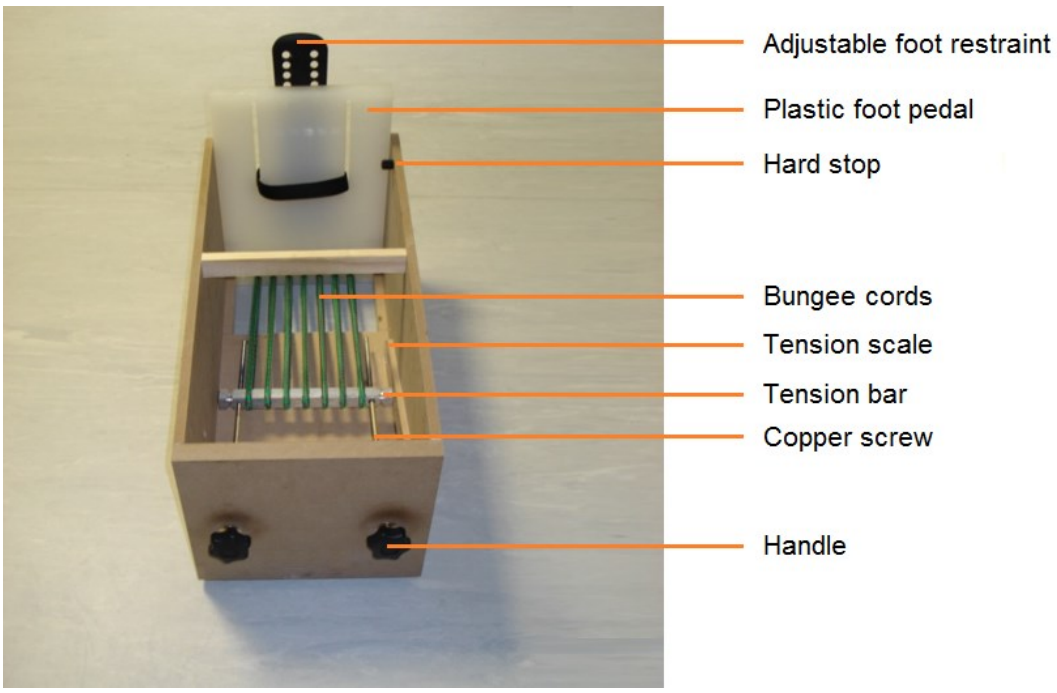

Figure 4-6. Plantarflexion exercise apparatus

Bungee cord tension was adjusted by turning the handles clockwise to move the aluminium bar further from the footplate; the device was calibrated using a strain gauge and the required position of the bar to give the three desired work rates marked on a scale. This allowed the tension to be adjusted quickly under direct vision during the 7-minute rest periods. The whole plantarflexion apparatus was arranged so as to abut the end of the MRI scan table, and strapped in place on using a combination of inelastic polypropylene webbing and a proprietary putty-like pressure-sensitive adhesive tac (Blu-Tack®, Bostik, Leicester, UK). This configuration guaranteed that no movement of the apparatus could occur during exercise. The 'hard stop' aided consistent depression of the pedal, allowing the participant and investigators audible feedback to ensure that exercise was being performed in a satisfactory manner.

Data for this portion of the protocol were analysed off-line by a blinded investigator. This individual did not know the gender or age of the participant, nor to which visit the data related. Spectral peaks were fitted using the automated AMARES algorithm within the jMRUI software package. Absolute concentrations of phosphorus metabolites were calculated from the spectral data using an established method (Golding *et al.* 1995, Kemp

et al. 2007, Formenti *et al.* 2010, Kemp *et al.* 2015). This approach (i) assumed the cytosolic concentration of ATP at rest to be 8.2 mmol/L; (ii) took the creatine kinase reaction to be at equilibrium; and (iii) corrected for measured pH:

$$pH = 6.75 + \log((\sigma - 3.27) \div (5.63 - \sigma))$$

Where σ is the chemical shift, in parts per million, of the Pi peak (Figure 4-7).

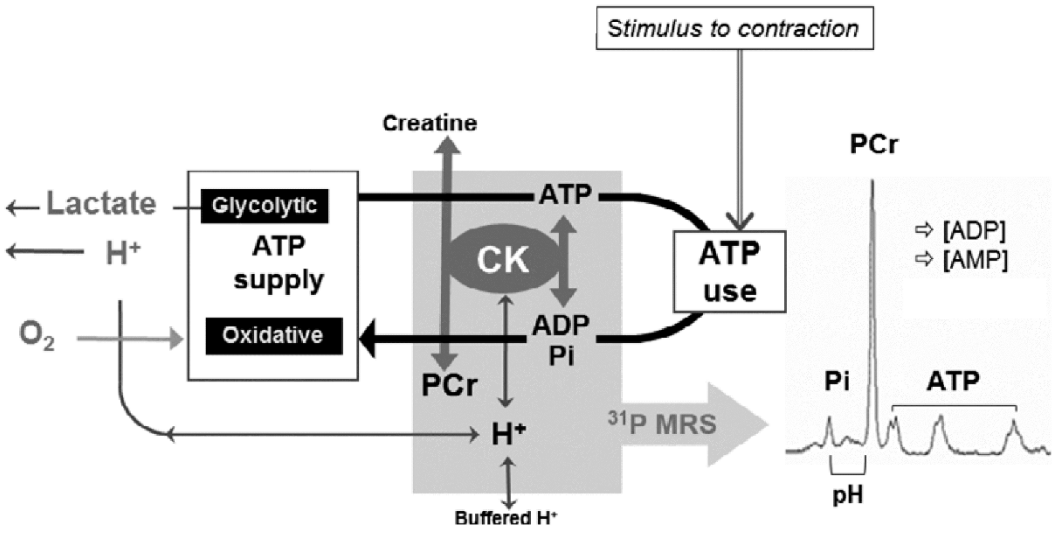

Figure 4-7. Skeletal muscle metabolism as interrogated using ^{31}P-MRS

The energetics of contraction are simplified to ATP supply and demand, buffered by the creatine kinase (CK) reaction which equilibrates PCr, creatine (Cr), ATP and ADP. The shaded rectangle represents the metabolic domain from which data can be derived using ^{31}P-MRS. The resting muscle spectrum to the right of the figure shows the peaks of Pi and PCr and the three peaks of ATP, from which the cytosolic pH (and the free concentrations of ADP and AMP) may be derived. Adapted from (Kemp *et al.* 2015) with permission.

The kinetics of PCr resynthesis were modelled for the recovery period after each plantarflexion exercise bout. A monoexponential relationship was derived for each set of

recovery data (Meyer 1988), using a least-squares-fit approach to determine the time-constant (τ), and expressing [PCr] as a function of time (t) (Khushu *et al.* 2010):

$$[PCr]_t = [PCr]_0 + ([PCr]_{Rest} - [PCr]_0) \cdot (1 - e^{(-t/\tau)})$$

Which, if [PCr] values are normalised such that $[PCr]'_{Rest} = 1$, simplifies to:

$$[PCr]'_t = 1 - e^{(-t/\tau)} \cdot (1 - [PCr]'_0)$$

Additionally, the maximum theoretical rate of mitochondrial ATP synthesis (Q_{MAX}) was extrapolated from a combination of the end-exercise [ADP] and initial rate of PCr resynthesis, as follows:

$$Q_{MAX} = V \cdot (1 + (K_m \div [ADP])^n)$$

Where V is the initial rate of PCr resynthesis, K_m is the [ADP] at which oxidative ATP synthesis is taken to be half maximal (25 μmol/L) and n (2.2) is a Hill coefficient that describes the relationship between V and [ADP] (Jeneson *et al.* 1996, Trenell *et al.* 2006, Edwards *et al.* 2012). Figure 4-8 shows a typical fit achieved using this approach.

4.3.3.2. Cardiopulmonary exercise testing

Following completion of the ^{31}P-MRS section of the experimental protocol, participants were escorted to CCRF for the second experimental component of the study morning. First, a 22-gauge venous cannula (Venflon™, Becton Dickinson) was inserted aseptically into a large antecubital fossa vein (typically ipsilateral to the non-dominant hand), from which 20 ml of blood were drawn and processed as described for *Study A*. Thereafter, large skeletal-muscle-mass exercise was investigated using a stepped CPET to volitional fatigue on an electronically braked cycle ergometer (ergoselect 100, ergoline GmbH, Germany),

ensuring a constant work-rate at each level, independent of cadence. The saddle height was carefully adjusted for participant comfort and the same setting used on both experimental days. The participant wore a close-fitting facemask with respired gases sampled continuously through a catheter and analysed by indirect calorimetry using a clinical-grade CPET platform (Metalyzer® 3B CPET System, CORTEX Biophysik GmbH, Germany).

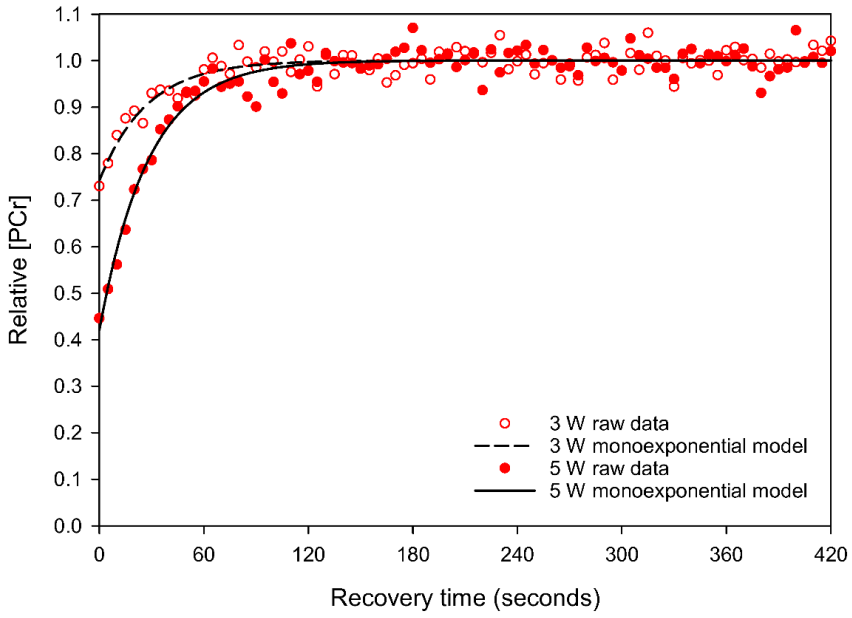

Figure 4-8. Monoexponential fitting of PCr recovery data

Typical experimental data, in this case taken from an iron-deficient female, following cessation of plantarflexion exercise at 3 W and 5 W, on the second study day. [PCr] is expressed relative to resting values and a monoexponential function (black line) has been fitted for each workload. Modelling the recovery in this way allows τ and Q_{MAX} to be derived.

At the outset, resting data were collected over a 2-minute period. Work was then begun at 50 W, for three minutes, increased thereafter by 25 W increments at 3-minute intervals. Participants were asked to select a cadence that felt comfortable for them – typically around

sixty revolutions per minute – and increase this as they wished as the work rate increased. Venous blood was sampled via the indwelling cannula during the final thirty seconds of each exercise interval and finally upon cessation of exercise. Samples were immediately analysed for lactate concentration using a Lactate Pro™ near-patient testing device (Arkray Inc. Japan), which had previously been clinically validated (Pyne *et al.* 2000, Goodwin *et al.* 2007, Baldari *et al.* 2009, Tanner *et al.* 2010). At the time of blood sampling, participants were asked to rate their perception of exertion by pointing to a fifteen-point rating of perceived exertion (RPE) scale, from 6 (rest) to 20 (maximum exertion) (Borg 1970).

After a rest period of fifteen minutes following completion of maximal CPET, participants were returned to the ergometer and two minutes of resting data recorded. Next, they were asked to exercise for a further 20 minutes at a work rate equivalent to 65% $\dot{V}O_{2MAX}$, determined by interpolation of the data just obtained. Participants were allowed to drink water *ad libitum* prior to exercise and during the rest period. During submaximal exercise, blood was sampled at rest, after two minutes, five minutes and every five minutes thereafter. On the second study day arrangements were identical, but the same work rate was used during the submaximal CPET as on the first study morning.

Data obtained during CPET were processed off-line by a single investigator who had been involved in supervising the cycle ergometer exercise testing but who did not know the iron status of the participant nor whether they had been randomised to receive iron or saline. This generated, for each time point, averages for the preceding 30 seconds, that is, the final half minute of each exercise period. Then, using these data, the lactate threshold during maximal CPET was determined as described by Wasserman and colleagues (Beaver *et al.* 1985). A plot of the logarithm of venous [lactate] against the logarithm of $\dot{V}O_2$ was generated and the point of intersection of the lines describing two phases of the lactate rise

during incremental exercise determined, as illustrated in Figure 4-9. For completeness, the lactate threshold was also determined using a plot of the logarithm of venous [lactate] against work intensity.

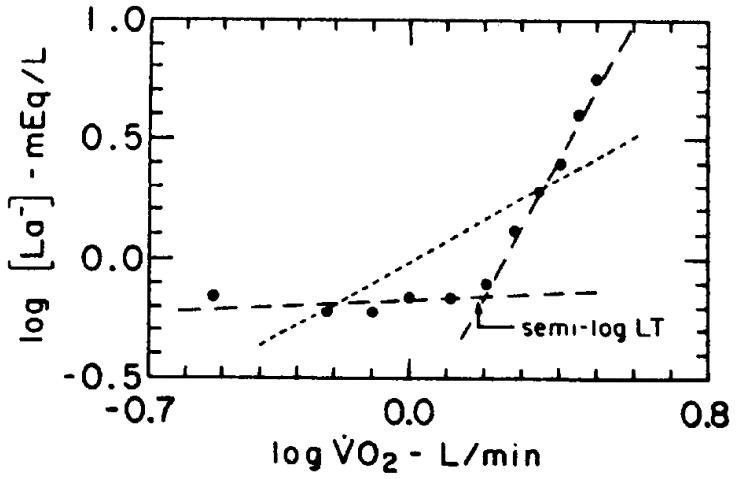

Figure 4-9. Determination of lactate threshold using a log-log plot

The dashed lines are of linear regression for two visually distinct segments, with the lactate threshold defined as the point at which these intersect. The dotted line is single regression line model of all data points. The arrow indicates the lactate threshold (LT) as previously determined using a different visual method (a semi-log plot), to highlight that LT may be underestimated if that technique is used rather than a log-log plot. Adapted from (Beaver *et al.* 1985).

4.3.3.3. Infusion and analysis of blood samples

At the end of the first study morning, participants received either 0.9% saline or ferric carboxymaltose (Ferinject®, Vifor Pharma, Switzerland) 15 mg/kg (maximum 1 g); each IV infusion was of 250 ml total volume and given over 15 minutes. Allocation was in a 1:1 fashion and was performed by an investigator who had no other role in the experimental component of the study; block randomisation was used according to iron status and sex. Participants were blindfolded, and the infusion, infusion line and infusion site obscured

using an opaque plastic drape. Blood samples were processed as for *Study A*. The only significant difference was that the ELISA used to measure hepcidin was more modern (Hepcidin 25 HS, DRG, Marburg, Germany).

4.3.4. Statistics

The primary outcome measure was PCr depletion during graded exercise of a small skeletal muscle mass, assessed with ^{31}P-MRS. Secondary outcome measures included blood lactate and cardiorespiratory parameters during large skeletal muscle mass exercise, assessed with CPET. Statistical analyses followed the approach described for *Study A* (section 2.3.2).

4.3.5. Study approval

The study was conducted in accordance with the Declaration of Helsinki and received ethical approval from the National Research Ethics Service NHS Oxford B Research Ethics Committee (reference: 13/SC/0439). Though not a CTIMP, the study was nevertheless registered prospectively with ClinicalTrials.gov (reference: NCT02308449). The study sponsor was the University of Oxford. All participants provided written informed consent.

4.4. Results

4.4.1. Baseline characteristics of participants in *Study B*

Thirteen ID individuals were recruited and matched with controls, as illustrated in Figure 4-10. Table 4-2 gives baseline characteristics of the participants. Mean ferritin in the ID group was 8.3 μg/L and transferrin saturation was 10.6%, indicating absolute iron deficiency. Plasma sTfR values in the ID group were around twice those of IR participants. Hepcidin was very heavily suppressed in the ID group. None of the participants in either group had an elevated CRP.

Characteristic	ID Group (n=13)	IR Group (n=13)	*P*-value
Female sex – no. (%)	12 (92)	12 (92)	
Age – years	23 (21.5 - 38)	24 (22 - 25.5)	0.59
Body mass index – kg/m²	21.8 (20.8 - 24.1)	21.1 (20.7 - 24.1)	1
Smoking status – ex:never	1:12	1:12	1†
Resting S_pO_2 – %	98.8 ± 0.9	98.5 ± 1.1	0.45
Systolic blood pressure – mmHg	119 ± 11	119 ± 9	0.99
Diastolic blood pressure – mmHg	73 ± 10	79 ± 8	0.08
FEV_1 – % predicted	106 ± 12	104 ± 9	0.75
Weekly aerobic exercise – hours	5.0 (2.0 - 7.5)	4.0 (1.0 - 8.5)	0.70
Plasma CRP – mg/L	0.4 (0.3 - 2.6)	0.5 (0.3 - 1.6)	0.96
Serum ferritin – µg/L	8.3 ± 3.1	58.0 ± 38.2	
Serum TSat – %	10.6 ± 3.9	35.2 ± 7.6	
Serum iron – µmol/L	8.4 ± 3.2	22.0 ± 3.6	**< 0.001**
Serum transferrin – g/L	3.6 ± 0.6	2.9 ± 0.5	**0.003**
Plasma sTfR – nmol/L	38.4 (32.7 - 50.9)	21.8 (18.4 - 25.0)	**< 0.001**
Plasma hepcidin – µg/L	2.0 (1.3 - 2.4)	13.9 (9.9 - 29.5)	**< 0.001**
Haemoglobin concentration – g/dl	12.7 (11.5 - 13.3)	13.6 (13.5 - 14.0)	**< 0.001**
Haematocrit – %	39.7 ± 3.2	42.0 ± 1.7	**0.028**
Mean cell volume – fl	86.9 (83.0 - 91.3)	92.4 (89.9 - 94.1)	**0.010**
Serum vitamin B12	323 (262 - 515)	398 (259 - 496)	0.98
Serum folate	6.7 (5.9 - 10.3)	8.8 (7.0 - 14.1)	0.17

Table 4-2. Participant characteristics on enrolment to *Study B*

Values are means ± SD, for normally distributed data, and comparisons by t-test; or medians (IQR), if not normally distributed, in which case comparisons are by MWU test. †, Chi-squared test; CRP, C-reactive protein. Note that values for hepcidin are not directly comparable with those from *Study A* given in Table 2-1 as the assay used differs. *P*-values for comparisons where statistically significant differences are present appear in bold.

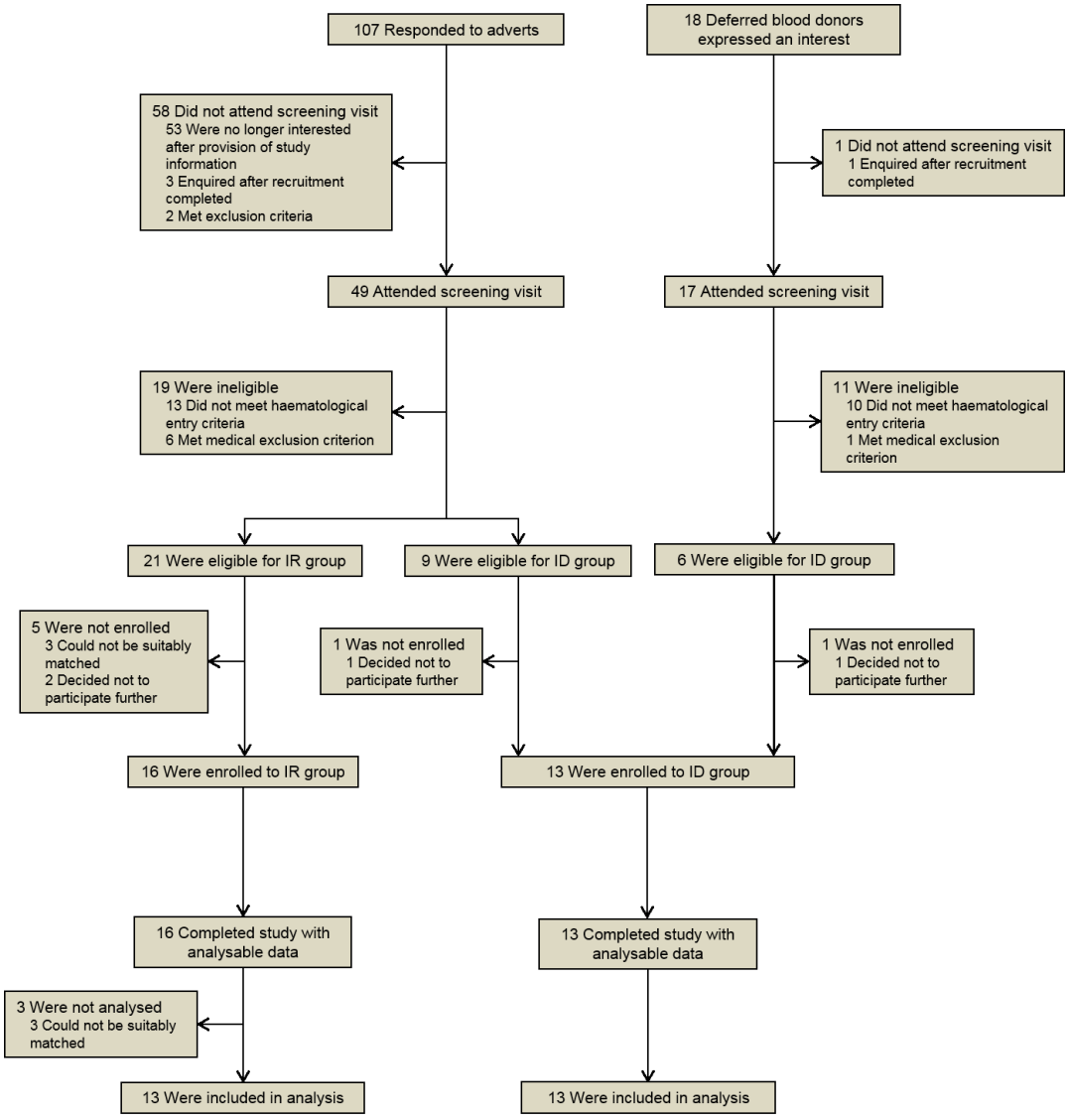

Figure 4-10. Study recruitment flow diagram for *Study B*

There were 18 expressions of interest from deferred blood donors and 107 responses to advertisements, which, in contrast to *Study A*, also specifically invited individuals to respond if they believed they might be iron deficient. Thirteen participants were enrolled to the ID group and sixteen to the IR group. No ID participants could be recruited as suitable matches for three young men enrolled to the IR group early in the course of recruitment.

4.4.2. Haematological parameters over the course of *Study B*

Table 4-3 shows the manner in which haematological parameters and iron indices changed between the first and second study days, according to whether participants received IV iron or saline at the conclusion of the first study morning.

	ID Group (n=13)				IR Group (n=13)				*P*-value for interactions (RM-ANOVA)	
	Iron (n=7)		Saline (n=6)		Iron (n=7)		Saline (n=6)		Day & allocation	Day, status & allocation
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2		
Hb – g/dl	13.1 ± 1.1	13.0 ± 1.3	12.7 ± 0.6	12.5 ± 0.7	14.3 ± 0.7	13.7 ± 0.9	14.1 ± 0.4	13.9 ± 0.6	0.66	0.43
MCV – fl	84.4 ± 7.6	87.1 ± 6.8	86.0 ± 3.9	86.0 ± 4.3	91.9 ± 5.7	91.4 ± 2.6	90.9 ± 3.2	91.3 ± 2.3	0.47	0.09
Ferritin – µg/L	7.9 ± 3.5	561 ± 433	6.9 ± 2.4	10.6 ± 2.8	56.3 ± 35.5	840 ± 331	29.0 ± 14.3	38.7 ± 16.2	**< 0.001**	0.32
TSat – %	12.3 ± 4.9	37.9 ± 11.2	13.7 ± 5.5	11.0 ± 2.8	29.0 ± 8.8	62.1 ± 20.1	23.0 ± 5.5	24.3 ± 12.5	**< 0.001**	0.73
Iron – µmol/L	9.3 ± 2.6	24.0 ± 9.1	11.1 ± 4.8	8.7 ± 2.0	17.8 ± 4.8	31.2 ± 10.4	16.2 ± 4.3	16.5 ± 8.2	**< 0.001**	0.54
Transferrin – g/L	3.61 ± 0.77	2.90 ± 0.48	3.65 ± 0.49	3.68 ± 0.44	2.83 ± 0.34	2.32 ± 0.26	3.17 ± 0.40	3.13 ± 0.39	**< 0.001**	0.27
sTfR – nmol/L	40.3 ± 12.0	33.4 ± 7.6	41.8 ± 13.1	40.9 ± 14.0	24.3 ± 3.5	20.7 ± 4.2	22.5 ± 2.5	21.7 ± 2.0	**0.007**	0.30
Epo – mIU/ml	17.7 ± 9.2	13.6 ± 3.9	13.8 ± 4.7	21.3 ± 7.8	8.0 ± 2.8	7.6 ± 4.0	7.7 ± 2.4	7.2 ± 2.1	**0.016**	**0.015**
Hepcidin – µg/L	2.1 ± 1.2	38.5 ± 28.9	1.5 ± 0.5	2.1 ± 1.0	17.2 ± 22.6	85.5 ± 54.6	7.3 ± 3.3	14.6 ± 14.7	**< 0.001**	0.30

Table 4-3. Haematological and iron parameters over the course of *Study B*

Values are means ± SD. *P*-values for comparisons where statistically significant differences are present appear in bold.

Irrespective of iron status on enrolment, those randomised to receive iron showed a significant increase in ferritin, serum iron, TSat and hepcidin; transferrin and sTfR fell significantly. In the case of erythropoietin, the effect of IV iron differed according to iron status, with a significant fall in erythropoietin in the ID participants given iron compared with those receiving saline, in whom erythropoietin instead rose considerably. These effects were not associated with any significant changes in [Hb].

4.4.3. ^{31}P-MRS during small muscle mass exercise

The changes in [PCr] and pH during calf muscle exercise on the first study morning are illustrated in Figure 4-11. All participants were able to complete the 3 W exercise bout, but one IR participant ended the 4 W period early, and one ID and two IR participants ended the 5 W period prematurely. PCr levels on the second study morning in the ID group are illustrated in Figure 4-12, and those in the IR group, in Figure 4-13.

An analysis of the effect of iron status, workload, and iron infusion based on monoexponential fitting of data from each recovery period is given in Table 4-4. For individuals stopping work prematurely, recovery kinetics were modelled from cessation of exercise. By chance, both [PCr] at end exercise and τ were significantly different in individuals randomised to receive iron, irrespective of iron status on enrolment. Specifically, individuals receiving iron had depleted PCr less at end exercise and recovered more quickly; these differences are evident from inspection of Figure 4-12 and Figure 4-13. The only significant effect detected was of workload, with end exercise [PCr] and pH tending to fall with successive bouts of exercise, and Q_{MAX} rise. No effect of iron status was clearly evident, nor an effect of IV iron.

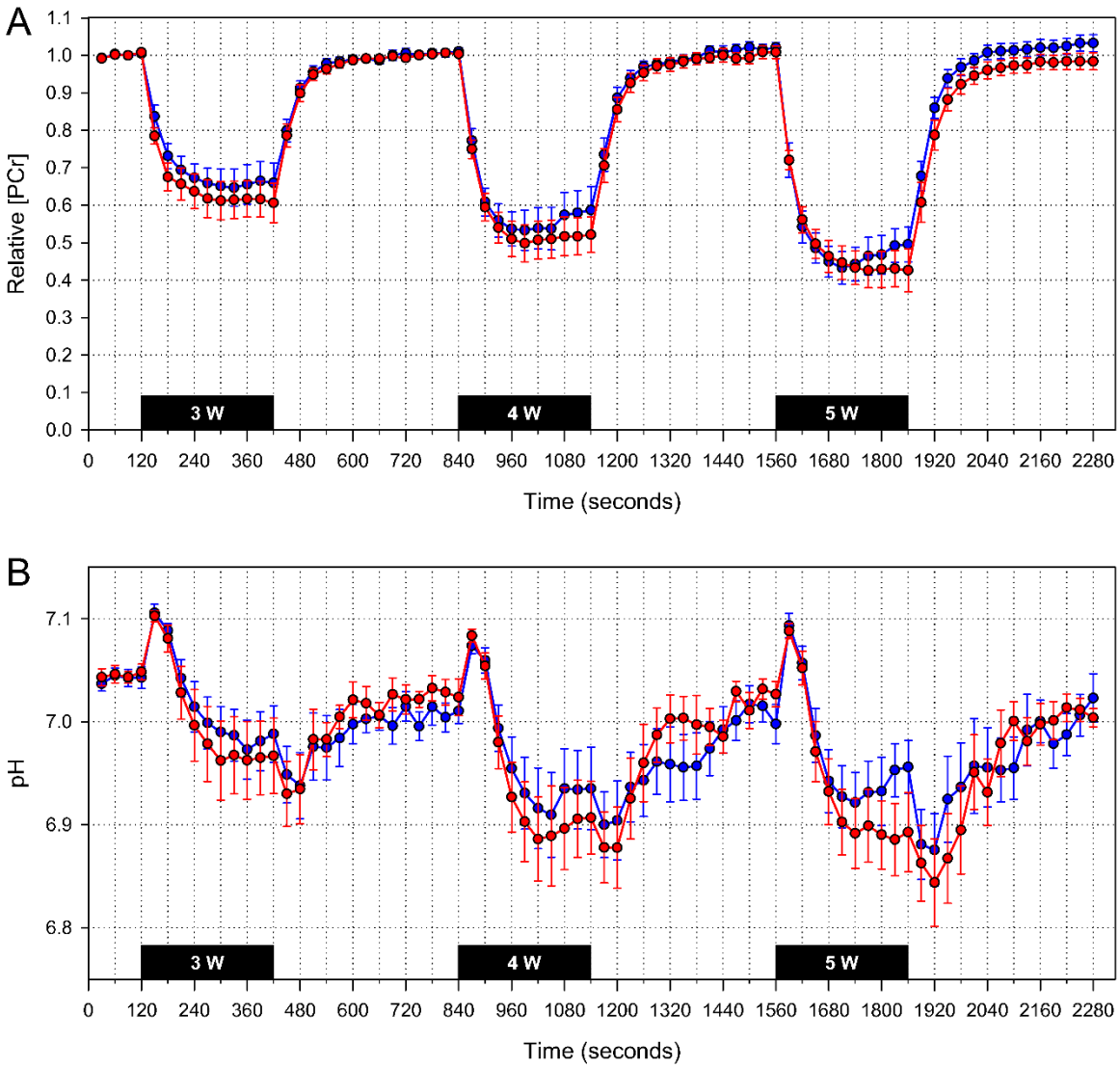

Figure 4-11. ^{31}P-MRS data from the first study morning

(A) [PCr] and (B) pH during the calf muscle exercise protocol. [PCr] is expressed as a fraction of the mean value during the initial 2-minute rest period. Data for the ID group are shown in red, and those for the IR group in blue. The solid black bars indicate the 5-minute periods of plantarflexion at 1 Hz. All participants completed the 3 W bout. During the 4 W bout, one IR participant ceased exercise prematurely at the 1050-second point. During the 5 W bout, one ID participant ceased exercise at the 1820-second point, and two IR participants ceased exercise prematurely, one at the 1720-second point and the other at the 1775-second point. For illustrative purposes, recovery data for participants unable to complete a given 5-minute exercise period are shifted to align with cessation of exercise in the other participants; an 'early recovery' artefact is thus apparent in the IR group near the end of the 4 W and 5 W bouts; values for the rest periods include data for all participants. Values are 30-second means; error bars show SEM.

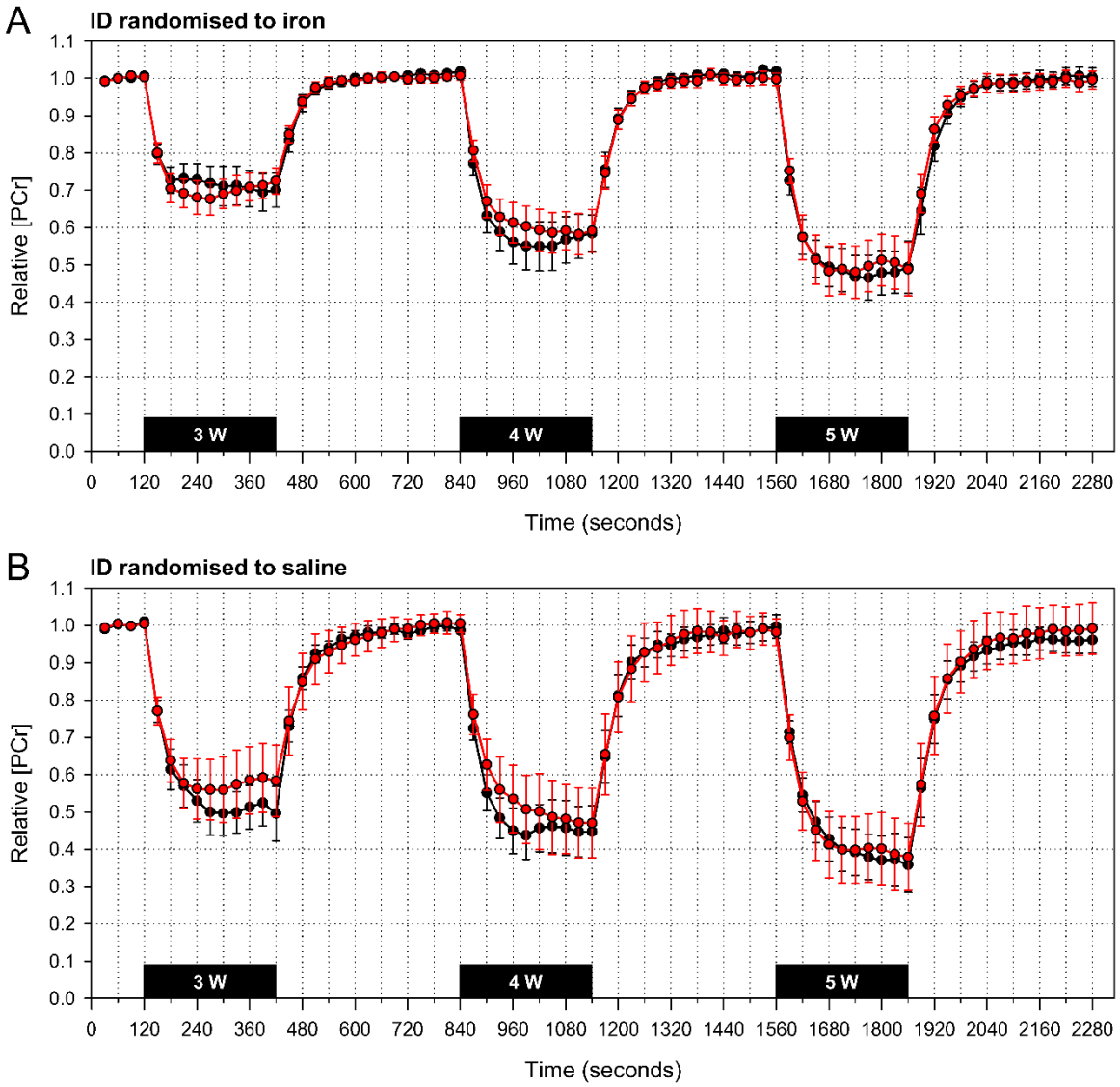

Figure 4-12. PCr metabolism in the ID group on both study mornings

Data are for ID participants randomised to (A) iron and (B) saline. [PCr] is expressed as a fraction of the mean value during the initial 2-minute rest period. Data for the first study morning are shown in black, and those for the second morning in red. On the first study morning, during the 5 W bout, one ID participant randomised to receive iron ceased exercise prematurely at the 1820-second point. For this participant, recovery data are shifted to align with planned cessation of exercise. Otherwise all ID participants completed all exercise bouts. Though PCr depletion was progressively more pronounced with increasing workload in both groups, it is evident that those ID participants randomised to receive iron maintained a higher [PCr] during each exercise bout on the first study morning. Values are 30-second means; error bars show SEM.

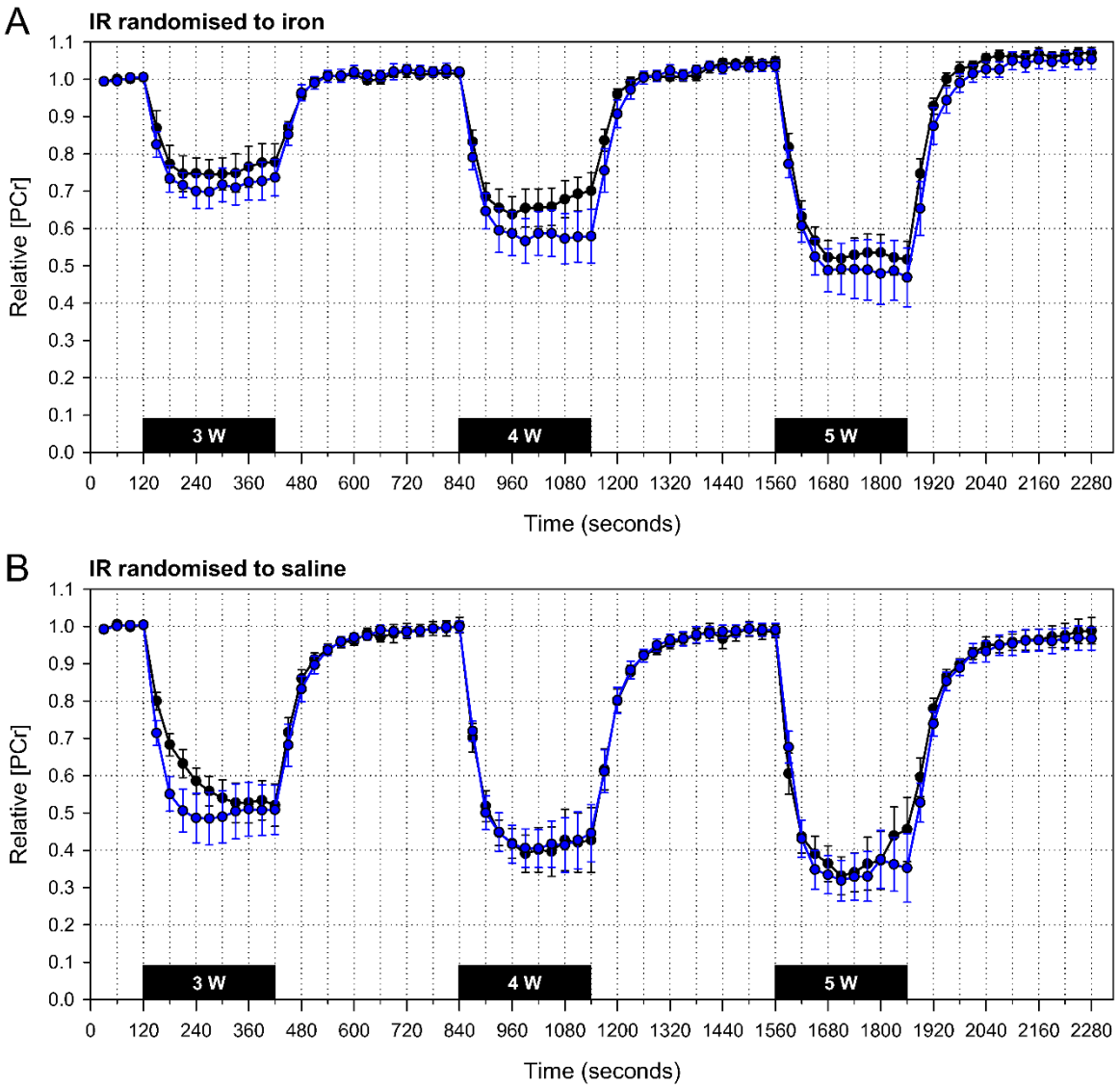

Figure 4-13. PCr metabolism in the IR group on both study mornings

Data are for IR participants randomised to (A) iron and (B) saline. [PCr] is expressed as a fraction of the mean value during the initial 2-minute rest period. Data for the first study morning are shown in black, and those for the second morning in blue. On the first morning, one participant randomised to receive saline ceased exercise at 1050 seconds during the 4 W bout, and two stopped during the 5 W bout, one at 1720 seconds and the other at the 1775-second point. On the second morning these latter two again ceased exercise during the 5 W bout, one at 1775 seconds and the other at the 1815-second point. For these participants, recovery data are shifted to align with planned cessation of exercise. As for the ID group, those ID participants randomised to receive iron maintained a higher [PCr] during each exercise bout on the first study morning. Values are 30-second means; error bars show SEM.

	ID Group (n=13)				IR Group (n=13)				
	Iron (n=7)		Saline (n=6)		Iron (n=7)		Saline (n=6)		
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	*P*-value (RM-ANOVA)
[PCr] 3 W	0.72 ± 0.11	0.74 ± 0.10	0.57 ± 0.15	0.62 ± 0.23	0.74 ± 0.10	0.73 ± 0.09	0.60 ± 0.12	0.53 ± 0.19	Status – 0.99 **Allocation – 0.024**
[PCr] 4 W	0.59 ± 0.15	0.61 ± 0.18	0.45 ± 0.17	0.47 ± 0.22	0.68 ± 0.10	0.63 ± 0.12	0.44 ± 0.19	0.44 ± 0.22	**Workload – < 0.001** Day & allocation – 0.60
[PCr] 5 W	0.50 ± 0.20	0.52 ± 0.21	0.38 ± 0.19	0.38 ± 0.24	0.50 ± 0.14	0.50 ± 0.18	0.44 ± 0.20	0.32 ± 0.23	Day, status & allocation – 0.48
pH 3 W	6.99 ± 0.13	7.00 ± 0.06	6.95 ± 0.16	6.91 ± 0.25	7.04 ± 0.03	7.04 ± 0.04	6.92 ± 0.11	6.98 ± 0.10	Status – 0.75 Allocation – 0.15
pH 4 W	6.92 ± 0.11	6.94 ± 0.11	6.90 ± 0.15	6.86 ± 0.24	6.98 ± 0.10	6.97 ± 0.11	6.86 ± 0.15	6.88 ± 0.07	**Workload – < 0.001** Day & allocation – 0.90
pH 5 W	6.90 ± 0.09	6.90 ± 0.14	± 6.88 0.18	6.87 ± 0.24	6.95 ± 0.11	6.90 ± 0.14	6.87 ± 0.14	6.86 ± 0.09	Day, status & allocation – 0.37
τ 3 W	35 ± 14	32 ± 4	40 ± 14	51 ± 29	32 ± 16	29 ± 8	43 ± 16	49 ± 23	Status – 0.79 **Allocation – 0.047**
τ 4 W	36 ± 13	35 ± 6	41 ± 15	52 ± 31	43 ± 16	33 ± 11	43 ± 16	46 ± 15	Workload – 0.16 Day & allocation – 0.14
τ 5 W	40 ± 15	35 ± 9	45 ± 19	50 ± 19	45 ± 15	41 ± 14	45 ± 15	47 ± 19	Day, status & allocation – 0.34
Q_{MAX} 3 W	0.33 ± 0.08	0.37 ± 0.18	0.41 ± 0.17	0.25 ± 0.07	0.41 ± 0.24	0.44 ± 0.17	0.31 ± 0.03	0.49 ± 0.37	Status – 0.32 Allocation – 0.72
Q_{MAX} 4 W	0.43 ± 0.16	0.44 ± 0.29	0.48 ± 0.16	0.42 ± 0.32	0.47 ± 0.18	0.48 ± 0.12	0.43 ± 0.11	0.54 ± 0.28	**Workload – < 0.001** Day & allocation – 0.16
Q_{MAX} 5 W	0.43 ± 0.09	0.51 ± 0.24	0.46 ± 0.12	0.36 ± 0.07	0.53 ± 0.11	0.52 ± 0.18	0.38 ± 0.09	0.59 ± 0.30	Day, status & allocation – 0.08

Table 4-4. Parameters derived from monoexponential fitting of [PCr] recovery data

Values are given for (i) [PCr], phosphocreatine concentration at end exercise expressed as a fraction of the resting level to which it subsequently recovered; (ii) pH at end exercise; (iii) τ, measured in seconds; and (iv) Q_{MAX}, measured in mM of ATP per second. Values are means ± SD. *P*-values for comparisons where statistically significant differences are present appear in bold.

4.4.4. Large skeletal muscle mass exercise

4.4.4.1. Maximal CPET

Table 4-5 gives values for a number of parameters at the point of volitional fatigue during the maximal CPET. None of these variables differed in a statistically significant way according to iron status. All participants achieved a respiratory exchange ratio (RER) > 1 on both study mornings.

A statistically significant differential effect of IV iron on maximal power output was detected, with the performance of ID individuals receiving iron tending to improve and the performance of IR individuals receiving iron tending to worsen, but the size of this effect was extremely small – of the order of only a one percent change. The only other effect detected was for IV iron to increase the oxygen pulse ($\dot{V}O_2$ divided by heart rate) irrespective of iron status. The size of this effect was considerably larger – of the order of a ten percent increase compared to individuals receiving saline. There was a trend for IV iron to reduce minute ventilation ($\dot{V}E$) at maximum power output irrespective of iron status but this did not reach statistical significance ($P = 0.10$).

Data relating to lactate kinetics are presented in Table 4-6. None of these variables differed according to iron status. Irrespective of iron status, individuals randomised to receive IV iron showed an increase in lactate threshold however determined. The size of this effect was in excess of a ten percent increase compared to individuals receiving saline. There was also a trend for IV iron to reduce peak lactate irrespective of iron status, but this did not reach statistical significance ($P = 0.12$).

	ID Group (n=13)				IR Group (n=13)				
	Iron (n=7)		Saline (n=6)		Iron (n=7)		Saline (n=6)		
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	*P*-value (RM-ANOVA)
Power (W/kg)	2.94 ± 0.93	2.96 ± 0.90	2.92 ± 0.67	2.86 ± 0.72	3.21 ± 0.49	3.14 ± 0.51	3.02 ± 0.51	3.06 ± .55	Iron status – 0.49 Day & allocation – 0.67 **Day, status & allocation – 0.024**
$\dot{V}O_2$ (ml/kg)	38.0 ± 11.3	38.0 ± 10.9	39.0 ± 8.2	36.1 ± 8.3	41.3 ± 7.7	41.4 ± 9.8	39.6 ± 4.7	39.3 ± 5.6	Iron status – 0.45 Day & allocation – 0.10 Day, status & allocation – 0.22
RER	1.15 ± 0.08	1.16 ± 0.09	1.14 ± 0.07	1.14 ± 0.06	1.15 ± 0.05	1.12 ± 0.02	1.12 ± 0.04	1.12 ± 0.06	Iron status – 0.40 Day & allocation – 0.84 Day, status & allocation – 0.33
$\dot{V}E$ (L/kg/ min)	1.65 ± 0.67	1.59 ± 0.66	1.60 ± 0.30	1.64 ± 0.22	1.83 ± 0.45	1.80 ± 0.45	1.51 ± 0.27	1.56 ± 0.31	Iron status – 0.74 Day & allocation – 0.10 Day, status & allocation – 0.83
Oxygen pulse (ml/kg/ beat)	0.199 ± 0.05	0.207 ± 0.05	0.212 ± 0.05	0.205 ± 0.05	0.220 ± 0.05	0.230 ± 0.06	0.215 ± 0.03	0.208 ± 0.03	Iron status – 0.50 **Day & allocation – 0.020** Day, status & allocation – 0.91
RPE	18.6 ± 1.5	18.3 ± 1.1	18.0 ± 1.8	17.8 ± 2.6	19.0 ± 1.0	18.9 ± 1.1	18.3 ± 1.0	18.5 ± 1.0	Iron status – 0.37 Day & allocation – 0.61 Day, status & allocation – 0.82

Table 4-5. Variables measured at the point of volitional fatigue during the maximal CPET

P-values are given for (i) the effect of iron status, (ii) the interaction between day and allocation (i.e. the effect of IV iron), and (iii) the interaction between day, status, and allocation (i.e. whether the effect of IV iron differed according to iron status). No statistically significant effect of allocation was detected for any CPET variable, in contrast to the ^{31}P-MRS experiments. RER, respiratory exchange ratio; $\dot{V}E$, minute ventilation; oxygen pulse is defined as $\dot{V}o_2$ divided by heart rate. *P*-values for comparisons where statistically significant differences are present appear in bold.

	ID Group (n=13)				IR Group (n=13)				
	Iron (n=7)		Saline (n=6)		Iron (n=7)		Saline (n=6)		
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	*P*-value (RM-ANOVA)
Peak lactate (mmol/L)	6.2 ± 2.4	5.7 ± 1.9	6.4 ± 1.6	7.3 ± 2.3	6.7 ± 1.9	6.4 ± 2.3	6.3 ± 2.0	6.5 ± 2.3	Iron status – 0.90 Day & allocation – 0.12 Day, status & allocation – 0.46
$\dot{V}O_2$ lactate threshold (ml/kg)	20.6 ± 6.9	23.0 ± 8.0	22.5 ± 6.9	22.7 ± 7.6	24.9 ± 6.3	28.0 ± 6.9	24.0 ± 4.7	24.0 ± 4.9	Iron status – 0.24 **Day & allocation – 0.039** Day, status & allocation – 0.72
Work lactate threshold (W/kg)	1.36 ± 0.59	1.54 ± 0.66	1.70 ± 0.63	1.62 ± 0.70	1.81 ± 0.48	1.98 ± 0.40	1.58 ± 0.69	1.57 ± 0.68	Iron status – 0.46 **Day & allocation – 0.002** Day, status & allocation – 0.52

Table 4-6. Venous lactate kinetics during maximal CPET

P-values are given for (i) the effect of iron status, (ii) the interaction between day and allocation, and (iii) the interaction between day, status, and allocation. The peak lactate is that measured at volitional fatigue. Lactate thresholds were determined with respect to both $\dot{V}O_2$ and power output. No statistically significant effect of allocation itself was detected for any variable. *P*-values for comparisons where statistically significant differences are present appear in bold.

4.4.4.2. Submaximal CPET

Physiological variables during the period of submaximal exercise on the first study morning are illustrated in Figure 4-14. All participants completed the 20-minute period of exercise, and both groups exercised at very similar levels of $\dot{V}O_2$, close to the target of 65% of $\dot{V}O_{2MAX}$ throughout.

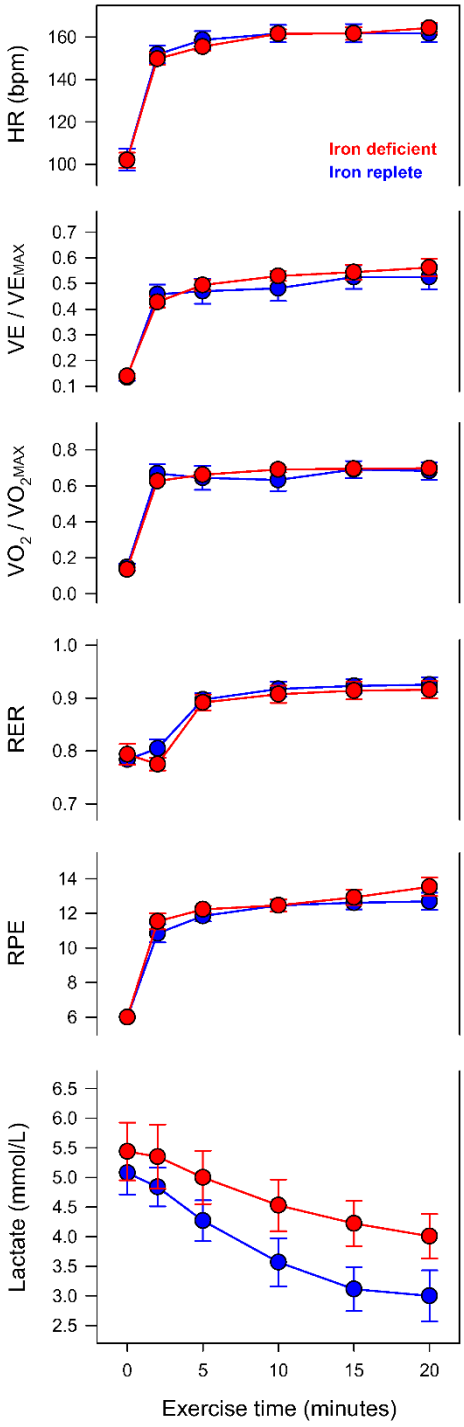

Figure 4-14. Submaximal CPET on the first study morning

Data for the ID group are shown in red, and those for the IR group, blue. $\dot{V}E$ and $\dot{V}O_2$ are expressed as a fraction of the value at cessation of the preceding maximal CPET. Data for $\dot{V}E$, $\dot{V}O_2$ and RER are missing for one IR participant at the 10, 15 and 20-minute time points, and a single lactate value missing for one ID participant at the 2-minute time point, due to technical issues. Values for parameters other than [lactate] are means for the previous 30 seconds; error bars show SEM.

The only variable with grossly different behaviour between groups during submaximal exercise on the first study morning was venous [lactate], as shown in more detail in Figure 4-15. In a mixed effects model, lactate fell significantly over the course of the rest and submaximal exercise period ($P < 0.001$ for effect of time), but the rate of decline in lactate was slower in the ID group (estimated shortfall in rate of net lactate clearance in ID compared with IR: 2.6 mmol/L/h; 95% CI 0.8 – 4.4 mmol/L/h; $P = 0.005$).

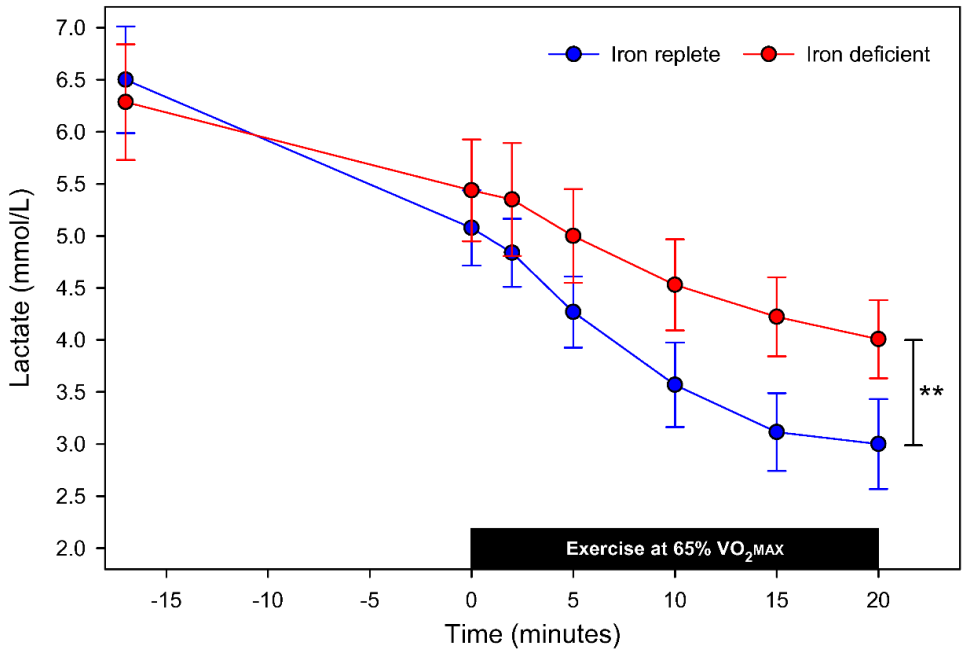

Figure 4-15. Venous lactate concentration following maximal CPET on the first study morning

The lactate value at -17 minutes was that determined at volitional fatigue during the preceding maximal CPET. Following a 15-minute interval, participants returned to the cycle ergometer and measurements of cardiorespiratory variables were made during two minutes of rest. A single lactate value was missing for one ID participant at the 2-minute time point due to a technical issue. Values are means; error bars show SEM. **, $P < 0.01$ for interaction of iron status and time.

The effect of IV iron infusion on cardiorespiratory variables on the second study morning is illustrated for ID participants in Figure 4-16 and for IR participants in Figure 4-17.

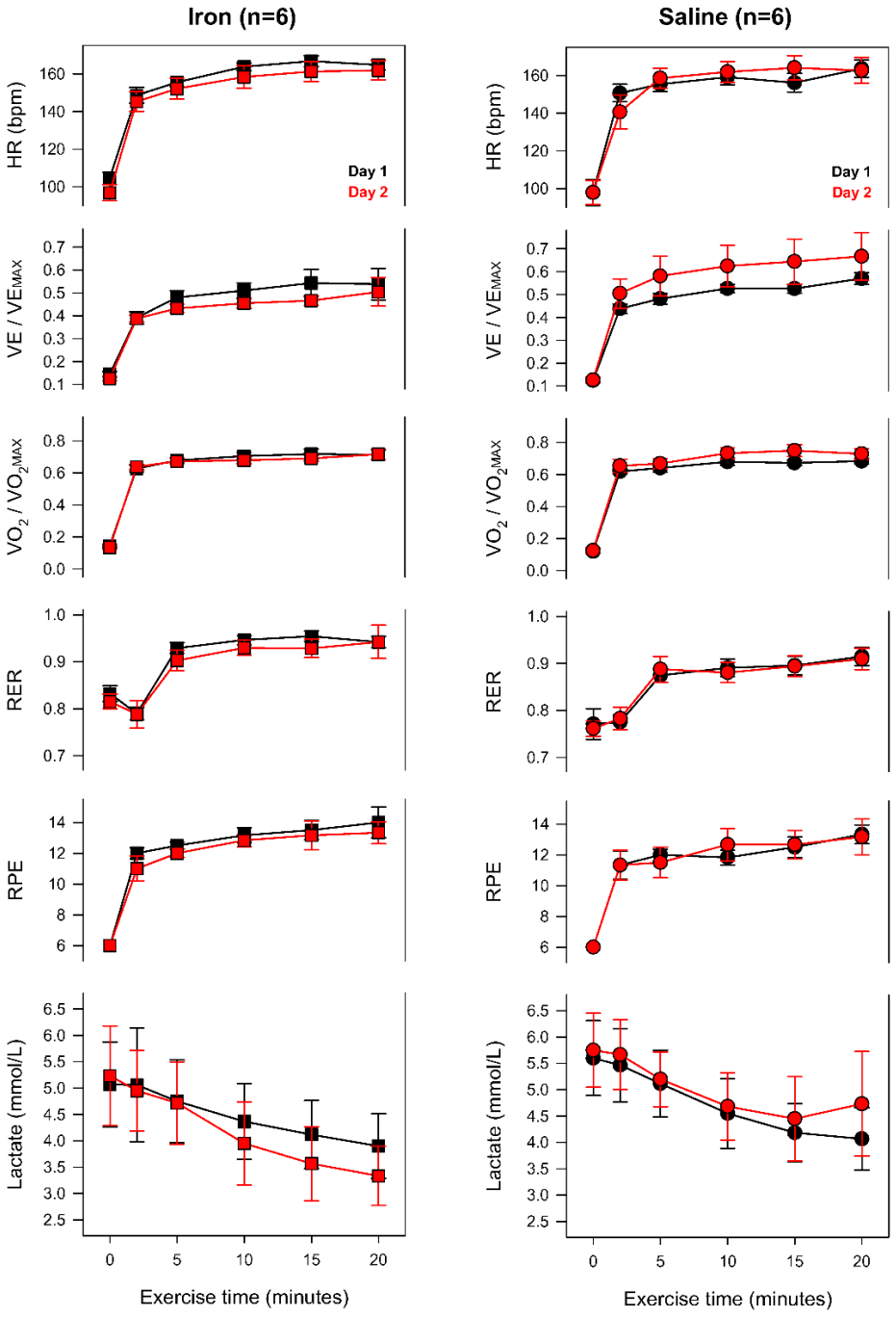

Figure 4-16. Submaximal CPET on both study mornings in the ID group

Data for the first day are shown in black, and those for the second, red. Squares represent participants randomised to iron, circles saline. $\dot{V}E$ and $\dot{V}O_2$ values for both days are expressed relative to maximal values on the first day. One participant randomised to iron became presyncopal on return to the ergometer following the rest period on day 2 and did not perform submaximal exercise; data from both days for this individual are excluded from this figure. Values for parameters other than [lactate] are means for the previous 30 seconds; error bars show SEM.

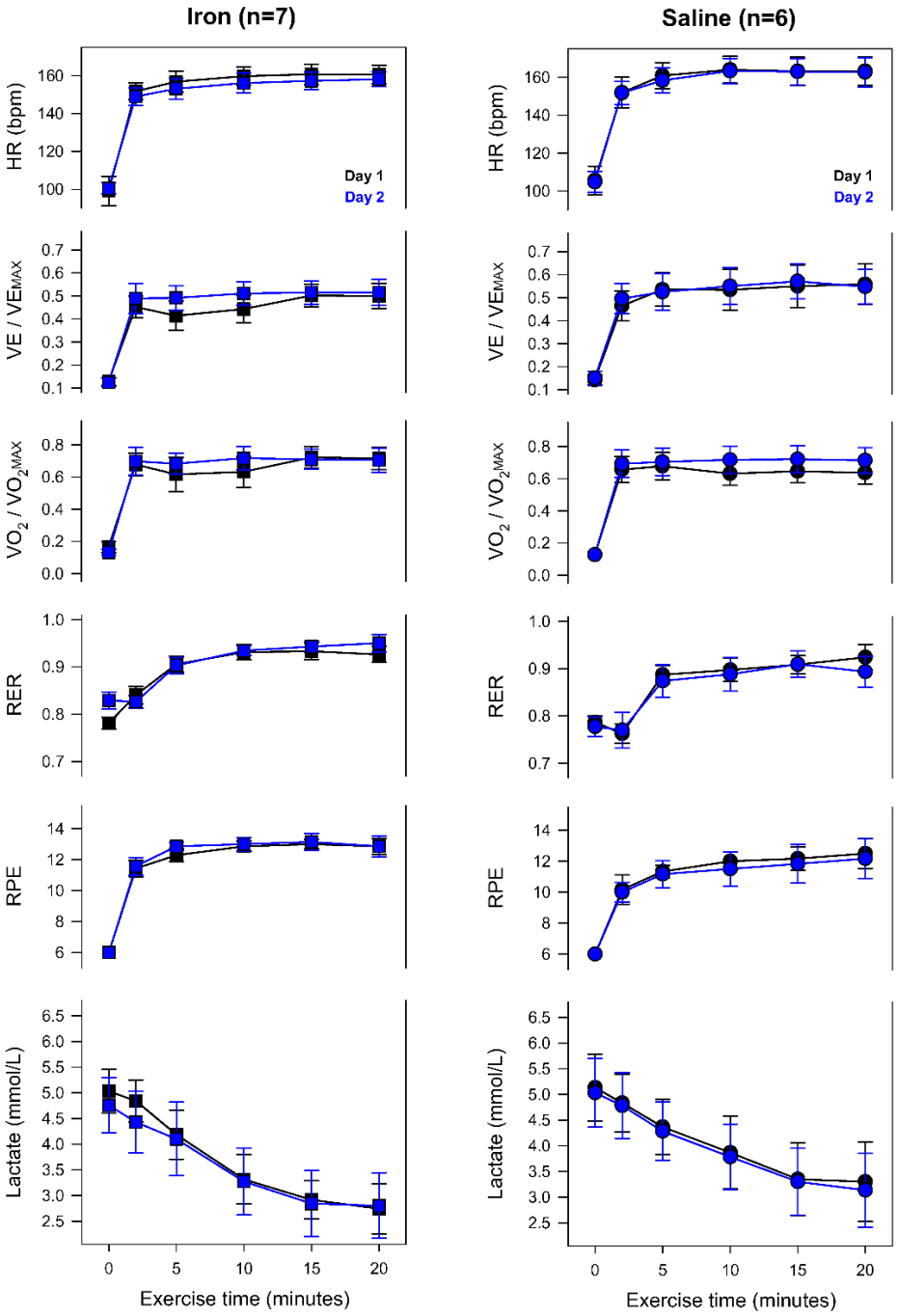

Figure 4-17. Submaximal CPET on both study mornings in the IR group

Data for the first day are shown in black, and those for the second, blue. Squares represent participants randomised to iron, circles saline. $\dot{V}E$ and $\dot{V}O_2$ values for both days are expressed relative to maximal values on the first day. Data on the first day for $\dot{V}E$, $\dot{V}O_2$ and RER at the 10, 15 and 20-minute time points are missing for one participant randomised to receive saline due to a technical issue. Values for parameters other than [lactate] are means for the previous 30 seconds; error bars show SEM.

Mixed effects modelling also indicated a significant interaction between iron status, allocation, day and time; specifically, IV iron had an effect to accelerate net lactate clearance only in the ID group (estimated increase in rate of net lactate clearance in ID group given iron: 5.9 mmol/L/h; 95% CI 0.7 – 11.0 mmol/L/h; $P = 0.028$).

4.5. Discussion

4.5.1. Main findings

The main finding of *Study B* is of abnormal whole-body aerobic metabolism in iron-deficient individuals in the absence of good evidence for a defect in mitochondrial oxidative phosphorylation. When iron-deficient participants were exercised at 65% $\dot{V}O_2MAX$ they exhibited significantly impaired net lactate clearance compared with their iron-replete counterparts. What this implies is that iron deficiency either impairs lactate disposal during aerobic exercise or augments its production – or a combination of the two. This effect cannot simply be explained by a marginally lower [Hb] in the ID group. For one thing, a submaximal work rate was used that was tailored to the $\dot{V}O_2MAX$ of each individual. Moreover, following infusion of IV iron, the rate of net lactate clearance increased significantly in iron-deficient participants in the absence of any increase in [Hb]. No effect of IV iron on lactate kinetics during submaximal CPET was seen in iron-replete volunteers.

Maximal power output and $\dot{V}O_2MAX$ determined during the incremental CPET to volitional fatigue did not differ significantly according to iron status. Ever since the seminal work of Hill and colleagues almost a century ago (Hill *et al.* 1924a, Hill *et al.* 1924b), physiologists have been working to establish those factors that contribute most significantly to determining $\dot{V}O_2MAX$. The argument boils down to whether it is oxygen *delivery* to the

mitochondrion, or the ability of this organelle to *consume* oxygen, that is the liming factor. Several authorities have concluded, based in part on observations regarding relationships with CO and [Hb], that oxygen delivery is paramount (Richardson *et al.* 1999, Bassett and Howley 2000, Saltin and Calbet 2006). However, mitochondrial volume density in the skeletal muscle of humans (and other animals) is very closely related to $\dot{V}O_2MAX$ (Hoppeler *et al.* 1973, Weibel and Hoppeler 2005), as is mitochondrial oxidative enzyme activity (Costill *et al.* 1976, Blomstrand *et al.* 1997). In fact, a recent study reported that the relationship between $\dot{V}O_2MAX$ and muscle fibre oxidative capacity held for professional cyclists, healthy untrained individuals and patients with CHF, with measured $\dot{V}O_2MAX$ being around 90% of mitochondrial oxidative capacity (van der Zwaard *et al.* 2016). These observations support the notion of symmorphosis:

> *"The allometric variation of maximal metabolic rate is directly related to the scaling of the total effective surfaces of mitochondria and capillaries…the scaling of maximal metabolic rate is determined by the energy needs of the cells active during maximal work. The vascular supply network is adapted to the needs of the cells at their working limit. We conjecture that the optimization of the arterial tree by fractal design is the result rather than the cause…" (Weibel and Hoppeler 2005)*

The relevance of this to the present study is that the absence of impairment in $\dot{V}O_2MAX$ or maximal power output during CPET in iron-deficient individuals is as would be expected given the absence of a defect in mitochondrial oxidative phosphorylation during ^{31}P-MRS of exercising calf muscle. That absolute values for $\dot{V}O_2MAX$ and maximal power output during CPET were slightly lower in ID participants may, though, be explained by the marginally lower [Hb].

The difference in lactate kinetics during submaximal exercise might reasonably be interpreted as indicating that for iron-deficient participants, 65% $\dot{V}O_{2MAX}$ represented a level of exertion closer to the lactate threshold than was the case for iron-replete individuals, or even above it in some cases. The question therefore arises as to why no difference was detected in lactate threshold during the maximal CPET (Table 4-6). In fact, it can be seen that in terms of $\dot{V}O_2$, the lactate threshold occurred on the first study day at a mean of 55.7% $\dot{V}O_{2MAX}$ in the ID group, and 60.2% $\dot{V}O_{2MAX}$ in the IR group. In terms of power output, the lactate threshold occurred on the first study day at 50.0% maximal power output in the ID group and 53.5% maximal power output in the IR group. These differences did not reach statistical significance, but they are certainly of a size consistent with clinical significance; within group heterogeneity was high, which may have led to a type II error.

Interestingly, irrespective of iron status, administration of IV iron led to a sizeable increase in the lactate threshold during the incremental CPET whether assessed by $\dot{V}O_2$ or power output. Why, then, was no difference detected in lactate kinetics during submaximal exercise in iron-replete individuals given iron? The explanation may be that because the IR group were generally exercising further below their lactate thresholds than the ID group, lowering the threshold further did not translate into a detectable effect.

Another finding of interest is of an increased oxygen pulse, irrespective of iron status, at $\dot{V}O_{2MAX}$, following IV iron infusion. This parameter is an index of stroke volume in healthy individuals (Stringer *et al.* 1997) and finds clinical utility in identifying the onset of myocardial ischaemia – which is associated with a plateau or fall in SV – in patients with suspected flow-limiting coronary artery disease (Belardinelli *et al.* 2003, Chaudhry *et al.* 2009). A final observation of note is the suppression of serum erythropoietin in the iron-deficient participants given IV iron. This occurred before any increase in [Hb], and

effectively confirms the suspicion in *Study A* that iron deficiency acts directly to elevate circulating erythropoietin levels.

4.5.2. Advances over previous studies

The main strength of the present study lies in the combination of three features. First, the dual assessment of metabolism using ^{31}P-MRS of a small muscle mass and whole body exercise in the same individual. Secondly, the use of a high dose of IV iron in a double-blind manner. Thirdly, recruitment of individuals with profound iron deficiency in the absence of marked anaemia, with repeated assessment following administration of IV iron but prior to any change in [Hb].

In comparison, no previous study has used both CPET and ^{31}P-MRS; the majority have employed CPET, being primarily concerned with the improvement in athletic performance following iron supplementation. To this author's knowledge, only one small study has previously used ^{31}P-MRS to investigate the effects of iron status in humans, and this recruited a small, highly heterogeneous group of anaemic hospital-inpatients (Thompson *et al.* 1993b). The dose of IV iron used in *Study B* was twice that employed in the study of elite athletes discussed earlier (Burden *et al.* 2015b), making it exceedingly unlikely that an inadequate dose could have led to a significant effect on high energy phosphate metabolism being missed.

Any study that uses oral iron supplementation inevitably suffers from several significant drawbacks. The bioavailability is variable and absorption confounds the findings. It is impossible adequately to blind participants since those receiving iron invariably note dark stools with or without unpleasant gastrointestinal symptoms (Tolkien *et al.* 2015). Oral iron must be given for such a length of time that [Hb] unavoidably rises significantly by the time

tissue iron stores have increased. Indeed, given that iron will preferentially be directed to erythropoiesis if IDE is present, it is almost inevitable that with oral iron therapy [Hb] will climb in advance of a significant correction of the iron deficiency in other tissues.

4.5.3. Limitations

4.5.3.1. Technical considerations

A graphical representation of the window onto skeletal muscle metabolism that is provided by ^{31}P-MRS is given in Figure 4-7. The literature arguing the finer points of how best to interrogate skeletal muscle biology with this technique is enormous (Kemp and Radda 1994, Kushmerick 1995, Jeneson *et al.* 1997, Kemp *et al.* 2015) and largely irrelevant to the present study, since evidence of a large effect on mitochondrial function akin to that seen in CP patients (Figure 4-1) was absent. Of note though, pH may influence PCr recovery kinetics, so the suggestion of a lower cytosolic pH at all workloads in the iron-deficient participants is a concern, though the absolute differences, if real, appear to be smaller than those that would be required to confound significantly the findings (Kemp *et al.* 2015). Much of this effect results from the fact that hydrolysis of PCr consumes, and PCr resynthesis generates, H^+. This accounts for the sharp rise in cytosolic pH with the onset of calf muscle exercise evident in Figure 4-11, and also the somewhat counterintuitive initial fall in pH at cessation of each plantarflexion exercise bout.

One other issue that should be considered is whether the reproducibility of the technique is such that it compromised the ability of the study to detect a small effect. Using experimental apparatus very similar to that in *Study B*, Edwards *et al.* reported in trained male subjects that whilst measures of resting phosphorus metabolites were generally reproducible, exercise data were less reliable. For example, during exercise, PCr had a coefficient of variation of 27%, and that for PCr half-time was 40% (Edwards *et al.* 2012).

Finally, venous [lactate] was measured in *Study B* rather than arterial [lactate]. Some argue that arterial sampling is preferred, since with the venous approach the particular vascular bed drained may be a net consumer or producer of lactate, such that [lactate] varies with the site of sampling (Goodwin *et al.* 2007). Whilst this is true, repeated arterial sampling would have necessitated placement of an indwelling arterial catheter, which would likely have been too invasive to be acceptable for many participants. In any event, participants had a cannula placed in a large upper limb vein at similar sites, and the same site was used on both study days in a given participant. Venous [lactate] determined from upper limb blood sampling during lower limb exercise, as in our participants, has been shown to correlate very well with arterial [lactate] (Nordrehaug *et al.* 1991). Thus, the difference in the kinetics of net lactate clearance between groups on the first study day, and the differential response to IV iron, cannot conceivably be attributed to the measurement technique adopted.

4.5.3.2. Sex of participants

It proved very difficult to recruit iron-deficient males to the present study. Whist iron deficiency is recognised to be far more common in women, the reasons for this difficulty are not entirely clear, since the recruitment methods and entry criteria were near identical to those for *Study A*, to which male recruitment was satisfactory. The men that did volunteer for the present study were almost invariably not sufficiently iron deficient to meet the entry criteria. The mean age of participants in *Study B* was somewhat lower than in *Study A*, suggesting perhaps that the latter was more attractive to older volunteers and the former, younger volunteers, possibly because of the exercise component involved. If iron deficiency in men were to become more common with age, which is possible, this could explain the greater ease with which men were enrolled to *Study A*.

Whatever the origin of the difficulty, the result is that the present study includes only a pair of men compared with twelve pairs of women. Though it seems highly unlikely that men and women could differ fundamentally with respect to mitochondrial oxidative phosphorylation and aerobic exercise physiology, this paucity of male participants must be acknowledged when considering the generalisability of the results.

4.5.3.3. Heterogeneity of participants

The range of $\dot{V}O_2MAX$ values for participants in the current study was quite marked, and this heterogeneity may have made it more difficult to detect baseline difference between the ID and IR groups, particularly in the lactate thresholds during maximal CPET. Though participants were nominally matched for exercise habits, behaviours in this respect were, as would be anticipated with such a range of $\dot{V}O_2MAX$ values, heterogeneous, with some participants exercising for only an hour a week and others ten times this amount. One strategy to overcome this issue would have been to recruit exclusively trained individuals. If we consider the study by Burden and colleagues, for example, we find a mean $\dot{V}O_2MAX$ of 73 ml/kg/min, and SD of 4.8 ml/kg/min, for their participants receiving IV iron (Burden *et al.* 2015b); clearly a much fitter and more homogenous group than in *Study B.*

However, there are real disadvantages to this strategy. Trained individuals are not representative of the 'healthy' general population, and there may be effects of athletic training on iron homeostasis that make this group unattractive for a physiological study of iron and metabolism. A frequently encountered view in the exercise literature is that increased hepcidin following exercise (Peeling *et al.* 2014) contributes to iron deficiency in this population. Another – not mutually exclusive – possibility, is that athletes partition iron differently in some way. It may be that case that there is abundant iron to be found within skeletal muscle in trained individuals who, as a consequence, have serum ferritin

levels that in untrained individuals would be taken to indicate iron deficiency. Such a phenomenon effect might explain why Burden *et al*. found no effect on exercise outcomes of interest using 500 mg IV ferric carboxymaltose in trained men and women with serum ferritin values < 30 μg/L and < 20 μg/L, respectively (Burden *et al.* 2015b). Values for TSat (~ 30 %), transferrin (~ 2.6 g/L) and sTfR (~ 21 nmol/L) were not dissimilar in that study to those in the healthy IR groups in both *Study A* and *Study B*. This observation rather suggests that the trained participants in that study did not have iron deficiency as this author would understand it.

In order to avoid the sort of issues that might arise if a group of more 'consistent' fitness were to be recruited, the only way to overcome the problem of heterogeneity of fitness would have been to increase the sample size.

4.5.3.4. Haemoglobin concentration

As in *Study A*, [Hb] in the ID group was slightly lower than that in the IR group, and again it must be considered whether this difference could have confounded the findings. In the case of the MRS data, the small muscle mass exercise used is likely to have been effectively independent of oxygen delivery (Formenti *et al.* 2010). More importantly, though, because in *Study B* participants were randomised to receive iron or saline, and no changes in [Hb] had occurred by the time of the second study visit, we can be confident that the effects seen are not haemoglobin mediated. As already mentioned, the absolutely lower $\dot{V}O_{2MAX}$ and power output in the ID group may, though, be accounted for by this difference.

As an aside, it might be asked why [Hb] had not increased by the time of the second study day in ID participants given iron. The answer may be that at this level of IDE, the stimulus is not as great as with more marked anaemia, in which setting the typical rise in [Hb]

following IV iron has been reported in a meta-analysis to be just over 1 g/dl at one week (Moore *et al.* 2011). In support of this possibility, in a group of patients with an [Hb] of 9.2-11.9 g/dl, anaemia had not yet improved significantly 8 days after administration of IV iron (Lyseng-Williamson and Keating 2009).

4.5.3.5. Haematological parameters over the course of the study

The recruitment strategy and definitions of ID and IR adopted again performed very well insofar as two groups were generated that upon enrolment differed very significantly with respect to iron status, despite the absence of marked anaemia in the ID group. As in *Study A*, it was notable that in the ID group serum iron – and therefore TSat – was higher on the first study day than it had been at the screening visit, though this effect was less marked. Reasons for this phenomenon have already been considered in Chapter 2.

4.5.4. Underlying mechanisms

In 1924, Otto Warburg described the tendency of neoplastic cells to metabolise glucose to lactate irrespective of the availability of oxygen to support oxidative metabolism (Warburg 1924). Though in some ways this could be seen as metabolically inefficient, it is in fact a property of healthy proliferating cells, and contemporary studies suggest that it may be explained by a beneficial effect in promoting the uptake and assimilation of nutrients (Vander Heiden *et al.* 2009).

In *Study B*, our iron-deficient human participants effectively behaved in a Warburg-like manner; apparently having a greater preference to metabolise carbohydrate to lactate during aerobic exercise than their iron-replete counterparts. This is the opposite effect to that which has been described in endurance athletes, who seem to exhibit downregulation of the HIF pathway (Lindholm *et al.* 2014), and in animals with tissue-specific deletion of skeletal

muscle HIF-1α (Mason *et al.* 2004). Just as in *Study A*, it cannot be proved from this human physiology study that iron deficiency is acting through the HIF pathway to promote glycolysis, but collectively these observations strongly imply this to be the case. That IV iron supplementation altered this behaviour over the course of days further suggests an action via HIF – or other iron-sensitive transcription factor pathways – to alter the activity of glycolytic enzymes.

With respect to the observed increase in oxygen pulse with IV iron administration, an action of iron to increase SV might be explained by improved diastolic LV filling, an increase in ejection fraction, or a combination. It is known that as exercise intensity increases, LV diastolic volume increases and compliance decreases (Sullivan *et al.* 1991), at least in part due to competition with the RV for limited space within the inelastic pericardium (Reeves *et al.* 1990). The magnitude of this effect is attested by studies of pericardectomy in animals, in which both SV and $\dot{V}O_{2MAX}$ increase considerably after surgery (Stray-Gundersen *et al.* 1986, Hammond *et al.* 1992). It may be the case that at maximal exercise a fall in S_vO_2 promotes HPV, raising PVR and therefore RV afterload, thus compromising LV diastolic filling. It is certainly true that particularly fit individuals show greater increases in PASP during exercise (Bossone *et al.* 1999) perhaps in part related to enhanced oxygen extraction by exercising skeletal muscle and consequently a lower S_vO_2 (Van Thienen and Hespel 2016).

However, in euoxic conditions, pulmonary vasodilators have been found not to affect maximum power output or $\dot{V}O_{2MAX}$ (Hsu *et al.* 2006, Naeije *et al.* 2010), which would argue against IV iron administration increasing SV via an action on the pulmonary vasculature. Having said this, pulmonary vasodilators tend to increase systemic venous return, potentially counteracting any decrease in afterload obtained through a decrease in

PVR (Naeije and Chesler 2012). Iron might not have such an effect since it is not a conventional pulmonary vasodilator; rather as we have seen it is an HPV-inhibitor. Notably, Cheng found that a dose of iron similar to that used in the present study did indeed have an effect to reduce the increase in PASP seen during exercise in a group of individuals older than 50 years (Cheng 2015). In the absence of any invasive or echocardiographic measurements of haemodynamic parameters in the present study, though, it is difficult to reach firm conclusions regarding a mechanism for this finding. One final possibility is that the increase in oxygen pulse in fact reflects increased tissue oxygen extraction rather than increased SV, though it is not obvious how such an action would be brought about.

4.5.5. Clinical implications of findings

However the effect of iron deficiency on human metabolism is mediated, an action to promote glycolysis and augment lactate production is likely to be of very considerable clinical significance. A thorough review on the subject of exercise, lactate and the anaerobic threshold, now two decades old, articulates very clearly why this is the case:

> *"The point at which lactate begins to accumulate in the blood, causing an increase in ventilation, is important to document clinically. Irrespective of the underlying mechanism or specific model that describes the process, the physiologic changes associated with lactate accumulation have significant import for cardiopulmonary performance. These include metabolic acidosis, impaired muscle contraction, hyperventilation, and altered oxygen kinetics, all of which contribute to an impaired capacity to perform work. Thus, any delay in the accumulation of blood lactate which can be attributed to an intervention…may add important information concerning the efficacy of the intervention." (Myers and Ashley 1997)*

Thus, the apparently lower anaerobic threshold in iron-deficient but otherwise healthy individuals, and the effect of IV iron to delay lactate accumulation irrespective of iron status, both suggest that a mechanism by which iron deficiency may be deleterious in cardiopulmonary disease, and iron supplementation helpful, involves an effect on skeletal muscle metabolism.

The relevance of these findings specifically to COPD is considered in the next chapter. Revisiting the seminal study by Anker and colleagues of IV iron in patients with CHF, though, we see that dyspnoea – as measured by New York Heart Association class – improved significantly in those patients given iron (Anker *et al.* 2009). That is, patients tended to be less breathless at a given level of exertion; 6-minute walking distance (6MWD) also rose in the group given iron. Interestingly, in a study of patients with IPAH, it was reported that whilst peak $\dot{V}O_2$ was entirely unchanged with administration of IV iron (mean 0.97 ± 0.22 L/min before vs 0.97 ± 0.26 L/min after), the anaerobic threshold rose significantly and patients were able to exercise at 75% of peak $\dot{V}O_2$ for over 50% longer than prior to receiving iron; there was also a trend for 6MWD to improve (Ruiter *et al.* 2015).

The findings from this latter study reinforce a key message of *Study B*, that viewing $\dot{V}O_{2MAX}$ as the key outcome of interest risks missing important physiological effects that are of clinical relevance. It is clear that $\dot{V}O_{2MAX}$ is a very useful indicator of cardiopulmonary fitness, and predicts, for example, the likelihood of morbidity and mortality following major surgical procedures (Stringer *et al.* 2012, Levett and Grocott 2015). However, it seems unlikely that patients with chronic cardiopulmonary disease spend much of the average day at, or even close to, $\dot{V}O_{2MAX}$. Endurance at a given workload may well be of more importance from the perspective of the patient when considering the

impact on the individual's activities of daily living. Returning to the analysis of Myers and Ashley, we see that this is also a point that has been made previously:

> *"A substantial body of evidence is available demonstrating that lactate accumulation occurs later (shifting to a higher percentage of $\dot{V}O_{2MAX}$) after a period of endurance training. In athletes, the level of work that can be sustained prior to lactate accumulation, visually determined, is an accurate predictor of endurance performance. Presumably, these concepts have implications related to vocation/disability among patients with cardiovascular and pulmonary disease…"*
> *(Myers and Ashley 1997)*

As this quote implies, athletes expend very considerable time and energy attempting to raise both their $\dot{V}O_{2MAX}$ and lactate threshold, and a sizeable literature exists surrounding the best strategy to achieve this. Thus, *Study B* may also have implications for individuals interested in optimising athletic performance. The findings imply that the pseudo-hypoxic signal of iron deficiency in humans does not seriously impair skeletal muscle mitochondrial function, but does promote a metabolic phenotype that may be deleterious to endurance exercise. Supplementing iron might downregulate anaerobic glycolysis and provide a stimulus for oxidative metabolism, which could therefore be advantageous:

> *"Runners appear to set a race pace which allows the utilization of the largest possible $\dot{V}O_2$ which just avoids the exponential rise in plasma lactate." (Farrell et al. 1979)*

Conversely, it is even conceivable that in physically fit individuals, some degree of NAID might improve performance in events that rely entirely upon anaerobic energy provision, such as sprinting.

4.6. Conclusions

Study B appears to exclude a clinically meaningful direct effect of iron deficiency on mitochondrial oxidative phosphorylation in otherwise healthy individuals. Additionally, it suggests a significant effect of iron deficiency to promote glycolysis by exercising skeletal muscle under aerobic conditions. Given what is known regarding the processes that regulate skeletal muscle metabolism, these observations are in keeping with an effect mediated at least in part by an action on the HIF pathway.

Chapter 5. Iron deficiency in chronic obstructive pulmonary disease

5.1. Introduction

So far this thesis has considered the consequences of iron deficiency for individuals who are otherwise healthy. Abnormalities have been demonstrated in the pulmonary vascular response to hypoxia, and skeletal muscle exercise physiology. It has been argued that these findings may be of relevance to the association of iron deficiency with adverse outcomes in the setting of cardiopulmonary disease, reflecting a deleterious upregulation of hypoxia-sensing pathways by iron deficiency.

Chronic obstructive pulmonary disease is the name given to a spectrum of chronic lung diseases, including those that would previously have been termed chronic bronchitis and emphysema. In view of the physiological consequences of iron deficiency demonstrated in

previous chapters, COPD is of particular interest since hypoxaemia, PH, and skeletal muscle dysfunction are all features of the disease. The final question that shall be addressed by this thesis is whether iron deficiency plays a role in COPD.

5.1.1. Historical aspects

Many early reports of patients suffering from what we would today term COPD are to be found in the literature (Figure 5-1).

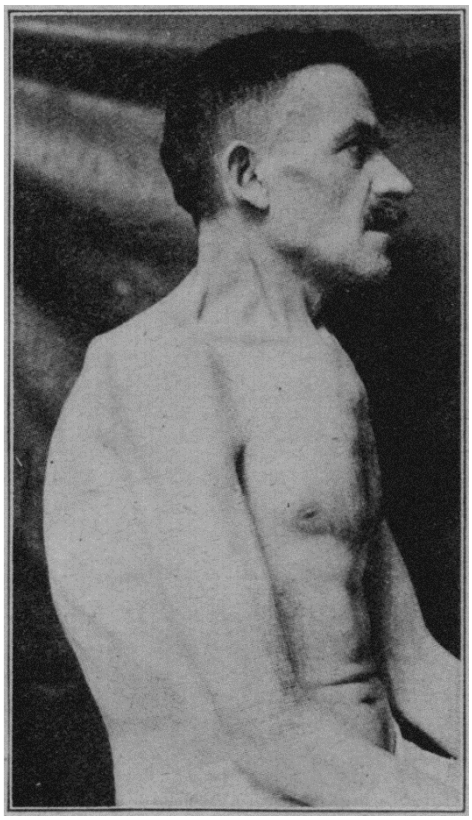

Figure 5-1. Early photograph of a 49-year-old male with "severe pulmonary emphysema"

The increased anteroposterior diameter of the thorax from chronic airflow obstruction is clearly evident. The patient also appears cachectic. Reproduced with permission from (Scott 1920). Copyright© (1920) American Medical Association. All rights reserved.

Such reports typically describe well the clinical features of severe, untreated disease:

> *"His complaint was shortness of breath and cough. Inspection showed the typical emphysematous thorax, with cyanosis of the face and hands…The patient was apparently having much difficulty in ventilation, and expiration lasted about three times as long as inspiration."*
> *(Scott 1920)*

The term chronic obstructive pulmonary disease first appeared in the 1960s (Briscoe and Nash 1965) and has become established as the description of choice for a collection of related lung conditions (Petty 2006). Traditionally, a distinction was drawn between emphysema and chronic bronchitis. The former was described in *histopathological* terms:

> *"Emphysema is a condition of the lung characterized by increase beyond the normal in the size of air spaces distal to the terminal bronchiole either from dilatation or from destruction of their walls."*
> *(Fletcher et al. 1959).*

The latter, in contrast, had a *clinical* definition:

> *"Chronic bronchitis is a clinical disorder characterised by excessive mucous secretion in the bronchial tree. It is manifested by chronic or recurrent productive cough. Arbitrarily, these manifestations should be present on most days for a minimum of three months in the year, and for not less than two successive years."*
> *(American Thoracic Society 1962)*

Some authors stressed the importance of nosology in chronic lung disease in the period during which the terminology we use today was beginning to evolve (Scadding 1963). Unfortunately, it is clear that there are still shortcomings in the approach taken today, including conflation of the *definition of*, and the *diagnostic criteria for*, COPD (Snider

2003). Further discussion of this issue would not be germane to this thesis. However, it is important to recognise that, in addition to representing a false dichotomy, neither the description of emphysema nor that of chronic bronchitis is based on any physiologic criteria. On the other hand, there may be considerable disadvantages of the use of an umbrella term such as COPD to describe what are in reality a number of somewhat different diseases with some shared features (Lynch *et al.* 2015, Woodruff *et al.* 2015).

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) was formed in 1998 with the aim of promoting COPD awareness and improving prevention and treatment of the condition. An initial consensus report – the Global Strategy for the Diagnosis, Management, and Prevention of COPD – was produced in 2001 (Pauwels *et al.* 2001) and subsequently revised five years later (Rabe *et al.* 2007). The 2011 update (Vestbo *et al.* 2013) is current at the time of writing. These documents have sought methodically to examine the evidence relating to the definition, diagnosis and management of COPD, and it is the recommendations set out by GOLD that shall be followed here.

5.1.2. Definition, aetiology and pathogenesis of COPD

The symptoms of COPD include chronic progressive dyspnoea, wheeze and cough, which may be productive. Patients experience episodic acute worsening of these symptoms, termed exacerbations, which are disabling, may necessitate hospital admission, and appear to drive disease progression (Hurst *et al.* 2010, Tanabe *et al.* 2011, Halpin *et al.* 2012).

The pathogenesis is extremely complex, but can be understood as an injurious sequence involving initiation, progression, and consolidation of pulmonary tissue damage. Oxidative stress, inflammation, extracellular matrix proteolysis, and apoptotic and autophagic cell death interact in such a way that the initial injurious stimulus leads to self-propagating

processes (Tuder and Petrache 2012). In Westernised countries, exposure to cigarette smoke is the commonest initiating factor, though a significant minority of patients with COPD are non-smokers (Tan *et al.* 2015); in these individuals the initiating factor may be an industrial or infectious exposure, or another lung disease such as asthma. The definition of COPD in the most recent iteration of the GOLD guidance provides a summary:

> *"COPD…is characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients. The chronic airflow limitation characteristic of COPD is caused by a mixture of small airways disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from person to person. Chronic inflammation causes structural changes and narrowing of the small airways. Destruction of the lung parenchyma, also by inflammatory processes, leads to the loss of alveolar attachments to the small airways and decreases lung elastic recoil; in turn, these changes diminish the ability of the airways to remain open during expiration."*
> *(Vestbo et al. 2013)*

The cellular interactions underlying the pulmonary pathology in COPD are illustrated in Figure 5-2. Though the primary site of injury is the lung, it is increasingly recognised that the extrapulmonary features of COPD are extremely important in contributing to morbidity and mortality. These include exercise intolerance, skeletal muscle dysfunction, osteoporosis, psychiatric disorders (such as anxiety and depression), and cardiovascular disease (Decramer *et al.* 2008, Fabbri *et al.* 2008). Whether it is correct to think of all these complications as extrapulmonary features of COPD, as opposed to comorbidities, is a

matter of debate. Cardiovascular disease risk, for example, may be increased by the chronic inflammation arising in COPD, as well as the shared aetiological factor of tobacco smoke.

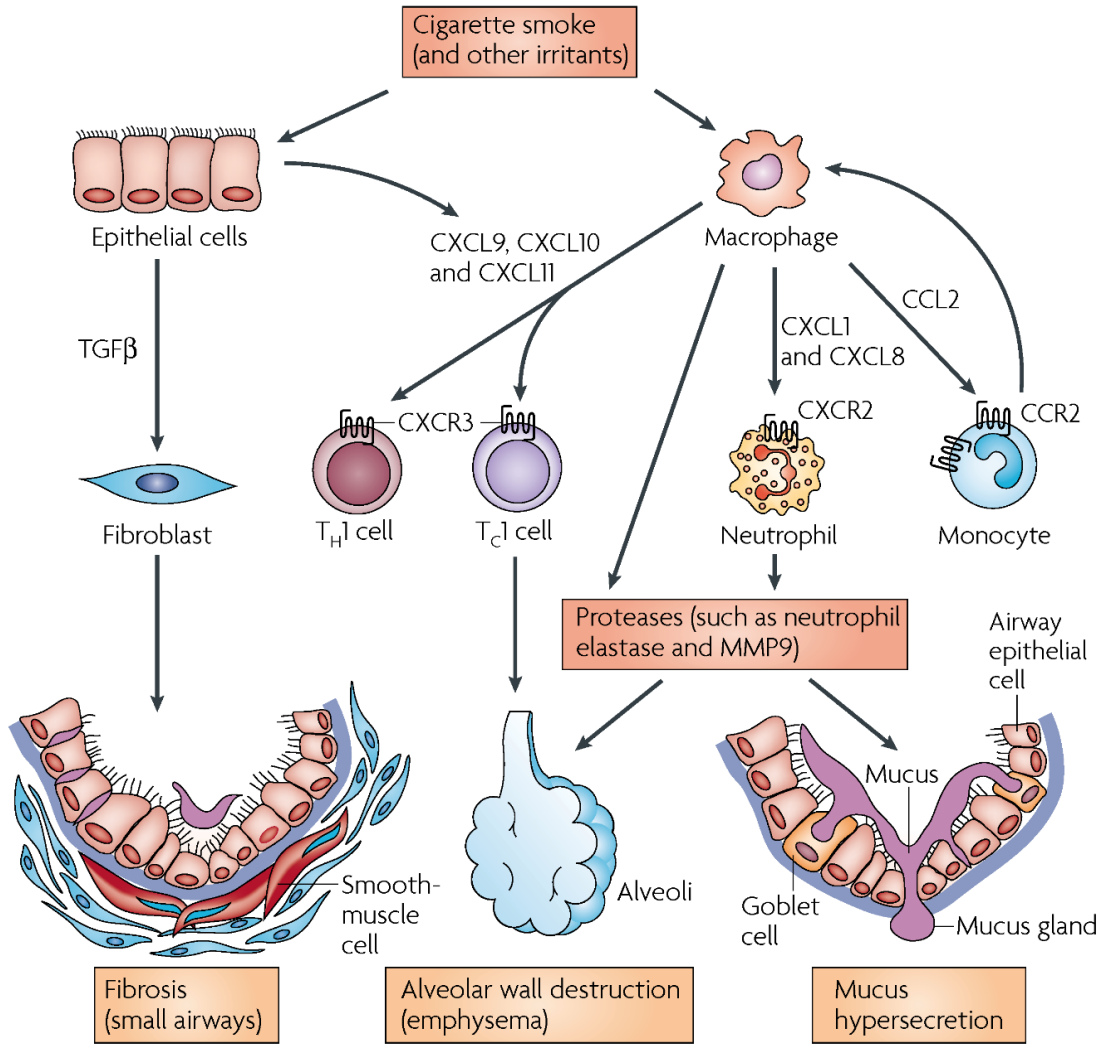

Figure 5-2. Cellular interactions and signalling molecules in the pathogenesis of COPD

Injurious stimuli activate epithelial cells and macrophages, which release chemotactic factors recruiting inflammatory cells. These secrete proteases degrading elastin; neutrophil elastase also promotes mucus hypersecretion. Transforming growth factor-β (TGFβ) stimulates fibroblast proliferation, causing small airway fibrosis. CCL2, CC-chemokine ligand-2; CCR2, CC-chemokine receptor-2; CXCL, CXC-chemokine ligand; MMP9, matrix metalloproteinase-9. T_C1, type 1 cytotoxic T-cell; T_H1, type 1 helper T-cell. Reproduced with permission from (Barnes 2008).

Recognition that COPD is not simply a disease the manifestations of which are confined to the lungs is vital when considering how iron deficiency may influence the natural history of the condition. For instance, iron deficiency could be particularly disabling if it were adversely to affect skeletal muscle function independently of any pulmonary effects. Some have gone so far as to propose that the pathogenesis of COPD be considered in terms of a network connected by the vasculature, with the lungs as an "external sensor" generating "danger signals", the endothelium as an "internal sensor" and potential target tissue, and bone marrow and adipose tissue as key effector tissues producing both inflammatory and repair signals (Agusti *et al.* 2013). Irrespective of the conceptual framework adopted, a general effect of iron deficiency on hypoxia-sensitive pathways could clearly have a number of possible anatomical sites of action in the condition.

5.1.3. Diagnosis of COPD

There are four GOLD core recommendations (Vestbo *et al.* 2013) regarding the diagnosis of COPD (FVC, forced vital capacity):

(i) *A clinical diagnosis of COPD should be considered in any patient who has dyspnoea, chronic cough and/or sputum production, and a history of exposure to risk factors for the disease.*

(ii) *Spirometry is required to make the diagnosis in this clinical context; the presence of a post-bronchodilator FEV_1/FVC less than 0.70 confirms the presence of persistent airflow limitation and thus of COPD.*

(iii) The goals of COPD assessment are to determine: (1) the impact of the disease on the patient's health status, (2) the severity of airflow limitation, and (3) the risk of future exacerbations, in order to guide therapy. The risk of future exacerbations is estimated by the severity of airflow limitation and the history of previous exacerbations.

(iv) Comorbidities, including cardiovascular disease, skeletal muscle dysfunction, metabolic syndrome, osteoporosis, depression, and lung cancer, occur frequently in patients with COPD. Comorbidities should be actively looked for, and treated appropriately if present.

It is of note that the justification in the guidance for the spirometric definition provided in point (ii) is not pathophysiological, rather it is defended as being simple and independent of reference values, such that it is convenient for a "busy nonspecialist clinician". Indeed, discussions of the utility of spirometry in COPD appear frequently to reach the conclusion that though its limitations are numerous, it is the "best available tool" (Doherty 2008). In patients meeting this arbitrary spirometric criterion who are also felt on clinical grounds to have COPD, the severity of the disease is further classified based on the severity of airflow limitation (Table 5-1).

FEV_1 (% predicted)	Severity of airflow limitation	Classification
> 80	Mild	GOLD 1
51 – 80	Moderate	GOLD 2
31 – 50	Severe	GOLD 3
≤ 30	Very severe	GOLD 4

Table 5-1. GOLD classification of COPD severity based on airflow obstruction (Vestbo *et al.* 2013)

The values are those obtained after the patient has taken any appropriate bronchodilator therapy.

5.1.4. Hypoxaemia

The pathophysiological importance of hypoxaemia in COPD is attested by two seminal studies that demonstrated the utility of long-term oxygen therapy (LTOT) as a treatment when the condition is complicated by chronic arterial hypoxaemia. The potential utility of correcting hypoxaemia with supplemental oxygen had been recognised previously (Levine *et al.* 1967), but these were the first well-controlled studies of oxygen as a therapy, and led to a widespread change in practice.

The first, a multicentre North American study of 203 patients followed for at least a year, randomly allocated individuals either to continuous oxygen therapy or to 12 hours per day of nocturnal oxygen therapy. Mortality in the nocturnal oxygen therapy group was almost twice as high as that in those randomised to continuous therapy (Noctural Oxygen Therapy Trial Group 1980). The second, a smaller multicentre UK study of 87 patients, randomly allocated participants either to 2 L/min nasal oxygen for at least 15 hours per day, or to usual care. Over a follow-up period of several years, LTOT was found to reduce mortality significantly (Medical Research Council 1981) (Figure 5-3). Many of the patients in these two studies had evidence of PH and consequent RVF, indeed the latter was a prerequisite for enrolment into the Medical Research Council study.

More recent studies have confirmed that stable hypoxaemia severe enough to merit therapy with LTOT is an adverse prognostic factor (Martinez *et al.* 2006). Similarly, severity of hypoxaemia on admission to hospital with an acute exacerbation of COPD (AECOPD) was identified as a predictor of poor outcome in a systematic review of 37 studies involving nearly two hundred thousand patients (Singanayagam *et al.* 2013).

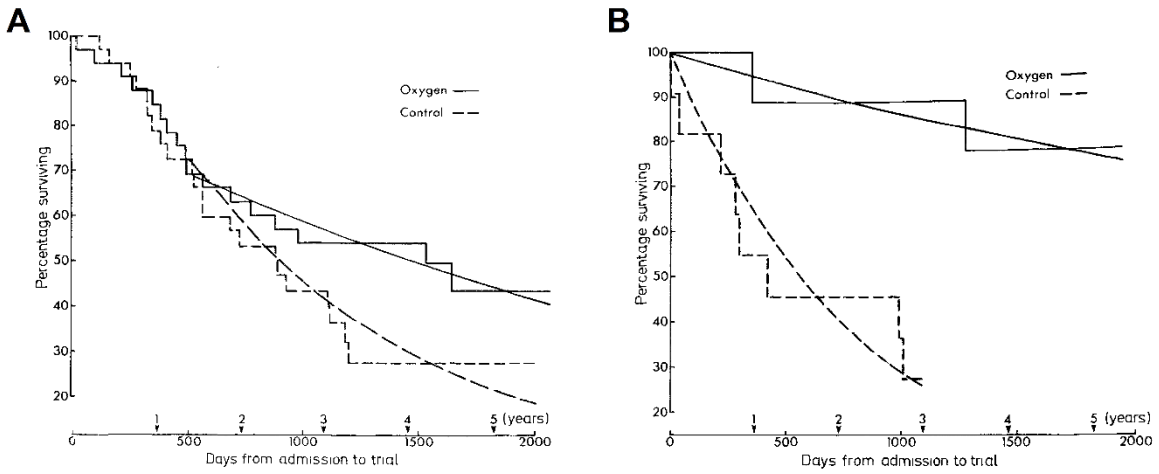

Figure 5-3. Mortality in COPD patients in the Medical Research Council study of LTOT

(A) Data for males. Smoothed curves indicate expected survival from 500 days using an annual mortality risk in the control group (n=33) of 29.4% and in the LTOT group (n=33) of 11.9%. (B) Data for females. Smoothed curves indicate expected survival from commencement of the study using an annual mortality risk in the control group (n=12) of 36.5% and in the LTOT group (n=9) of 5.7%. Adapted from (Medical Research Council 1981) with permission.

Despite its clear importance in the condition, the prevalence of hypoxaemia in COPD is uncertain, and reported rates vary considerably between studies. For example, in a trial of the bronchodilator tiotropium bromide in nearly six thousand patients with predominantly GOLD stage 2 and 3 disease, only just over two percent were using any form of supplemental oxygen therapy at enrolment (Tashkin *et al.* 2008). On the other hand, a study of over six hundred patients with predominantly GOLD stage 3 and 4 disease reported over eighty percent to be using some form of oxygen therapy (Martinez *et al.* 2006). However, the use of prescription of oxygen therapy as an index of hypoxaemia is not ideal, since factors other than arterial oxygen tension may influence decisions regarding provision of oxygen in clinical practice, and physician behaviour may vary across healthcare systems.

West has extensively investigated and very clearly described the fundamental mechanisms that underlie the development of hypoxaemia in lung disease (West 2011a). He recognises four distinct mechanisms causing hypoxaemia: (i) $\dot{V}_A/\dot{Q}$ inequality, (ii) shunt, (iii) diffusion impairment, and (iv) hypoventilation. Several or all of these may operate in a single condition, and the relative contributions may vary between patients with the same disease.

5.1.4.1. Ventilation-perfusion inequality and shunt

Attempts have been made to measure the distributions of $\dot{V}_A$ and $\dot{Q}$ *in vivo* using the multiple inert gas elimination technique (MIGET) (Wagner *et al.* 1974b, Wagner *et al.* 1974c). Briefly, several gases of differing solubilities are allowed to equilibrate in solution and then infused intravenously. Once a steady state has been established, the concentrations in mixed arterial blood and mixed expired gas are determined. The curves relating arterial concentration and expired concentration to solubility are then transformed into an almost continuous distribution. The technique has not escaped criticism; Olszowka pointed out soon after MIGET was described that, theoretically, a number of considerably different distribution patterns could produce indistinguishable elimination data (Olszowka 1975). Subsequently, it was argued that the limit of resolution using MIGET was likely to be only three perfused compartments: shunt and two alveolar compartments – a tripartite distribution (Lee *et al.* 1987), rather than the 49 compartments that West and Wagner had envisaged.

Notwithstanding these limitations, examination of data derived using MIGET is helpful conceptually when considering the mechanisms that lead to arterial hypoxaemia. If this technique is applied to a healthy human breathing air, the distributions of $\dot{V}_A$ and $\dot{Q}$ are seen to be very well matched (Figure 5-4). There are no regions that are poorly perfused despite adequate ventilation, nor are there any regions that are perfused in the absence of

significant ventilation. When at its most extreme, this latter phenomenon is referred to as 'shunt', that is, the perfusion of a totally unventilated lung region ($\dot{V}_A/\dot{Q} = 0$). The effect is identical to that seen when blood passes right-to-left through an intracardiac defect, or a pulmonary arteriovenous malformation.

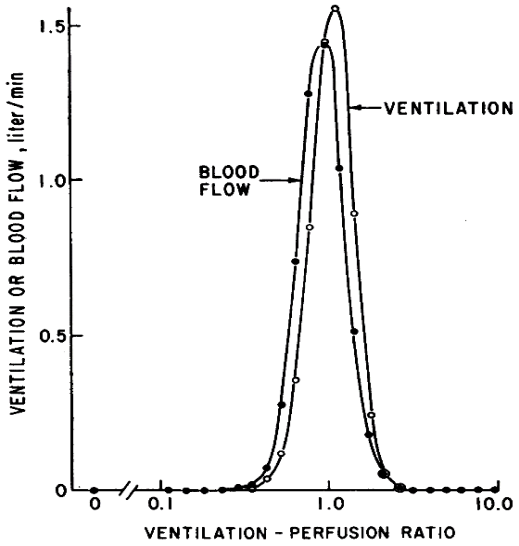

Figure 5-4. Typical distribution of ventilation-perfusion ratios in a healthy young individual

Data were obtained using MIGET in a 22-year-old man. Such a distribution is described as lognormal, with small dispersion. Adapted from (Wagner *et al.* 1974a) with permission.

Considering now the abnormal lung, ventilation-perfusion inequality could take one of two forms, which will have different clinical manifestations. Figure 5-5A illustrates the scenario in which a significant proportion of lung units are perfused in the absence of appreciable ventilation, whilst Figure 5-5B illustrates the converse, where a significant number of well-ventilated but poorly perfused regions exist. In the former case, hypoxaemia would be anticipated to be severe, as a significant proportion of pulmonary blood flow will escape

effective oxygenation. In the latter case, hypoxaemia would not be anticipated to be so severe, but the physiological dead space will be considerably increased. These two forms of $\dot{V}_A/\dot{Q}$ inequality may also coexist (Figure 5-5C). These distributions are not theoretical; all the data in Figure 5-5 were in fact derived from patients with COPD.

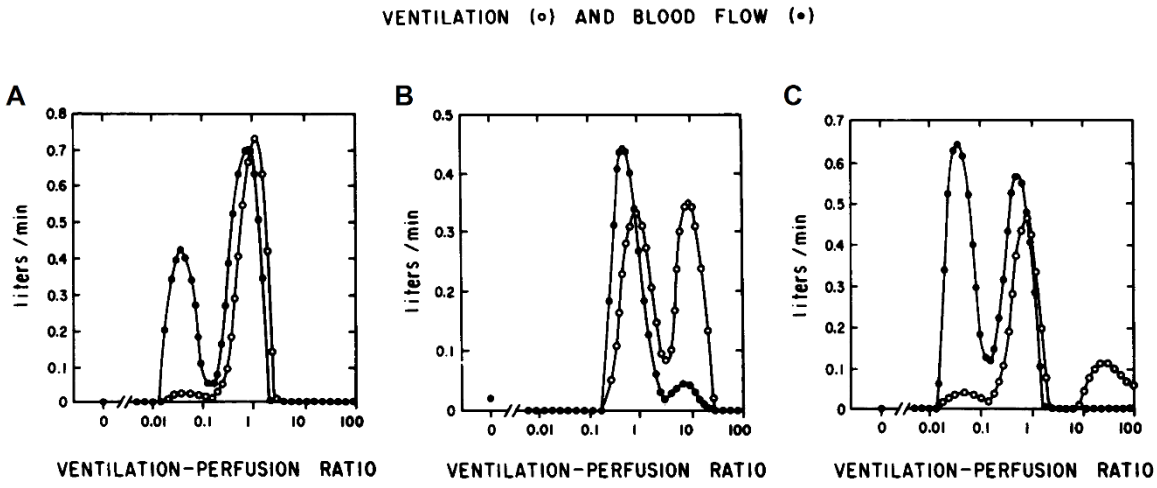

Figure 5-5. Pathological distributions of ventilation-perfusion ratios

(A) Perfusion of a significant proportion of lung regions that are poorly ventilated ($\dot{V}_A/\dot{Q}$ = 0.01-0.1). (B) Ventilation of a significant proportion of lung units that are poorly perfused ($\dot{V}_A/\dot{Q}$ around 10); there is also a small shunt in this case (approximately 20 ml/min). (C) Coexistence of both types of $\dot{V}_A/\dot{Q}$ inequality. The three patients from whom these data were obtained all suffered from COPD. Adapted from (Wagner *et al.* 1977) with permission.

Of course, to think exclusively in these terms ignores the physiological responses that occur to resist changes in arterial oxygen and carbon dioxide tensions. These act to mitigate the effects of pathological $\dot{V}_A/\dot{Q}$ inequality, though for reasons that shall be discussed they are insufficient completely to correct hypoxaemia and hypercapnia in COPD.

Ventilation-perfusion inequality appears to be the main mechanism causing hypoxaemia in COPD (Wagner *et al.* 1977, Sandek *et al.* 2001a), arising due to the progressive destruction of alveoli and pulmonary capillaries (Kent *et al.* 2011). Significant $\dot{V}_A/\dot{Q}$ inequality is reported early in the course of the disease, at least from a spirometric standpoint (Barbera *et al.* 1990). There does not appear to be a simple relationship between severity of radiologically measured parenchymal destruction and hypoxaemia (Sandek *et al.* 2002). The explanation for this observation is presumably that the extent to which loss of pulmonary capillaries occurs in tandem with loss of alveoli will determine the abundance of lung units where $\dot{V}_A/\dot{Q} < 1$, and this will vary from patient to patient.

5.1.4.2. Diffusion impairment

It might be expected that impaired diffusion (D) of gases across the alveolar epithelium into the pulmonary capillary would be a common mechanism by which hypoxaemia occurred in chronic lung disease. In fact, the only pathological situation in which diffusion limitation has been shown convincingly to contribute in this way is during exercise in patients with interstitial lung disease (ILD) (Young and Bye 2011), in whom the blood-gas barrier may be thickened. The reason becomes clearer if one considers that for diffusion impairment to manifest as hypoxaemia or hypercapnia it must prevent equilibration of capillary gas tensions with those in the alveolus before the erythrocyte completes its journey through the pulmonary capillary.

It has been estimated that the mean time taken for an erythrocyte to transit through the pulmonary capillary bed is 0.75 seconds at rest, falling perhaps to slightly less than half this duration during strenuous exercise (Roughton 1945, Johnson *et al.* 1960). In health, equilibration of oxygen tension appears to take place within 0.25 seconds (Staub *et al.* 1961), leading to the oft repeated physiological ‘rule of thumb’ that equilibration occurs

around one third of the way along the pulmonary capillary (Wagner and West 1972, West and Wagner 1980). Thus, even in ILD, studies typically find either no or only a minor contribution from diffusion impairment to resting hypoxaemia, and a significant contribution is evident only when CO increases during exercise (Jernudd-Wilhelmsson *et al.* 1986, Agusti *et al.* 1991, Wagner 1992).

A study directly comparing patients with ILD to those suffering from COPD found no regions with low D/$\dot{Q}$ ratios in the lungs of COPD patients, only the expected abnormalities of $\dot{V}_A/\dot{Q}$ distribution (Yamaguchi *et al.* 1997), with regions of low and high $\dot{V}_A/\dot{Q}$ ratios as already described. Furthermore, a study of high-dose inhaled corticosteroids in COPD patients found that, although diffusing capacity for carbon monoxide (DL_{CO}) was significantly increased after two months of therapy, no change in resting P_{aO_2} was evident (Sandek *et al.* 2001b). However, care is needed in interpreting the results of studies using DL_{CO} as an index of blood-gas barrier function, since other physiological factors contribute to this measurement, including blood volume within the pulmonary capillaries.

5.1.4.3. Hypoventilation

The hypoxaemia that results from a fall in alveolar minute ventilation will always be accompanied by a rise in P_{aCO_2}. This can be predicted from the simplified form of the alveolar gas equation (Fenn *et al.* 1946, West 2011a):

$$P_{AO_2} = P_{IO_2} - (P_{ACO_2}/R)$$

Where R is the respiratory exchange ratio, and it is assumed that $P_{ACO_2} = P_{aCO_2}$, that is, the carbon dioxide gradient between alveolar capillary blood and alveolar gas is negligible. The effect of a controlled reduction in alveolar minute ventilation on P_{aCO_2} in animal experiments is illustrated in Figure 5-6.

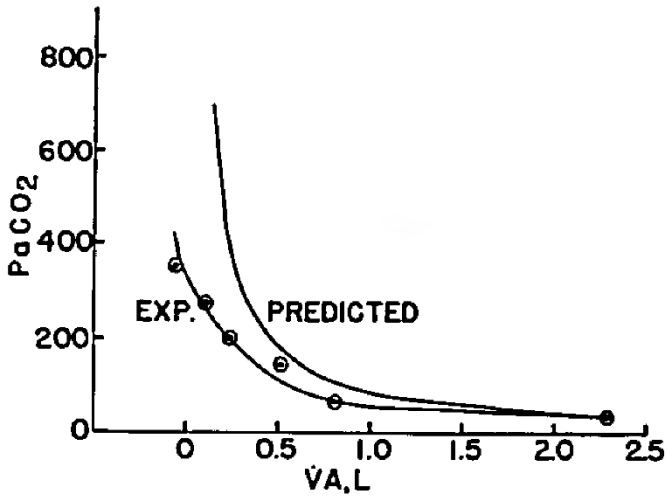

Figure 5-6. Relationship between alveolar ventilation and partial pressure of carbon dioxide

Data are from mechanically ventilated curarized dogs anaesthetised with pentobarbital and given inspired oxygen sufficient to maintain S_pO_2 > 85%. Two lines are shown, one that is predicted from the alveolar gas equation, and the other describing the experimentally obtained values, which are shown as dotted circles. The investigators attributed the discrepancy between the observed and predicted behaviours to an overestimation of the extent to which $\dot{V}_A$ was reduced by mechanical hypoventilation in their experiments. They assumed a constant physiological dead space, but noted that this might have fallen with marked tidal volume reductions. P_aCO_2 values are in mmHg. Reproduced with permission from (Earle *et al.* 1958).

Hypoventilation does not appear to be a primary cause of hypoxaemia in COPD. Indeed, early work (Prime and Westlake 1954) and more recent studies (Alvisi *et al.* 2003, Moore *et al.* 2009) indicate that resting ventilation in COPD patients is equal to or greater than that seen in healthy subjects. Of course, this is to be expected, since the hypercapnia that would otherwise result from $\dot{V}_A/\dot{Q}$ inequality is opposed by augmented ventilation as a consequence of chemoreceptor stimulation. This maintains a normal P_aCO_2 but is not sufficient to counteract the effect of $\dot{V}_A/\dot{Q}$ inequality on P_aO_2 owing to differences in the shape of the dissociation curves describing the relationships for the O_2 and CO_2 affinity of haemoglobin (West 2011a). In this situation, lowering the P_ACO_2 of well-perfused regions

of lung by increasing ventilation will cause pulmonary capillary blood CO_2 concentration in these regions to fall considerably. On the other hand, increasing P_{AO_2} of these well-perfused regions will not alter pulmonary capillary blood O_2 concentration to anything like the same extent, since these lung units are operating on a relatively flat part of the curve that describes the relationship between P_{AO_2} and pulmonary capillary blood O_2 concentration (Figure 1-2).

Having said all this, it has long been recognised that the ventilatory response to CO_2 may be blunted in some patients with COPD (Reinhardt 1912, Scott 1920), particularly those with chronic hypercapnia (Brodovsky *et al.* 1960, Lane and Howell 1970, Gelb *et al.* 1977). The ventilatory response to hypoxia itself may also be reduced (Flenley *et al.* 1970, Bradley *et al.* 1979), and there is some evidence that that mechanical loading and control of breathing may also be abnormal in the condition (Duranti *et al.* 1995). A further factor that is increasingly important in contributing to hypoxaemia in COPD, by disturbing respiratory mechanics, is obesity (Franssen *et al.* 2008). Thus, failure to increase ventilation sufficiently to compensate and maintain eucapnia, or in response to hypoxaemia, could perhaps be considered a form of 'relative hypoventilation', and might promote more severe hypoxaemia than would otherwise be encountered. Respiratory embarrassment from obesity might similarly impede a corrective increase in alveolar ventilation.

5.1.5. Pulmonary hypertension

5.1.5.1. Significance of pulmonary hypertension in COPD

Pulmonary hypertension and consequent RVF – termed cor pulmonale – have long been recognised as complications of chronic lung diseases (Fishman 1976b), with the presence of cor pulmonale clearly predicting a worse outcome in COPD (Traver *et al.* 1979). The introduction of the pulmonary artery catheter (Swan *et al.* 1970) made it more

straightforward for studies to examine invasively pulmonary haemodynamics in patients with COPD using RHC. The prognostic significance of the abnormalities thus detected was recognised early on, with one study reporting an inverse relationship between PVR and survival (Burrows *et al.* 1972), and another finding that an mPAP > 20 mmHg carried a worse prognosis over several years of follow-up (Weitzenblum *et al.* 1981).

Perhaps the only study that failed to report a statistically significant association between severity of PH and outcome in COPD did in fact find a trend towards higher mPAP and PASP in non-survivors (Kawakami *et al.* 1983), but recruited a relatively small number of patients, limiting its power to detect an association. More recent work confirms that severity of PH is a strong predictor of adverse outcomes in COPD (Doi *et al.* 2003, Cuttica *et al.* 2010, Andersen *et al.* 2012) including in those treated with LTOT (Oswald-Mammosser *et al.* 1995) and patients admitted to hospital with an AECOPD (Stone *et al.* 2011).

In addition to the prognostic value of a single measurement of pulmonary haemodynamic variables, early studies examined the manner in which these changed over time (Boushy and North 1977, Schrijen *et al.* 1978, Weitzenblum *et al.* 1978). One such study using repeated RHC at an interval of around three years in patients with COPD and arterial hypoxaemia, suggested that the annual increase in mPAP was rather small – of the order of 1 mmHg/year (Weitzenblum *et al.* 1979). In patients showing the greatest increases, P_aO_2 tended to fall and P_aCO_2 rise during the intervening period. Of course, these observations do not allow the conclusion that worsening hypoxaemia *causes* an increase in mPAP, though they do highlight a relationship that has been seen in subsequent studies (Sims *et al.* 2009, Andersen *et al.* 2012). In terms of the functional consequences, even after controlling for severity of airflow obstruction and other possible confounders, PH is

associated with greater impairment of exercise performance in patients with severe COPD (Sims *et al.* 2009).

5.1.5.2. Severity of pulmonary hypertension associated with COPD

Though in clinical practice COPD is thought of as a disease that commonly leads to cor pulmonale, studies of large numbers of patients with the condition suggest that PH complicating COPD is typically not severe (Figure 5-7).

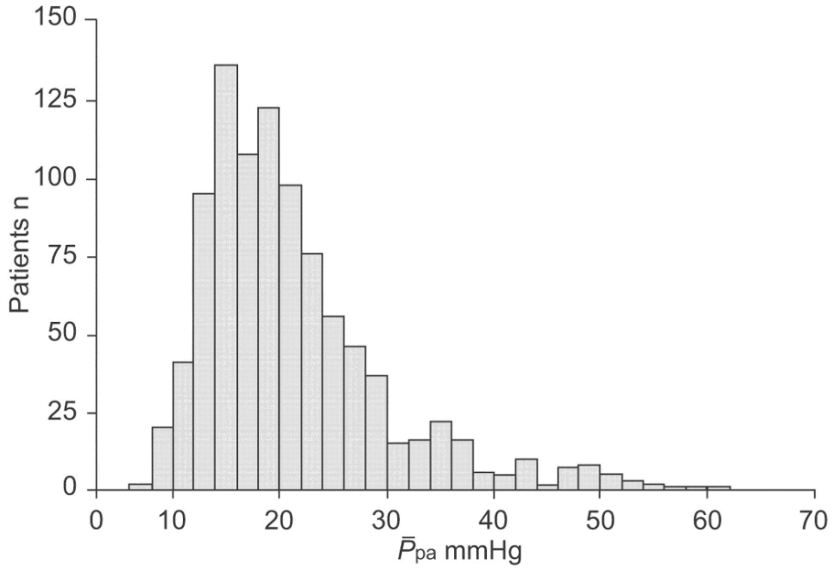

Figure 5-7. Histogram showing distribution of resting PAP in patients with COPD

Data are from a cohort (n=998) with airflow limitation ranging from mild to very severe; values were determined by RHC. $\bar{P}_{pa}$, mPAP. Reproduced from (Chaouat *et al.* 2008) with permission of the European Respiratory Society ©.

In a retrospective study of 27 patients with severe PH (mPAP ≥ 40 mmHg), identified from a French registry of almost one thousand patients who had undergone RHC, 16 were found to have a comorbid disease capable of causing PH, leaving only 11 individuals (1.1% of

the registry) in whom COPD was the only identifiable cause (Chaouat *et al.* 2005). The authors noted that these individuals were phenotypically unusual in having severe hypoxaemia despite only mild to moderate airway obstruction; hypocapnia and a very low DL_{CO} were also features. This prompted the observation that severe PH in the setting of COPD, with no other possible underlying cause, was not dissimilar to IPAH. Indeed, it has been argued that these patients may effectively be individuals with IPAH who happen to have developed concomitant COPD from exposure to tobacco smoke (Chaouat *et al.* 2008).

5.1.5.3. Aetiology of pulmonary hypertension in COPD

Though it would be tempting to assume that PH in COPD is primarily driven by chronic hypoxia, this is an oversimplification. For instance, hypoxaemia did not predict presence of PH in a group of 151 patients with severe emphysema (mean $FEV_1/FVC = 0.38$), and in those patients with an mPAP > 20 mmHg there was no correlation between invasively measured right heart haemodynamic variables and severity of resting hypoxaemia (Oswald-Mammosser *et al.* 1991). Similarly, in a study of 120 patients with severe emphysema (mean FEV_1 = 27% predicted) who underwent RHC, over ninety percent had an mPAP > 20 mmHg (Scharf *et al.* 2002) but, though mPAP correlated inversely with P_aO_2, multiple stepwise regression revealed that the latter was not an independent predictor of the former. Also, in a study of 117 COPD patients with PH who were not hypoxaemic at rest, the only variable that was found to be associated with the severity of PH was [Hb] (Lee *et al.* 2011). The direction of the relationship was for a lower [Hb] to be associated with more severe PH, the opposite of that found in the studies of acute isovolaemic haemodilution discussed earlier (section 2.5.3.1). Iron status was not assessed in this study, making it impossible to know if this association was, in fact, explained by iron deficiency.

Related to the effects of HPV in COPD is the phenomenon of hypercapnic pulmonary vasoconstriction. In those patients with COPD who develop hypercapnic ventilatory failure, this may reasonably be expected additionally to contribute to PH. Human studies appear to have grossly neglected this issue in compassion with the attention HPV has received, and its pathophysiological importance is unclear. One study that did examine the effect of lowering P_aCO_2 in men with COPD and hypercapnia used the respiratory stimulant almitrine, which unfortunately itself augments HPV (Dull *et al.* 1983).

In addition to hypoxia-driven pulmonary vasoconstriction, elevation of PVR in COPD is the result of a complex interaction between mechanical factors and pulmonary vascular remodelling (Singh *et al.* 2016). Emphysematous destruction of the pulmonary vascular bed might be expected to contribute very significantly to resting PH, but a clear relationship between radiological evidence of parenchymal destruction and PAP is not reported (Biernacki *et al.* 1989). Instead, inflammation may be a more important factor, with a role similar to that in promoting hypertrophy and fibrosis of the small airways (Figure 5-2). Given the very complex interactions between hypoxia and inflammation (Eltzschig and Carmeliet 2011), though, it has proved difficult to untangle the exact mechanisms.

Particularly noteworthy studies include one reporting that overexpression of IL-6 in transgenic mice causes muscularisation of the proximal arterial tree (Steiner *et al.* 2009), and another demonstrating not only that cultured human PASMCs increase expression of IL-6 mRNA in response to hypoxia, but also that circulating levels of IL-6 correlate with mPAP and severity of hypoxaemia in COPD patients with PH (Chaouat *et al.* 2009). Another study found an increase in circulating inflammatory cytokines – including IL-6 – in COPD patients exposed to hypoxia for two hours (Sabit *et al.* 2010). It may therefore be the case that a component of the contribution that inflammation makes to the development

of PH reflects an indirect action of hypoxia. The evidence from *Study A* suggesting a direct effect of hypoxia of this sort *in vivo* was discussed in Chapter 3. Figure 5-8 gives a simple overview of the mechanisms thought to contribute to the development of PH in COPD.

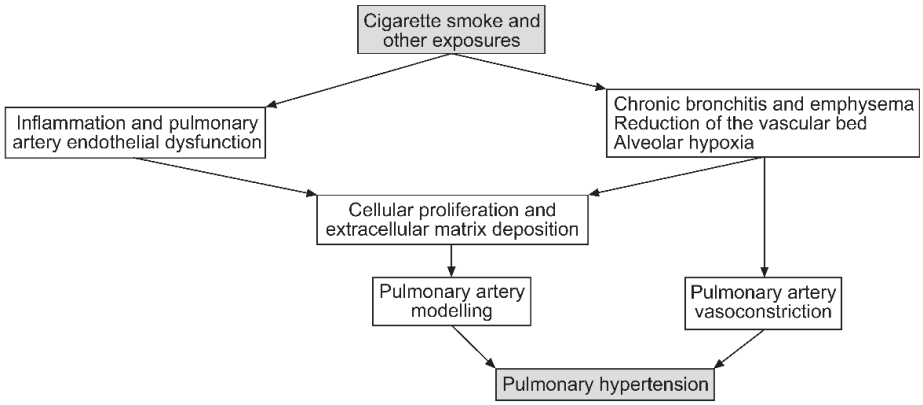

Figure 5-8. Overview of mechanisms underlying PH development in COPD

Adapted from (Chaouat *et al.* 2008) with permission of the European Respiratory Society ©.

5.1.5.4. Supplemental oxygen and pulmonary hypertension

Experiments examining the effects of supplemental oxygen in patients with COPD are very informative with respect to the role of hypoxia in the pathogenesis of PH, and tend to support the view that HPV is a major contributor (Minai *et al.* 2010). In one study, a significant fall in mPAP and PVR was reported in COPD patients given pure oxygen to breathe for only 20 minutes (Holt and Branscomb 1965), suggesting that active HPV contributes to PH even late in the course of the disease. Other studies report similar findings (Tschopp *et al.* 1985, Hunt *et al.* 1989).

In terms of the haemodynamic consequences of LTOT, studies have variously reported: a slowing of progression of PH (Weitzenblum *et al.* 1985); that the response of the pulmonary

circulation to LTOT predicts survival (Timms *et al.* 1985); and even arrest of progression of PH despite worsening of airflow limitation and hypoxaemia (Zielinski *et al.* 1998). If oxygen is removed from LTOT-treated patients, a progressive rise in mPAP is seen over several hours (Figure 5-9); invasive measurements indicate that this is due to increased PVR and not a rise in left atrial pressure or CO (Selinger *et al.* 1987).

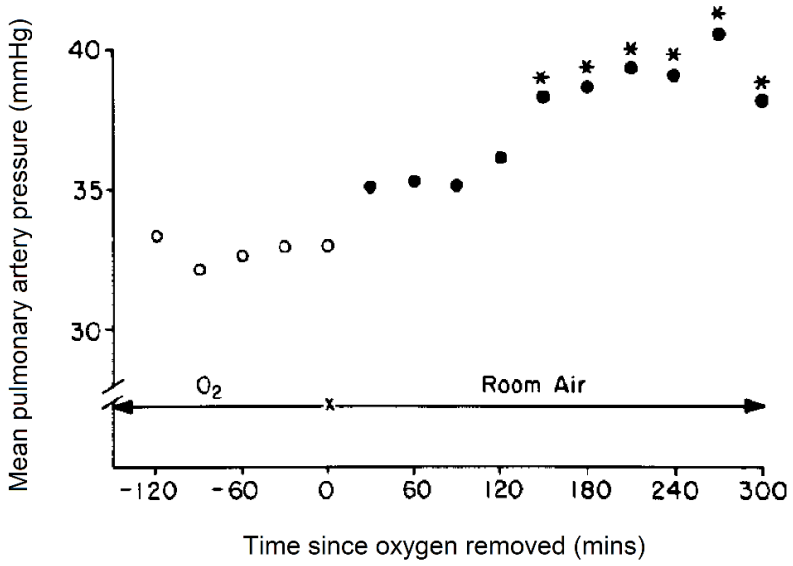

Figure 5-9. Effect on mPAP of removing oxygen from LTOT-treated patients with COPD

Patients (n=6) had been receiving continuous oxygen therapy at 1-3 L/min for at least four months, and had had stable disease for at least three months, prior to the experiment. None had evidence of LVF. Note the similarity of this response to the biphasic time course of HPV illustrated in Figure 2-2 and Figure 2-5. Empty circles, mean values breathing oxygen; filled circles, mean values breathing air; *, $P < 0.05$ compared with breathing oxygen. Modified from (Selinger *et al.* 1987) with permission.

In summary, a strong association between hypoxaemia and pulmonary haemodynamics is manifest, even if the complex pathobiological relationship between the two is incompletely

understood. Moreover, oxygen therapy clearly ameliorates PH in COPD, and this may be one of the mechanisms underlying the mortality benefit from LTOT.

5.1.6. Previous studies of iron homeostasis in COPD

The literature on iron homeostasis in COPD is dominated by work focusing on the importance of an abnormal [Hb] in the condition, be it anaemia or polycythaemia. The failure of the hypoxia associated with "pulmonary emphysema" invariably to cause significant polycythaemia was noted almost a century ago (Lemon 1929). A comprehensive work examining the effects of hypobaric hypoxia on erythropoiesis emphasised that this observation was plainly at variance with the expected outcome, given the behaviour seen in individuals exposed chronically to high altitude (Hurtado *et al.* 1945). A normal [Hb] in patients with chronic lung disease was viewed, very reasonably, as a sort of 'relative anaemia'. A number of investigators subsequently applied themselves to this problem:

> *"It seems most likely that the constant pressure of chronic inflammation combined with recurrent acute infections in the lung plays some part in preventing an appropriate erythropoietic response to hypoxaemia." (Vanier et al. 1963)*

> *"Chronic pulmonary disease with arterial hypoxia appears to be associated with a suboptimal response to the erythropoietic stimulus of hypoxia…possibly…attributed to infection, shortened red cell life span, azotaemia, severe anoxia, high arterial carbon dioxide tension, or a defective production of erythropoietin…It may be that pulmonary impairment in some unknown manner interferes with the stem cell response to erythropoietin or with normal erythroid maturation. Since it is possible that this picture is a variant of the anaemia seen in chronic inflammatory disease, studies on iron reutilisation and the degree of erythropoietic effectiveness are in progress." (Gallo et al. 1964)*

The suspicion that iron deficiency might explain this 'inappropriate normality' of [Hb] in patients with severe emphysema prompted one pair of investigators to administer intramuscular iron to a small group of such patients, the majority of whom showed a response in terms of a rise [Hb] or red cell mass (Fielding and Zorab 1964). It is a pity that this illuminating finding appears to have been largely forgotten in the decades since.

From contemporary studies it is abundantly clear that, as in a host of other chronic diseases, anaemia identifies COPD patients with a worse functional status and prognosis (Portillo *et al.* 2013). This is true both in the setting of admission with an AECOPD (Martinez-Rivera *et al.* 2012) and in the long term (Martinez *et al.* 2006, Cote *et al.* 2007, Boutou *et al.* 2013). Prior to the work described in this chapter, very few recent studies had considered iron homeostasis in COPD in its own right. This is perhaps surprising given how much attention the issue has received in CHF, and, for that matter, IPAH.

Two relatively recent studies claim explicitly to have examined iron deficiency in COPD. In the first (Schneckenpointner *et al.* 2014), 185 patients with "chronic respiratory failure" were prospectively studied. The authors reported that 18.4% were anaemic and that this proportion did not vary significantly depending on the presence or absence of COPD. Anaemic patients were older, and had higher serum creatinine and erythropoietin levels, but lower TSat, serum iron and vitamin B12 levels. A TSat $\geq$ 20% was associated with less dyspnoea and better subjective exercise capability, whilst lower [Hb] and serum iron levels predicted mortality. The authors concluded that iron status was an independent prognostic marker. However, the findings are somewhat difficult to interpret, for several reasons. First, patients with a variety of diagnoses were enrolled, including neuromuscular diseases and obesity-hypoventilation syndrome. Secondly, all patients were receiving "home mechanical ventilation", which the authors did not define. Thirdly, the key grouping

variable for classifying patients as iron deficient was TSat < 20%; those with TSat ≥ 20% were assumed to have normal iron homeostasis. The difficulty with such an approach, as employed by studies in the 1960s before a serum ferritin assay became available (McFarlane *et al.* 1967), has been discussed in Chapter 1. Furthermore, no measurements of sTfR or hepcidin were reported. Thus, it is difficult to conclude much with certainty about iron deficiency in COPD from this particular study.

The second study (Silverberg *et al.* 2014) included both a retrospective observational component and a small interventional arm. In the former, the hospital records of 107 patients suffering from an AECOPD were examined. The authors reported that 44% of AECOPD patients were anaemic on admission. Only 38% of the anaemic patients had TSat and serum ferritin measured; all were found to have evidence of iron deficiency, but none had any form of iron therapy administered. In the interventional component, 12 anaemic patients with COPD (11 of whom were iron deficient) were given a combination of erythropoiesis stimulating agents and IV iron. Dyspnoea and [Hb] improved with these interventions. The key limitation of this study is, again, that it is not possible to draw from it any real conclusions about iron deficiency in COPD, other than that it appears to be prevalent in anaemic patients, which is hardly surprising. The methodology used illustrates the problem of ascertainment bias that is encountered if a low [Hb] is taken as the trigger for measurement of iron indices. Equally, that a rise in [Hb] and subjective improvement in dyspnoea occurred with administration of erythropoietin and iron does not allow any firm conclusions to be drawn about the role of iron deficiency in its own right.

There have been a handful of other studies in which iron homeostasis has been considered in COPD. One investigated the effect of venesection on iron parameters in polycythaemic patients (Martinez *et al.* 1997), and found, as expected, a reduction in serum ferritin with

repeated venesection; no clinical outcomes were reported. Another viewed iron as a "trace element" and measured only serum iron levels (Karul *et al.* 2003). The authors concluded that there was "no need for routine evaluation of trace elements in clinically stable, regularly treated COPD outpatients." It does not appear that the futility of using an isolated measurement of serum iron to assess iron status was appreciated by these investigators. The same group published a very similar report subsequently (Karadag *et al.* 2004), again reporting comparable serum iron levels in COPD patients and controls.

5.2. Aims

Given the absence of data regarding the epidemiology of iron deficiency in COPD, a cross-sectional observational study of patients was undertaken, *Study C*, with the aim of determining the prevalence of iron deficiency in the condition. It was hypothesised that iron deficiency would be more common than in healthy controls, and that iron-deficient patients with COPD would differ phenotypically, particularly with respect to indices of symptom burden and functional performance.

5.3. Methods

The contributions of this author and other individuals to the design and execution of *Study C* are described in detail in Appendix A. In contrast to the interventional studies of healthy volunteers presented earlier, in order to explore the role of iron deficiency in COPD, a cross-sectional study design was used with the aim of establishing the prevalence and associations of iron deficiency in this setting. The study was conducted in accordance with the Declaration of Helsinki and approval was given by the NHS South Central Berkshire

Research Ethics Committee (reference 08/H0607/81). Written informed consent was given by all participants.

5.3.1. Patient recruitment

Patients included in this study were recruited to the Oxford Biomedical Research Centre COPD Cohort between August 2009 and April 2012. This cohort comprised a carefully phenotyped group of patients with a clinical diagnosis of COPD confirmed by a respiratory physician, and consistent spirometric findings of $FEV_1/FVC < 0.70$ and $FEV_1 < 80\%$ predicted, in accordance with GOLD. During the period of recruitment, patients attending a specialist COPD clinic at the Churchill Hospital, Oxford were invited to enrol in the cohort. A specific criterion for enrolment was the absence of other significant cardiopulmonary disease or comorbidity likely to limit life-expectancy to less than two years.

5.3.2. Study design and procedures

Study assessments were only undertaken during a period of disease stability, specifically when patients reported being exacerbation-free for at least four weeks. These included a medical history, record of exacerbations, clinical examination and spirometry. Resting pulse oximetry and arterial or capillary blood gas analysis were performed. Nocturnal mean S_pO_2 and proportion of time spent asleep with $S_pO_2 < 90\%$ were also determined (Pulsox®-300i, Konica Minolta Sensing Inc., Japan). The St George's Respiratory Questionnaire (SGRQ) (Jones *et al.* 1992), Hospital Anxiety and Depression (HAD) score (Zigmond and Snaith 1983) and Epworth Sleepiness Score (ESS) (Johns 1991) were also recorded.

All patients underwent two Shuttle Walk Tests (SWT) (Singh *et al.* 1992) with the better result used for analysis. The SWT requires the patient to walk a distance of 10 m around

two cones at progressively increasing speeds until voluntary fatigue, or the point at which the individual is observed to be unable to achieve the pace required. Whole blood was obtained from patients at the same visit. FBC, TSat, ferritin, CRP, erythropoietin, sTfR and hepcidin were measured in a similar manner as described for *Study A*.

5.3.2.1. Definition of iron deficiency

In *Study A* and *Study B*, definitions of iron deficiency and iron repletion were based on serum ferritin and TSat values. In this study of COPD patients, a similar approach of classifying participants as iron deficient (ID), iron replete (IR) or indeterminate was employed. However, the utility of serum ferritin as an index was limited because it is an acute-phase reactant (Rogers *et al.* 1990). Whilst in the presence of inflammation a low serum ferritin remains diagnostic of iron deficiency, a normal serum ferritin cannot be used to exclude it. These issues were discussed in Chapter 1, and the effect of inflammation in this regard is illustrated in Figure 1-14.

Accordingly, in *Study C*, sTfR was used as an additional grouping variable to establish iron status. Thus, iron deficiency was defined as any one or more of (i) sTfR > 28.1 nmol/L; (ii) TSat < 16%; or (iii) serum ferritin < 12 μg/L. Patients were classified as iron replete if they did not meet the criteria for iron deficiency, and had TSat ≥ 20%. Patients who did not meet the definition of iron deficiency but who had 16 ≤ TSat < 20% were viewed as falling into an indeterminate group.

5.3.3. Control group

Though a key aim was to compare the phenotypes of ID and IR patients with COPD, in order to determine with certainty whether iron deficiency is more prevalent in the condition, it was felt desirable to have a healthy control group in whom to investigate the performance

of the definitions used. This healthy control group was identified from individuals participating in a different study examining iron and exercise physiology in adults aged over 50 years (Cheng 2015). Patients in this study were younger on average and mostly female, so COPD patients were matched 2:1 with age- and sex-appropriate controls to generate a suitable control group.

5.3.4. Statistical analysis

Data were analysed using SigmaPlot (version 13.0, Systat Software). The Chi-squared test was used to compare proportions. Non-normally-distributed data comparisons were made using a MWU test and normally distributed data compared using a t-test. To explore the effect of possible confounders, particularly FEV_1, analysis of covariance (ANCOVA) was used. Spearman's rank correlation coefficient (ρ) was calculated to investigate correlations between non-normally-distributed variables, including analysis of correlations with iron status rank (treating sTfR and TSat of equal importance). With respect to this latter approach, all COPD patients were ranked separately according to TSat and sTfR, and an overall rank determined by summing the values of the individual ranks; this served to combine the two indices and allow further exploration of possible confounders.

5.4. Results

One hundred and thirteen COPD patients made up the cohort. Sixty-five percent were male and the mean (SD) age was 67.6 (8.8) years. The median FEV_1 was 41.0% predicted, consistent with severe COPD, and the median smoking history was 43.0 pack years. Most were ex-smokers and all but two of the patients were Caucasian. Five patients were receiving LTOT, and nine more had access to short-burst oxygen at home. The control

cohort consisted of 57 individuals, 65% of whom were male, with a mean (SD) age of 67.6 (6.1) years; all were Caucasian.

5.4.1. Prevalence of iron deficiency

Twenty COPD patients (17.7%) were ID, 81 (71.7%) IR, and 12 (10.6%) of indeterminate iron status. This contrasted with 3 (5.3%), 51 (89.5%) and 3 (5.3%), respectively, in the control healthy volunteer group (Figure 5-10). Iron deficiency was therefore significantly more prevalent in the COPD cohort than in healthy controls ($P = 0.029$, Chi-squared test).

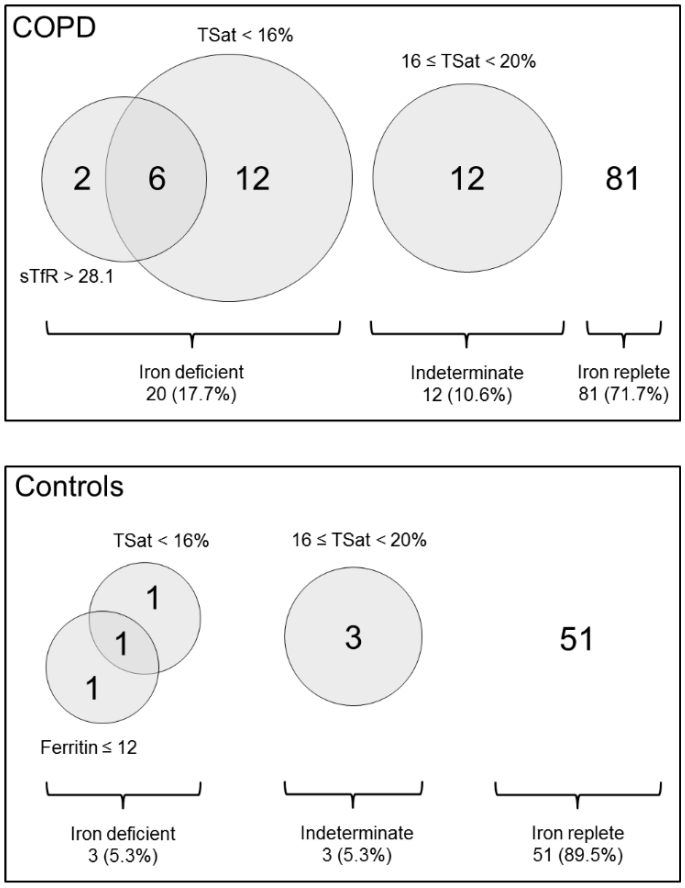

Figure 5-10. Iron status of COPD cohort patients and healthy controls

Venn diagrams showing proportions of COPD cohort (top) and healthy control group (bottom) who were iron deficient, of indeterminate iron status, or iron replete. No COPD patient had a serum ferritin < 12 µg/L and no healthy control participant had an sTfR > 28.1 nmol/L.

5.4.2. Characteristics of COPD patients

Characteristics of the COPD cohort patients are given in Table 5-2. There were no significant differences between the ID and IR groups in sex, smoking status, median number of pack years smoked or access to oxygen therapy. Though the difference in FEV_1 appeared to be of a magnitude consistent with clinical significance, it did not reach statistical significance. Similarly, further analysis revealed that in the cohort as a whole there was not a significant correlation between FEV_1 and any index of iron status, be it sTfR alone (ρ = -0.068, P = 0.48), TSat alone (ρ = 0.11, P = 0.24) or overall iron status rank (ρ = 0.096, P = 0.31), calculated as described earlier. However, the direction of trend for all these relationships was for more iron-deficient patients to have a lower FEV_1. In view of this, subsequent analyses explored FEV_1 as a possible confounder.

	All patients (n=113)	ID (n=20)	IR (n=81)	*P*-value ID vs IR
Male sex – n (%)	74 (65)	12 (60)	53 (66)	0.85
Age – years	67.6 ± 8.8	67.5 ± 9.9	67.5 ± 8.5	0.98
FEV_1 – % predicted	43.8 ± 17.1	39.7 ± 16.5	44.8 ± 17.0	0.24
Current smoker – n (%)	17 (15)	4 (20)	11 (13.6)	0.71
Pack years	44.0 (28.5 – 62.0)	44.0 (37.0 – 67.0)	45.0 (27.8 – 62.8)	0.80
BMI – kg/m^2	27.6 (21.7 – 30.3)	27.8 (21.6 – 31.9)	27.5 (21.7 – 30.6)	0.86
LTOT – n (%)	5 (4)	2 (10)	3 (4)	0.53
Any home O_2 – n (%)	14 (12)	5 (25)	8 (10)	0.15

Table 5-2. Characteristics of patients in the COPD cohort

Values are means ± SD, for normally distributed data, and comparisons by t-test; or medians (IQR), if not normally distributed, in which case comparisons are by MWU test. Proportions are compared using a Chi-squared test. FEV_1, forced expiratory volume in one second.

5.4.3. Relationships between iron homeostasis and inflammation

Thirty-seven COPD patients (33%) had a CRP greater than 8 mg/L, the upper limit of normal for the assay used. In contrast, none of the healthy volunteer controls had a raised CRP ($P < 0.001$, Chi-squared test). CRP was significantly higher in COPD patients with ID than those who were IR (median 10.5 vs 4.0 mg/L, respectively, $P < 0.001$; Figure 5-11A), and this relationship persisted after adjustment for differences in FEV_1 ($P = 0.028$). The ID group also had a higher self-reported rate of exacerbations in the year prior to enrolment (median 3 (IQR 2-6) vs 2 (IQR 1-4), respectively, $P = 0.024$). Again, this relationship persisted after adjustment for differences in FEV_1 ($P = 0.045$).

As would be expected, ferritin and hepcidin levels were both significantly lower in iron-deficient healthy controls than in their iron-replete counterparts (medians: ferritin 10.5 vs 76.3 μg/L, respectively, $P = 0.008$; hepcidin 3.3 vs 35.1 μg/L, respectively, $P = 0.007$). Similarly, ID COPD patients had lower ferritin and hepcidin levels than IR COPD patients (medians: ferritin 28.3 vs 79.9 μg/L, respectively, $P < 0.001$; hepcidin 21.4 vs 33.4 μg/L, respectively, $P = 0.013$; Figure 5-11B&C).

However, neither ferritin nor hepcidin was suppressed in ID COPD patients to the extent seen in iron-deficient healthy controls. Instead, consistent with their elevation by inflammation, the values for both were significantly higher in the ID COPD patients compared with the iron-deficient healthy controls (medians: ferritin 28.3 vs 10.5 μg/L, respectively, $P = 0.040$; hepcidin 21.4 vs 3.3 μg/L; respectively, $P = 0.020$). Furthermore, in the COPD cohort, hepcidin was significantly correlated with CRP ($\rho = 0.20$, $P = 0.039$), but this was not the case for the healthy control group ($\rho = 0.026$, $P = 0.85$), where CRP values were not elevated. Very similar positive correlations between hepcidin and ferritin

values were, however, found in both the COPD cohort ($\rho = 0.64$, $P < 0.001$) and the healthy control group ($\rho = 0.80$, $P < 0.001$) (Figure 5-11D).

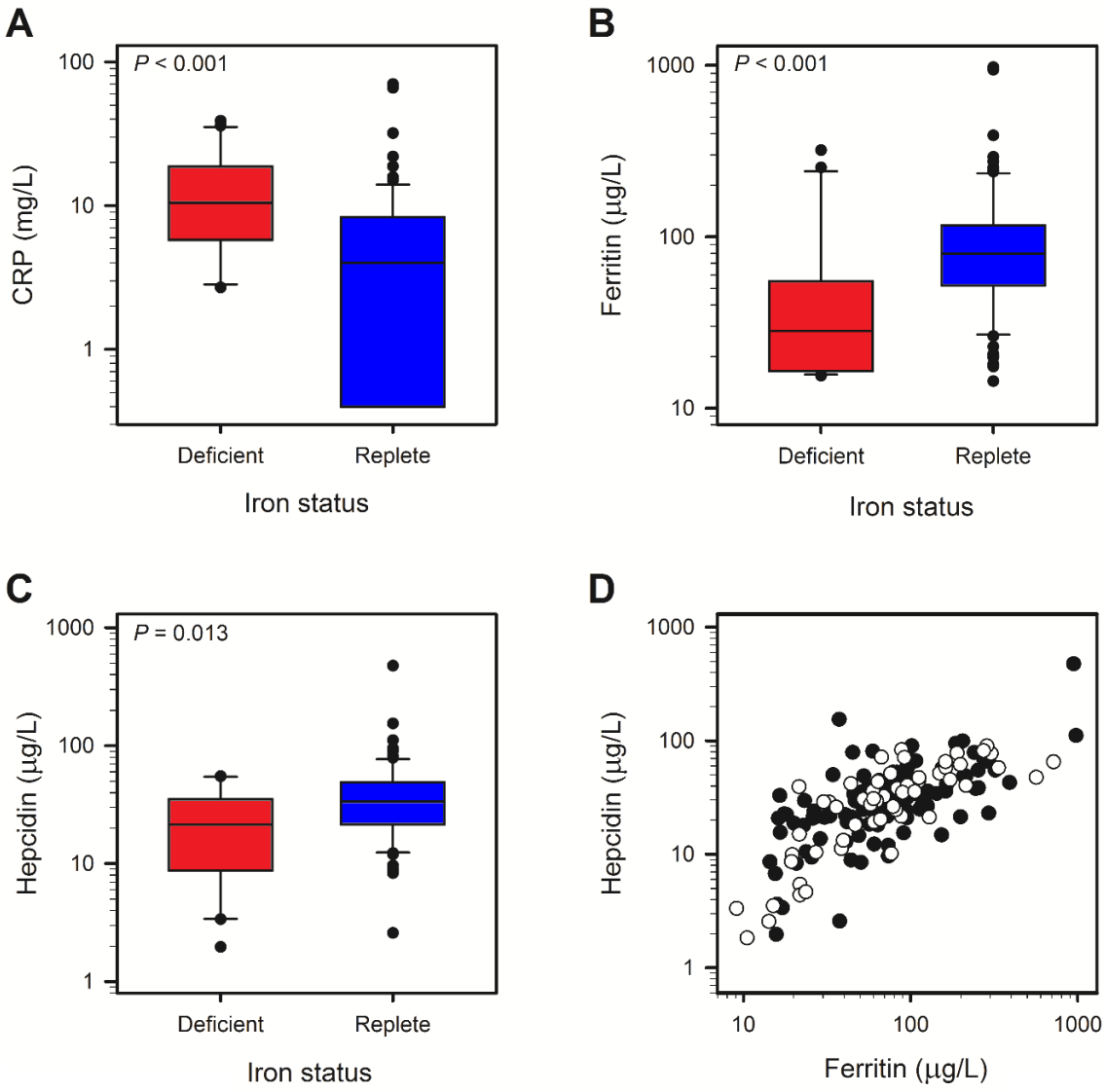

Figure 5-11. Inflammation and iron homeostasis in COPD patients and healthy controls

(A) Box plot showing distribution of CRP by iron status in the COPD cohort (boxes show IQR and median, whiskers are 10th and 90th centiles, circles are outliers). (B & C) Box plots showing distribution of results for ferritin and hepcidin by iron status in the COPD cohort. (D) Scatter plot showing relationship between hepcidin and ferritin for all patients in the COPD cohort (n=113; filled circles). The healthy control group (n=57; open circles) are shown for comparison; COPD did not obviously alter the relationship between hepcidin and ferritin. Note the log scales.

5.4.4. Hypoxaemia

Within the COPD cohort, resting daytime S_pO_2 was significantly lower in the ID group compared with IR patients (median 92 vs 95%, respectively, $P < 0.001$) (Figure 5-12A).

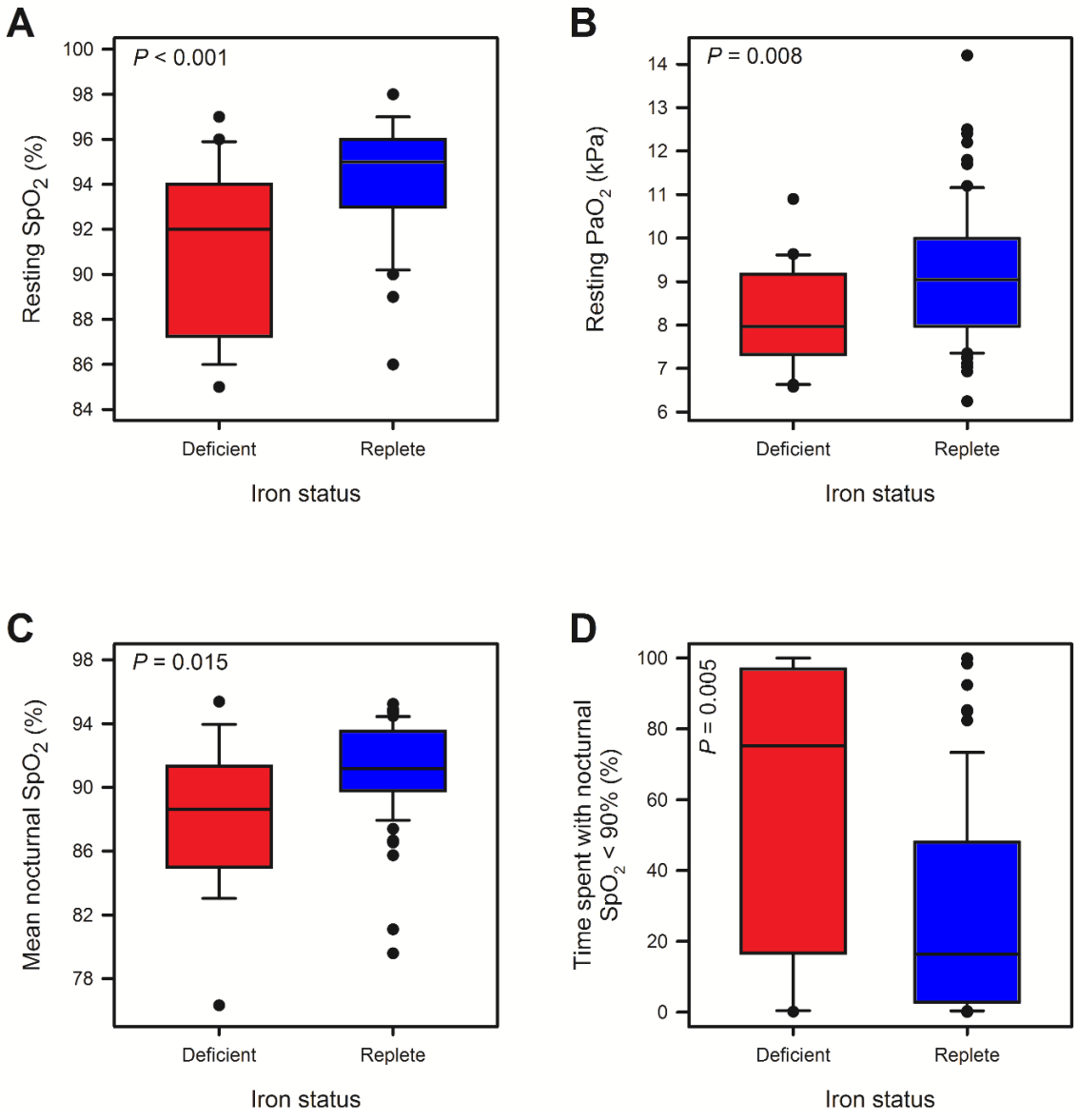

Figure 5-12. Daytime and noctunal hypoxaemia in COPD patients according to iron status

Box plots, constructed as for Figure 5-11, showing distribution of (A) resting S_pO_2, (B) resting P_aO_2 (C) mean nocturnal S_pO_2, and (D) percentage of nocturnal S_pO_2 recordings < 90%, all by iron status, in the COPD cohort.

This was also the case for resting P_aO_2 (median 7.97 vs 9.05 kPa, respectively, $P = 0.008$) and mean nocturnal S_pO_2 (median 88.6 vs 91.2%, respectively, $P = 0.015$). Additionally, the proportion of time spent overnight with an $S_pO_2 < 90\%$ was much higher in the ID group than the IR group (median 75.3 vs 16.4%, respectively, $P = 0.005$). All these relationships persisted after adjustment for FEV_1 ($P < 0.001$, $P = 0.007$, $P = 0.005$ and $P < 0.001$, respectively).

5.4.5. Erythropoiesis

Haemoglobin concentration did not differ according to iron status in COPD patients (means ID vs IR: 13.4 vs 13.8 g/dl, $P = 0.26$), despite a much higher serum erythropoietin level in the ID group (medians ID vs IR: 23.9 vs 13.5 mIU/ml, $P = 0.001$). However, MCV was lower in the ID group (means ID vs IR: 88.8 vs 93.1 fl; $P = 0.001$). These findings are illustrated in Figure 5-13.

5.4.6. Functional outcomes

Exercise performance, as measured by the distance achieved in the SWT, was poorer in the ID group (medians ID vs IR: 165 vs 240 m; $P = 0.035$; Figure 5-14). However, variance in FEV_1 appeared to explain a significant proportion of the variance in SWT performance, such that adjustment for FEV_1 weakened the relationship between SWT performance and iron status ($P = 0.081$). Differences in SGRQ scores, HAD scores and ESS between ID and IR groups did not reach statistical significance (ID vs IR: mean 33.5 vs 30.1, $P = 0.056$; median 13.5 vs 11, $P = 0.133$; and median 8 vs 5, $P = 0.279$; respectively), though in each case the trend was for scores consistent with greater symptom burden to be recorded in COPD patients with ID.

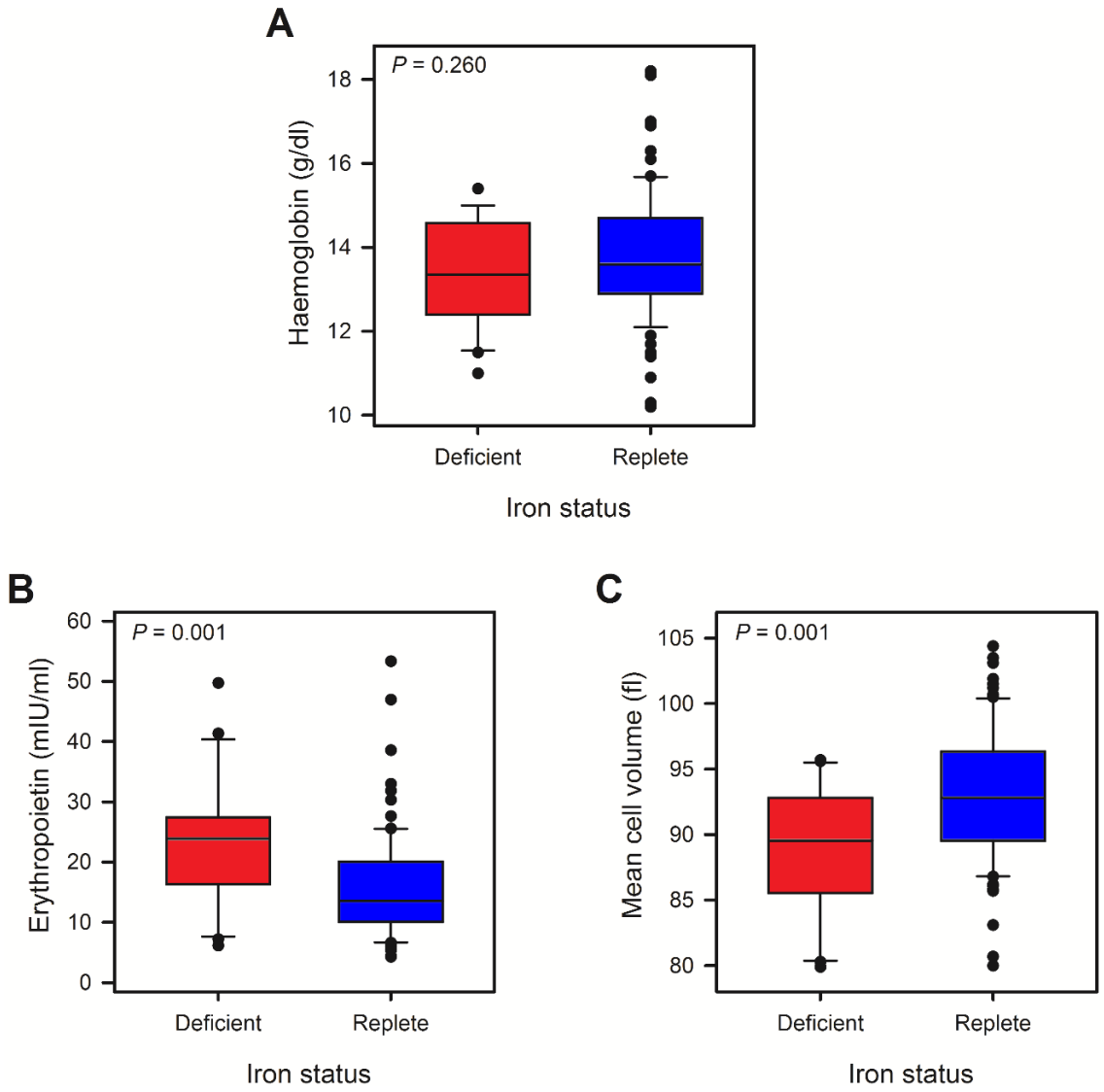

Figure 5-13. Erythropoiesis in the COPD cohort

Box plots, constructed as for Figure 5-11, showing distribution of (A) haemoglobin concentration, (B) serum erythropoietin, and (C) mean cell volume, by iron status, in the COPD cohort.

5.5. Discussion

5.5.1. Prevalence of iron deficiency in COPD

The first finding of this study is of a high prevalence of iron deficiency in COPD – close to 1 in 5 patients. Applying the same definition to a well-matched healthy volunteer control

group gave a prevalence of iron deficiency of only 5%. Non-invasive assessment of iron status is problematic in COPD, as in other chronic inflammatory conditions, since serum ferritin may be normal or raised despite inadequate iron stores. None of the COPD cohort patients had a serum ferritin that could be considered strictly diagnostic of absolute iron deficiency. However, the lower MCV in the ID group is consistent with IDE, indicating that this definition identified individuals with a significant unmet tissue iron demand.

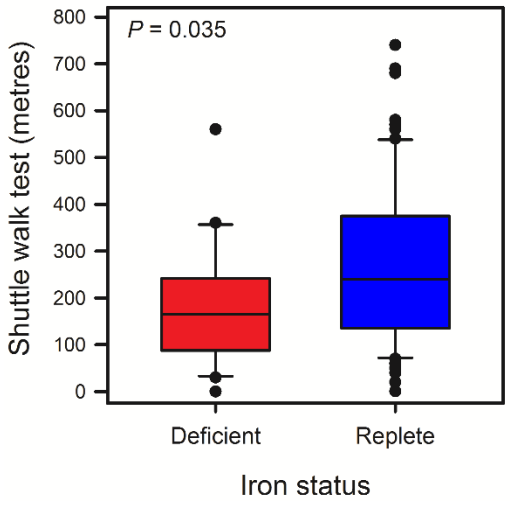

Figure 5-14. Exercise performance in COPD patients

Box plot, constructed as for Figure 5-11, showing distribution of SWT distance according to iron status in the COPD cohort. The value for each patient is that for the individual's best performance from two attempts at the test.

This study represents the first attempt to determine the prevalence of iron deficiency, rather than anaemia, in the setting of COPD. Since this work was performed, another published study has explored iron deficiency in COPD. In a group of 70 Spanish patients undergoing a pulmonary rehabilitation programme, the prevalence of NAID was reported to be 48%

(Barberan-Garcia *et al.* 2015), much higher than in the present study. However, those authors defined ID as a serum ferritin < 100 μg/mL, or of between 100 and 299 μg/mL with a TSat < 20%. Brief inspection of Figure 5-11B reveals that such a definition would have resulted in over half of the IR COPD patients in the present study being classified as ID.

A defence of this definition was not provided by the investigators, other than that it was that which had been adopted by another group investigating the effects of IV iron supplementation in patients with CHF (Okonko *et al.* 2008). The authors of that work cited two studies of sternal bone marrow iron content (Bolger *et al.* 2006, Nanas *et al.* 2006) as the basis for their system of classification:

> *"Nanas et al. measured the iron content of bone marrow biopsies in patients with advanced heart failure and anaemia; 73% of patients had reduced iron stores. The mean ferritin levels were 75 ng/ml in those who were iron deficient and 211 ng/ml in those not iron deficient. Bolger et al. reported a mean value of 87 ng/ml. These values are far above the 15 to 20 ng/ml cutoffs that are usually regarded as indicative of iron-deficiency anaemia. Consequently, we used higher ferritin cutoffs for iron deficiency to address its impact on exercise and symptoms in CHF." (Okonko et al. 2008)*

With respect to the manner in which ferritin levels behave in the setting of a chronic inflammatory disease, it could be argued that these two studies (Bolger *et al.* 2006, Nanas *et al.* 2006) serve only to reinforce that which had already been demonstrated several decades earlier by Finch and coworkers (Lipschitz *et al.* 1974, Finch *et al.* 1986), as illustrated in Figure 1-14. Aside from this, and the now somewhat laboured point regarding the use of a definition of iron deficiency derived from anaemic patients in those without anaemia, there is the much bigger issue of the validity of simply borrowing a definition that has fallen into use in the CHF literature (Anker *et al.* 2009, Klip *et al.* 2013, Ponikowski *et*

al. 2015), for another chronic disease entirely. If applied to COPD, such a definition is bound to identify a group of patients who are *relatively* iron-deficient compared with those who do not meet the criteria. However, this group will include many individuals who are certainly iron replete by established criteria, weakening the utility of such a definition, unless, that is, it is desired to categorise as many individuals as possible as iron deficient in order that they may be given an IV iron therapy within the terms of an existing product licence.

5.5.2. Hypoxaemia and iron status in COPD

One striking finding of the present study is the unanticipated association in COPD patients of significantly greater hypoxaemia with iron deficiency. This relationship was not simply explained by more severe airflow limitation, as measured by FEV_1. Marked daytime and nocturnal hypoxaemia were evident; these were entirely unexpected. Given that $\dot{V}_A/\dot{Q}$ inequality is the dominant mechanism driving hypoxaemia in COPD, one possible explanation is that globally upregulated HPV as a consequence of iron deficiency may further impair $\dot{V}_A/\dot{Q}$ matching and worsen hypoxaemia.

Obstructive sleep apnoea commonly occurs in patients with COPD (Soler *et al.* 2015) – an overlap syndrome. In terms of nocturnal hypoxaemia, it should therefore be considered whether a higher incidence of OSA in the ID group may have contributed to the findings. The ID group were not older, did not have a significantly higher BMI, were not more likely to be male, and were not more often current smokers; thus there was not a clear excess of risk factors for OSA (Young *et al.* 2004) in the group as a whole. Having said this, daytime sleepiness, as measured by the ESS, was higher in ID patients, albeit not statistically significantly so. Obstructive sleep apnoea is itself a chronic inflammatory disorder in which hepcidin-mediated iron sequestration has recently been implicated (Abakay *et al.* 2015, Liu

et al. 2015). Thus, in the present study it is possible that OSA contributed to nocturnal hypoxaemia.

Finally, it may simply be the case that iron deficiency identifies a group with more severe disease of a sort that is not fully captured by measuring FEV_1 alone. Greater systemic inflammation would seem to be the most likely confounding factor in this respect, and it is this topic to which attention shall now be turned.

5.5.3. Disturbance of iron homeostasis in COPD

As discussed in previous chapters, the major hormone that acts to determine iron availability and distribution is hepcidin, which is regulated by iron, erythropoietic drive and inflammation. The importance of inflammation in regulating iron availability in COPD is illustrated by the failure of the combination of iron deficiency and hypoxaemia completely to suppress hepcidin in the ID COPD patients. In contrast, plasma hepcidin was markedly suppressed in the few iron-deficient but euoxic healthy volunteer control participants.

This 'inappropriate expression' of hepcidin in COPD patients who appear by other criteria to be iron deficient has similarities with that seen in ACD (Weinstein *et al.* 2002, Ganz and Nemeth 2009). The main difference, of course, is that in the group of ID COPD patients, anaemia was uncommon and mean [Hb] was not lower than in the IR COPD patients despite a significantly lower ferritin and MCV. The ID patients had higher erythropoietin levels, consistent with appropriate sensing of hypoxaemia (possibly with a direct contribution from an effect of iron deficiency) but a failure of the marrow to respond accordingly. Thus it does appear that a tension exists in COPD between erythropoietic drive and iron availability, with inflammation-driven, hepcidin-mediated iron sequestration constraining a rise in haemoglobin.

As with work addressing iron deficiency generally in COPD, there are few published studies examining the role of hepcidin in the condition. Iron-loaded alveolar macrophages have been identified in lung tissue from humans with COPD, and associations demonstrated between the extent of iron loading and disease severity (Philippot *et al.* 2014). Interestingly, though, these authors viewed their findings as suggestive of a mechanism by which alveolar macrophages might provide protection against iron-induced oxidative stress in COPD, rather than a process simply reflecting ongoing inflammation and elevated hepcidin.

Other groups have measured circulating hepcidin in COPD. One study reported hepcidin levels to be lower in patients with moderate and severe COPD than in a healthy control group, and additionally a positive correlation between S_aO_2 and hepcidin in patients with severe disease (Duru *et al.* 2012). This led the authors to the rather opaque conclusion that:

> *"The serum hepcidin level that is decreased in severe COPD brings into mind that it may play a role in the mechanism to prevent hypoxemia."*
> *(Duru et al. 2012)*

The likely explanation for these confusing findings is that the ELISA used to measure hepcidin in that study in fact detected prohepcidin, a hepcidin precursor, circulating levels of which show no relationship whatsoever to markers of iron homeostasis or inflammation (Roe *et al.* 2007, Kemna *et al.* 2008). A more recent study found elevated hepcidin in COPD patients during exacerbations, and during disease stability, compared with healthy controls, and positive correlations of hepcidin with CRP, as in the present study (Tandara *et al.* 2015). However, no correlations were observed with hypoxaemia.

Returning to the study of NAID and pulmonary rehabilitation (Barberan-Garcia *et al.* 2015) it is of note that those authors found no difference in CRP levels between their COPD

patients with and without iron deficiency, nor a difference in the severity of resting hypoxaemia. This is in spite of broadly similar age, sex and spirometric characteristics of the COPD patients in that study and the COPD cohort in the present study. Those authors required a slightly longer period of disease stability prior to enrolment (6 weeks) and participants also had to be willing and able to exercise on a cycle ergometer and undergo a pulmonary rehabilitation programme. These differences would not, though, seem to provide an entirely satisfactory explanation for the absence of the very strong relationships found in the present study. Hepcidin levels were not reported.

In terms of whether iron deficiency might simply identify a group of COPD patients with more severe inflammation, which is the 'true' driver for hypoxaemia, it is of note that no difference was found in P_aO_2 between patients with normal and raised CRP levels in a study of 102 patients with clinically stable GOLD stage II-IV disease (Broekhuizen *et al.* 2006). On the other hand, another study of 130 patients found a strong negative association of CRP with P_aO_2, and for that matter exercise tolerance (de Torres *et al.* 2006). Some authors emphasise a potential role for tissue hypoxia driving inflammation (Baldi *et al.* 2008). Whilst it is clear from animal models that acute alveolar hypoxia does induce inflammation in a variety of tissues (Gonzalez and Wood 2010), and *Study A* supported the view that such an effect operates acutely in healthy humans, the extent to which this process continues in the chronic setting in humans with COPD is unclear.

Finally, it is interesting to note that several studies have implicated SNPs in the gene *IREB2*, which encodes IRP2, in the pathogenesis of COPD. Gene-expression and genetic-association results implicated *IREB2* as a COPD susceptibility gene in a study involving data from several cohorts of COPD patients and healthy controls (DeMeo *et al.* 2009). A subsequent study confirmed these findings and noted that the effect appeared to be

independent of lung function, since no association between FEV_1 and any of the SNPs in *IREB2* was evident (Chappell *et al.* 2011). Importantly, it was then shown that the effect of genetic variation within *IREB2* was not simply explained by an association with greater exposure to tobacco smoke, as appeared to be the case with SNPs elsewhere (Siedlinski *et al.* 2013). Most recently, a genome-wide association study in COPD patients found that SNPs near *IREB2* were associated with pulmonary artery enlargement, a radiological index of elevated PAP that is also predictive of future exacerbation risk (Lee *et al.* 2015).

5.5.4. Exacerbations

Iron-deficient COPD patients in the present study had a significantly higher number of self-reported exacerbations in the year preceding enrolment. Exacerbations are known to become more frequent as the severity of COPD increases (Hurst *et al.* 2010), but the relationship observed in the present study did not appear to be confounded by FEV_1. It may, though, be explained by the association of iron deficiency with higher levels of systemic inflammation in the group. A very large prospective cohort study of over six thousand patients with COPD, which sought to investigate potential biomarkers for exacerbation risk, found that simultaneously elevated levels of CRP, fibrinogen and white cell count were associated with increased risk of AECOPD, even in those with mild disease and without a history of exacerbation (Thomsen *et al.* 2013). Having said this, given that iron deficiency impairs various aspects of cellular immunity in animal models (Drakesmith and Prentice 2012), it is also possible that iron deficiency might predispose to a higher frequency of respiratory tract infections, a key trigger for exacerbations (Thomsen *et al.* 2013).

5.5.5. Functional consequences of iron deficiency in COPD

The present study suggests greater impairment of exercise capacity in iron-deficient COPD patients, though FEV_1 appeared to be a significant confounder. It is no surprise that

ventilatory mechanics are of considerable importance. Patients with even mild COPD show intolerance to exercise and significantly greater self-reported symptoms of dyspnoea than healthy controls, and this seems to be driven to an extent by increased dead space ventilation on exertion (Elbehairy *et al.* 2015).

The ID group managed only two-thirds the SWT distance of their IR counterparts. The SWT has been shown independently to predict survival in patients with COPD, with one study finding nearly a three-fold higher mortality during an average of four and a half years of follow-up when SWT distance fell below 170 m (Ringbaek *et al.* 2010). The median distance achieved by our ID participants was lower than this value. Aside from utility in predicting mortality, functional tests such as these reflect the degree to which a disease interferes with a patient's activities of daily living. Though no statistically significant differences were found in SGRQ or HAD scores, the outcomes were numerically worse in both cases in ID patients, of a magnitude potentially of clinical significance. Future studies recruiting much larger numbers of patients would be helpful to clarify whether a true association exists.

5.5.5.1. Iron homeostasis and pulmonary hypertension in COPD

If iron deficiency does exert an independent effect on exercise tolerance in COPD, how is this effect mediated? The complex interaction between hypoxaemia, exercise and PH in the condition (Christensen *et al.* 2004, Hilde *et al.* 2013) has already been highlighted. Irrespective of whether iron deficiency promotes hypoxaemia, or *vice versa*, both would be expected to contribute to HPV in COPD. Echocardiographic data were not collected as part of *Study C*, but a recently published report indicates that the relationship predicted from the results of *Study A* does indeed exist in patients with COPD (Plesner *et al.* 2016). This study found that iron deficiency (defined as serum ferritin < 100 μg/L) was associated in stable

non-anaemic COPD patients with an increase in TR jet velocity (3.02 vs 2.77 m/s, P = 0.01) equivalent to a ΔP_{MAX} of 36.6 mmHg in the iron-deficient patients and 30.7 mmHg in those who were iron replete. These investigators discussed some of the challenges in defining iron deficiency in COPD, including that simply applying the definition used in CHF appeared unsatisfactory. By dividing patients into four groups according to serum ferritin, they were also able to show a progressive increase in ΔP_{MAX} as patients became more iron deficient, and, furthermore, that a fall in DL_{CO} occurred concomitantly.

5.5.5.2. Iron status and skeletal muscle dysfunction in COPD

Skeletal muscle dysfunction is another very important factor in limiting functional performance in COPD (Gea *et al.* 2013, Maltais *et al.* 2014). The finding that treatment with LTOT seems to improve skeletal muscle metabolism (Jakobsson and Jorfeldt 1995) attests to a pathological role for tissue hypoxia, perhaps akin to the consequences for skeletal muscle of exposure to the hypobaric hypoxia of high altitude (Hoppeler *et al.* 1990). Additionally, though, smoking appears to have a direct injurious effect on skeletal muscle physiology, much of which may be reversed on cessation of tobacco use (Degens *et al.* 2015). Mitochondrial dysfunction appears to be of considerable importance in this respect (Meyer *et al.* 2013, Puente-Maestu *et al.* 2013).

In a murine model of cigarette smoke-induced experimental COPD, which examined lung mitochondria rather than those in skeletal muscle, animals deficient in IRP2 were protected, apparently as a result of reduced mitochondrial iron loading, which in wild-type animals contributed to mitochondrial dysfunction (Cloonan *et al.* 2016). Mice treated with a mitochondrial iron chelator, or those fed an iron-restricted diet, were also protected from cigarette smoke-induced COPD in this study, implying that iron may be important in the

injurious processes at the inception of COPD. Much further on in the course of the disease, when individuals begin to develop symptoms and come to medical attention, such processes may no longer be relevant, particularly if patients have ceased smoking. Caution is therefore required when considering the implications for COPD of the finding of no significant direct effect of iron deficiency on skeletal muscle mitochondrial oxidative phosphorylation, discussed in Chapter 4. It remains possible that the added insult of iron deficiency might be injurious to mitochondrial function if the mitochondria concerned are already abnormal as a consequence of chemical injury and hypoxia.

Given that *Study B* did find an effect of iron deficiency to promote glycolysis in otherwise healthy individuals, it might be asked whether such an effect is evident in COPD patients. Reviewing the literature, Remels and colleagues noted that a shift in skeletal muscle metabolic profile towards decreased oxidative metabolism and increased glycolysis did indeed appear to be a consistent finding in patients with COPD (Remels *et al.* 2015). Moreover, these investigators demonstrated this effect to be dependent on HIF-1α, which seemed to be upregulated as a consequence of inflammation, via a mechanism involving tumour necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB) signalling.

Not only does the study by Remels *et al.* show that iron status need not influence skeletal muscle metabolism in COPD via a direct disturbance of mitochondrial function in order to be of pathophysiological importance, it also highlights again the complex interaction between hypoxia-sensing pathways and inflammation. Returning to this topic once more, it is of interest that it was demonstrated *in vitro* some time ago that the PHDs play a role in the regulation of NF-κB signalling. Inhibition of PHD1, PHD2, or both, resulted in activation of NF-κB signalling in cell culture experiments; conversely, overexpression of PHD1 decreased cytokine-stimulated NF-κB activity (Cummins *et al.* 2006). These authors

suggested that this effect arose because IκB kinase-β – involved in regulating the NF-κB pathway – was a substrate for the PHDs. Subsequent work revealed just how complicated the links between these pathways are, with the finding that, in mice, basal NF-κB activity is required for HIF-1α protein accumulation during hypoxia in cultured cells, and in the brain and liver of hypoxic animals (Rius *et al.* 2008).

Taking all this together, it can be seen that the added insult of chronic inflammation in COPD serves to increase the potential for iron deficiency to act deleteriously via an action on the HIF pathway, in a manner not apparent in otherwise healthy individuals such as those studied in *Study A* and *Study B*.

5.5.6. Iron homeostasis in other chronic respiratory diseases

In adult patients with cystic fibrosis (CF), another chronic inflammatory respiratory disorder in which iron deficiency is common (Reid *et al.* 2002), an impaired erythropoietic response to hypoxaemia has also been described that appears to be associated with inflammation (Fischer *et al.* 2007). A three-month course of oral iron was shown not to increase [Hb] in a study of patients with CF and functional iron deficiency (defined as TSat < 16%) (Pond *et al.* 1996). However, a very small case series of adult CF patients given IV iron for anaemia refractory to oral iron found that [Hb] and MCV both rose very rapidly with therapy (Hoo and Wildman 2012). These findings are consistent with impaired gastrointestinal iron uptake coupled with iron sequestration, potentially mediated by hepcidin. Measurement of sTfR has also reported to be useful in CF, given that it is unaffected by the acute phase response (Khalid *et al.* 2007).

5.6. Conclusions

The significance of disturbed iron homeostasis in COPD is beginning to be explored, but anaemia continues to dominate the research agenda (Schneckenpointner *et al.* 2014, Silverberg *et al.* 2014, Copur *et al.* 2015, Ferrari *et al.* 2015, Guo *et al.* 2015, Sharma and Chakrabarti 2016, Vasquez and Logomarsino 2016). This is in spite of iron deficiency *per se* being the subject of intense research efforts in pulmonary vascular and cardiovascular diseases. The findings from *Study C* support the view that attention should be directed towards iron deficiency in COPD irrespective of the presence or absence of anaemia.

An observational study cannot establish the exact pathophysiological mechanisms at work, particularly with respect to the relationship between iron deficiency and hypoxaemia. Irrespective of the underlying mechanisms, though, both the high prevalence of iron deficiency in COPD and the novel association of iron deficiency with hypoxaemia are potentially of considerable clinical significance from an interventional perspective. Potential confounding effects of disease severity and inflammation should not be a deterrent to investigating whether correction of iron deficiency is of clinical benefit. Hypoxaemia in COPD occurs more commonly in those with more severe disease, but it does not follow that it is simply a marker; we know its correction improves morbidity and mortality from clinical trials of LTOT.

What is required next is an interventional study using IV iron to correct iron deficiency in COPD and assess whether this brings significant benefits. If iron replacement were to ameliorate hypoxaemia or inflammation – the latter effect having been observed in a select group of patients with CHF, as already mentioned (Toblli *et al.* 2015) – this would provide valuable insight into the pathophysiology of the condition. At least two studies of IV iron in COPD are now underway (NCT02416778, ISRCTN09143837). An additional

therapeutic option that appears attractive based on the results of the present study would be to antagonise hepcidin signalling. Hepcidin antagonists have been reported to be efficacious in animals (Cooke *et al.* 2013), and might prove valuable for combatting iron sequestration in COPD, provided that such agents do not have deleterious consequences for innate immunity or acquisition of iron by pathogens.

Chapter 6. Overall conclusions

By investigating otherwise healthy humans, *Study A* and *Study B* determined the effects of iron deficiency on physiological processes known to depend on oxygen-sensing pathways. First, absolute iron deficiency was seen to augment HPV – making the pulmonary vasculature behave as if it were exposed to more marked hypoxia than was actually the case. Next, iron-deficient individuals were found to have abnormally high circulating levels of erythropoietin, which fell in response to IV iron – implying that the kidney interpreted an iron-deficiency signal as an oxygen-deficiency signal. Finally, upregulation of glycolysis was evident in exercising iron-deficient humans – as if oxygen were less available to support skeletal muscle metabolism than was truly the case. It is difficult to reach a conclusion other than that the operation of a common pathway or pathways, sensitive to the abundance of both oxygen and iron, underlies these phenomena.

These studies were predicated on numerous previous experiments using manipulation of iron bioavailability in cells, animals and humans, as well as studies in genetically modified

animals, and humans with heritable disorders of oxygen sensing. What they demonstrate is that the effect of iron deficiency predicted from the molecular biology of the HIF-hydroxylases does play through to physiological significance. However, irrespective of the motivation for this work, it must be acknowledged that to view the HIF-hydroxylases as the sole or even most important point of intersection of iron and oxygen sensing in humans would be misguided. The IRE-IRP system adds another level of complexity, and the 2-OG-dependent dioxygenases that require iron as a cofactor are themselves a very large and diverse family of enzymes. Effects of iron deficiency on pathways other than HIF may explain, for example, why both the studies presented here and previous experiments have not found an effect of iron status on human ventilation. Similarly, that pVHL has important roles to regulate pathways other than HIF, may partly explain the augmented ventilation and defect of mitochondrial oxidative phosphorylation seen in CP patients, but not healthy humans with iron deficiency.

By whatever mechanisms iron deficiency is acting to alter physiological responses to hypoxia, we must ask what the clinical implications are. An early report of the consequences of profound dietary iron deficiency in animals observes:

> *"The pigs became dull and listless, their skins became intensely white…and…the breathing became pumping in character...Sudden death became a common event, until whole litters perished one after the other. On post mortem examination…The heart would be found so dilated as actually to fill almost the entire chest cavity, the lungs being crushed back and collapsed until they were confined in the angle between the bodies of the vertebrae and the ribs. There was great effusion into the pericardial cavity, as also into the pleural cavity, and the lungs were oedematous…The blood was extremely watery and pale." (McGowan and Crichton 1923)*

It might be argued that we have come a long way since this description, in that we now understand, of course, that the features described here of high-output cardiac failure arise due to the profound anaemia that inevitably develops if iron deficiency is left unchecked for a sufficient period of time, and that they might equally well arise from an anaemia of any cause. Yet, if we consider CHF in particular, we find overwhelming evidence of a direct deleterious effect of iron deficiency, independent of anaemia, and this increasingly seems to be the case for other chronic cardiopulmonary conditions. What are the mechanisms underlying this effect? This thesis strongly supports the possibility that the pseudo-hypoxic signal generated by iron deficiency may be harmful in conditions where there is already tissue hypoxia or frank hypoxaemia. The notion that iron deficiency may directly contribute to PH in IPAH and COPD is intuitively appealing, as is the possibly that impaired oxidative metabolism in the skeletal muscle of patients with chronic cardiopulmonary disease might be ameliorated by iron supplementation.

In *Study C*, hypoxaemia was found to associate with iron deficiency in COPD, highlighting the complexity of the interactions between hypoxia, iron homeostasis, and inflammation. Studies of IV iron supplementation in this and similar conditions are likely to shed light on the mechanisms underlying this association, and are urgently needed if the therapeutic potential of manipulation of iron status as a tool to treat human disease is to be realised.

Finally, the results of the experiments described in this thesis have general implications for any investigator concerned with the biology of iron and oxygen. This author would suggest that any experiment addressing the effects of hypoxia, whether it be in a single cell or whole organism, must pay proper attention to iron bioavailability. Equally, studies of iron biology will not reach secure conclusions if the very significant effects of hypoxia on the pathways that govern iron homeostasis are neglected.

Appendix A: Contributions

The studies in healthy volunteers and patients presented in this thesis involved invasive procedures and experimental techniques carrying certain risks. They could not have been designed and safely executed by a single individual. Additionally, certain aspects of the protocols, including randomisation and the requirement for blinded analysis of data, also necessitated the involvement of individuals other than the author of this thesis, in the interests of scientific rigor. Below are listed other individuals who have contributed to the execution of the studies presented herein. For *Study C*, unless otherwise stated, contributions are towards the work in patients with COPD rather than the healthy volunteer control cohort; the latter group were drawn from a separate study run by Dr Hung-Yuan Cheng, whom I thank for his allowing me access to their anonymised data. Notwithstanding these contributions, analysis and interpretation of data reported herein was undertaken entirely by the author under the supervision of Prof Peter A Robbins.

Study A

Dr Annabel H Nickol – Chief Investigator; conception and design of study; assistance in securing ethical approval.

Dr Hung-Yuan Cheng and Dr Keith L Dorrington – assistance recruiting participants; support running normobaric chamber apparatus.

Dr M Kate Curtis and Karen A Pollard – assistance with ELISAs.

Prof David J Roberts and Dawn Goodwin – recruitment of iron-deficient blood donors.

Prof Sir Peter J Ratcliffe – conception and design of study.

Study B

Maj David A Holdsworth – assistance running MRS and CPET experiments; blind processing of CPET data.

Dr Andrew W Johnson – blind processing of MRS data.

Dr Keith L Dorrington – assistance recruiting participants; randomisation.

Prof Damian J Tyler – programming sequence for acquisition of MRS data.

Prof David J Roberts and Dawn Goodwin – recruitment of iron-deficient blood donors.

Prof Sir Peter J Ratcliffe and Dr Pete J Cox – conception and design of study.

Study C

Dr Annabel H Nickol - Chief Investigator; conception and design of study; securing ethical approval, recruitment of participants.

Dr F Maxine Hardinge – conception and design of study; recruitment of participants.

Dr Hung-Yuan Cheng and Dr Keith L Dorrington – conception and design of study; recruitment of participants (healthy volunteer component).

Anne McGahey, Bethan M McFadyen and Tara Harris-Wright – Cohort patient care; patient assessments; collection of venous blood samples.

Dr M Kate Curtis, Dr Nayia Petousi, Shivani Khandwala, Karen A Pollard, Dr Andrew E Armitage, Prof Hal Drakesmith – assistance with ELISAs.

David P O'Neill – programming of MATLAB algorithm for blind matching of COPD patients with healthy controls.

Prof Sir Peter J Ratcliffe – conception and design of study.

Appendix B: Publications arising

Frise, M. C., Cheng, H. Y., Nickol, A. H., Curtis, M. K., Pollard, K. A., Roberts, D. J., Ratcliffe, P. J., Dorrington, K. L. and Robbins, P. A. (2016). Clinical iron deficiency disturbs normal human responses to hypoxia. *J Clin Invest* 126(6): 2139-2150. [Study A]

Frise, M. C. and Robbins, P. A. (2015). Iron, oxygen, and the pulmonary circulation. *J Appl Physiol (1985)* 119(12): 1421-1431. [Review]

Frise, M. C. and Robbins, P. A. (2015). The pulmonary vasculature - lessons from Tibetans and from rare diseases of oxygen sensing. *Exp Physiol* 100(11): 1233-1241. [Review]

Nickol, A. H., Frise, M. C., Cheng, H. Y., McGahey, A., McFadyen, B. M., Harris-Wright, T., Bart, N. K., Curtis, M. K., Khandwala, S., O'Neill, D. P., Pollard, K. A., Hardinge, F. M., Rahman, N. M., Armitage, A. E., Dorrington, K. L., Drakesmith, H., Ratcliffe, P. J. and Robbins, P. A. (2015). 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(7): e007911. [Study C]

Appendix C: Figure permissions

In all cases where figures have been reproduced, permission was sought from the copyright holder for use in the final version of this thesis, deposited physically at the Bodleian Library and electronically at the Oxford Research Archive (https://ora.ox.ac.uk). That this has been granted is made clear in each individual figure legend; the absence of any comment regarding permission indicates that this was not required to reproduce the material in this context (except in the case of Figure 1-14 – see below).

Figure 1-14 is redacted in the online version of this thesis as the fee requested by the copyright holder was regrettably prohibitive. The image, which appears as Figure 6 in (Finch *et al.* 1986), is freely accessible: www.ncbi.nlm.nih.gov/pmc/articles/PMC1307110

Figure 2-11 is reproduced with permission from *JAMA* (2009) 302(13):1444-1450; DOI: 10.1001/jama.2009.1404 – Copyright© (2009) American Medical Association. All rights reserved.

Figure 5-1 is reproduced with permission from *Arch Intern Med (Chic)* (1920) 26(5):544-560; DOI: 10.1001/archinte.1920.00100050037003 – Copyright© (1920) American Medical Association. All rights reserved.

Figures 5-7 and 5-8 are reproduced with permission of the European Respiratory Society©. *Eur Resp J* Nov 2008, 32 (5) 1371-1385; DOI: 10.1183/09031936.00015608

References

Abakay, O., Abakay, A., Palanci, Y., Yuksel, H., Selimoglu Sen, H., Evliyaoglu, O. and Tanrikulu, A. C. (2015) Relationship between hepcidin levels and periodic limb movement disorder in patients with obstructive sleep apnea syndrome. *Sleep Breath* 19(2):459-466

Abbrecht, P. H. and Littell, J. K. (1972) Plasma erythropoietin in men and mice during acclimatization to different altitudes. *J Appl Physiol* 32(1):54-58

Abe, T., Kitaoka, Y., Kikuchi, D. M., Takeda, K., Numata, O. and Takemasa, T. (2015) High-intensity interval training-induced metabolic adaptation coupled with an increase in Hif-1alpha and glycolytic protein expression. *J Appl Physiol (1985)* 119(11):1297-1302

Addison, G. M., Beamish, M. R., Hales, C. N., Hodgkins, M., Jacobs, A. and Llewellin, P. (1972) An immunoradiometric assay for ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *J Clin Pathol* 25(4):326-329

Adhikari, N. K., Dellinger, R. P., Lundin, S., Payen, D., Vallet, B., Gerlach, H., Park, K. J., Mehta, S., Slutsky, A. S. and Friedrich, J. O. (2014) Inhaled nitric oxide does not reduce mortality in patients with acute respiratory distress syndrome regardless of severity: systematic review and meta-analysis. *Crit Care Med* 42(2):404-412

Afshari, A., Brok, J., Moller, A. M. and Wetterslev, J. (2010) Inhaled nitric oxide for acute respiratory distress syndrome (ARDS) and acute lung injury in children and adults. *Cochrane Database Syst Rev* 7:CD002787

Agarwal, R., Vasavada, N., Sachs, N. G. and Chase, S. (2004) Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. *Kidney Int* 65(6):2279-2289

Agusti, A., Barbera, J. A., Wouters, E. F., Peinado, V. I. and Jeffery, P. K. (2013) Lungs, bone marrow, and adipose tissue. A network approach to the pathobiology of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 188(12):1396-1406

Agusti, A. G., Roca, J., Gea, J., Wagner, P. D., Xaubet, A. and Rodriguez-Roisin, R. (1991) Mechanisms of gas-exchange impairment in idiopathic pulmonary fibrosis. *Am Rev Respir Dis* 143(2):219-225

Aisen, P. (2004) Transferrin receptor 1. *Int J Biochem Cell Biol* 36(11):2137-2143

Aisen, P., Enns, C. and Wessling-Resnick, M. (2001) Chemistry and biology of eukaryotic iron metabolism. *Int J Biochem Cell Biol* 33(10):940-959

Aldenderfer, M. (2011) Peopling the Tibetan plateau: Insights from archaeology. *High Alt Med Biol* 12(2):141-147

Allemann, Y., Sartori, C., Lepori, M., Pierre, S., Melot, C., Naeije, R., Scherrer, U. and Maggiorini, M. (2000) Echocardiographic and invasive measurements of pulmonary artery pressure correlate closely at high altitude. *Am J Physiol Heart Circ Physiol* 279(4):H2013-2016

Allen, J., Backstrom, K. R., Cooper, J. A., Cooper, M. C., Detwiler, T. C., Essex, D. W., Fritz, R. P., Means, R. T., Jr., Meier, P. B., Pearlman, S. R., Roitman-Johnson, B. and Seligman, P. A. (1998) Measurement of soluble transferrin receptor in serum of healthy adults. *Clin Chem* 44(1):35-39

Alvisi, V., Mirkovic, T., Nesme, P., Guerin, C. and Milic-Emili, J. (2003) Acute effects of hyperoxia on dyspnea in hypoxemia patients with chronic airway obstruction at rest. *Chest* 123(4):1038-1046

American Thoracic Society (1962) Committee on diagnostic standards for non-tuberculosis respiratory disease: definition and classification of chronic bronchitis, asthma, and pulmonary emphysema. *Am Rev Respir Dis* 85:762-768

Andersen, K. H., Iversen, M., Kjaergaard, J., Mortensen, J., Nielsen-Kudsk, J. E., Bendstrup, E., Videbaek, R. and Carlsen, J. (2012) Prevalence, predictors, and survival in pulmonary hypertension related to end-stage chronic obstructive pulmonary disease. *J Heart Lung Transplant* 31(4):373-380

Anderson, C. P., Shen, M., Eisenstein, R. S. and Leibold, E. A. (2012) Mammalian iron metabolism and its control by iron regulatory proteins. *Biochim Biophys Acta* 1823(9):1468-1483

Anderson, S. A., Nizzi, C. P., Chang, Y. I., Deck, K. M., Schmidt, P. J., Galy, B., Damnernsawad, A., Broman, A. T., Kendziorski, C., Hentze, M. W., Fleming, M. D., Zhang, J. and Eisenstein, R. S. (2013) The IRP1-HIF-2alpha axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell Metab* 17(2):282-290

Andrews, S. C., Arosio, P., Bottke, W., Briat, J. F., von Darl, M., Harrison, P. M., Laulhere, J. P., Levi, S., Lobreaux, S. and Yewdall, S. J. (1992) Structure, function, and evolution of ferritins. *J Inorg Biochem* 47(3-4):161-174

Ang, S. O., Chen, H., Hirota, K., Gordeuk, V. R., Jelinek, J., Guan, Y., Liu, E., Sergueeva, A. I., Miasnikova, G. Y., Mole, D., Maxwell, P. H., Stockton, D. W., Semenza, G. L. and Prchal, J. T. (2002) Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nat Genet* 32(4):614-621

Anker, S. D., Comin-Colet, J., Filippatos, G., Willenheimer, R., Dickstein, K., Drexler, H., Luscher, T. F., Bart, B., Banasiak, W., Niegowska, J., Kirwan, B. A., Mori, C., von Eisenhart Rothe, B., Pocock, S. J., Poole-Wilson, P. A., Ponikowski, P. and Fair-HF Trial Investigators (2009) Ferric carboxymaltose in patients with heart failure and iron deficiency. *N Engl J Med* 361(25):2436-2448

Aragones, J., Schneider, M., Van Geyte, K., Fraisl, P., Dresselaers, T., Mazzone, M., Dirkx, R., Zacchigna, S., Lemieux, H., Jeoung, N. H., Lambrechts, D., Bishop, T., Lafuste, P., Diez-Juan, A., Harten, S. K., Van Noten, P., De Bock, K., Willam, C., Tjwa, M., Grosfeld, A., Navet, R., Moons, L., Vandendriessche, T., Deroose, C., Wijeyekoon, B., Nuyts, J., Jordan, B., Silasi-Mansat, R., Lupu, F., Dewerchin, M., Pugh, C., Salmon, P., Mortelmans, L., Gallez, B., Gorus, F., Buyse, J., Sluse, F., Harris, R. A., Gnaiger, E., Hespel, P., Van Hecke, P., Schuit, F., Van Veldhoven, P., Ratcliffe, P., Baes, M., Maxwell, P. and Carmeliet, P. (2008) Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nat Genet* 40(2):170-180

Arai, T. J., Henderson, A. C., Dubowitz, D. J., Levin, D. L., Friedman, P. J., Buxton, R. B., Prisk, G. K. and Hopkins, S. R. (2009) Hypoxic pulmonary vasoconstriction does not contribute to pulmonary blood flow heterogeneity in normoxia in normal supine humans. *J Appl Physiol (1985)* 106(4):1057-1064

Archer, S. L., Huang, J., Henry, T., Peterson, D. and Weir, E. K. (1993) A redox-based O2 sensor in rat pulmonary vasculature. *Circ Res* 73(6):1100-1112

Arosio, P. and Levi, S. (2002) Ferritin, iron homeostasis, and oxidative damage. *Free Radic Biol Med* 33(4):457-463

Arosio, P., Yokota, M. and Drysdale, J. W. (1976) Structural and immunological relationships of isoferritins in normal and malignant cells. *Cancer Res* 36(5):1735-1739

Asadi, A. K., Sa, R. C., Kim, N. H., Theilmann, R. J., Hopkins, S. R., Buxton, R. B. and Prisk, G. K. (2015) Inhaled nitric oxide alters the distribution of blood flow in the healthy human lung, suggesting active hypoxic pulmonary vasoconstriction in normoxia. *J Appl Physiol (1985)* 118(3):331-343

Aungst, C. W. (1966) A specific and sensitive method for the detection of ferritin in body fluids. *J Lab Clin Med* 67(2):307-313

Aziz, N. and Munro, H. N. (1987) Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region. *Proc Natl Acad Sci U S A* 84(23):8478-8482

Baez, S., Mazur, A. and Shorr, E. (1950) Hepatorenal factors in circulatory homeostasis. XX. Antidiuretic action of hepatic vasodepressor, VDM (ferritin). *Am J Physiol* 162(1):198-212

Bainton, D. F. and Finch, C. A. (1964) The diagnosis of iron deficiency anemia. *Am J Med* 37:62-70

Balanos, G. M., Dorrington, K. L. and Robbins, P. A. (2002) Desferrioxamine elevates pulmonary vascular resistance in humans: potential for involvement of HIF-1. *J Appl Physiol (1985)* 92(6):2501-2507

Balanos, G. M., Pugh, K., Frise, M. C. and Dorrington, K. L. (2015) Exaggerated pulmonary vascular response to acute hypoxia in older men. *Exp Physiol* 100(10):1187-1198

Balanos, G. M., Talbot, N. P., Dorrington, K. L. and Robbins, P. A. (2003) Human pulmonary vascular response to 4 h of hypercapnia and hypocapnia measured using Doppler echocardiography. *J Appl Physiol (1985)* 94(4):1543-1551

Balanos, G. M., Talbot, N. P., Robbins, P. A. and Dorrington, K. L. (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* 450(6):372-380

Baldari, C., Bonavolonta, V., Emerenziani, G. P., Gallotta, M. C., Silva, A. J. and Guidetti, L. (2009) Accuracy, reliability, linearity of Accutrend and Lactate Pro versus EBIO plus analyzer. *Eur J Appl Physiol* 107(1):105-111

Baldi, S., Pinna, G. D., Mombaruzzo, P., Biglieri, M., De Martini, A. and Palange, P. (2008) C-reactive protein correlates with tissue oxygen availability in patients with stable COPD. *Int J Chron Obstruct Pulmon Dis* 3(4):745-751

Barbera, J. A., Ramirez, J., Roca, J., Wagner, P. D., Sanchez-Lloret, J. and Rodriguez-Roisin, R. (1990) Lung structure and gas exchange in mild chronic obstructive pulmonary disease. *Am Rev Respir Dis* 141(4 Pt 1):895-901

Barberan-Garcia, A., Rodriguez, D. A., Blanco, I., Gea, J., Torralba, Y., Arbillaga-Etxarri, A., Barbera, J. A., Vilaro, J., Roca, J. and Orozco-Levi, M. (2015) Non-anaemic iron deficiency impairs response to pulmonary rehabilitation in COPD. *Respirology* 20(7):1089-1095

Barcroft, H. (1972) An enquiry into the nature of the mediator of the vasodilatation in skeletal muscle in exercise and during circulatory arrest. *J Physiol* 222(2):99P-118P

Barcroft, J. (1920) Presidential address [abridged]: On anoxæmia. *Lancet* 196(5062):485-489

Barnes, P. J. (2008) Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 8(3):183-192

Barrell, J. (1916) The influence of Silurian-Devonian climates on the rise of air-breathing vertebrates. *Proc Natl Acad Sci U S A* 2(8):499-504

Barrientos, T., Laothamatas, I., Koves, T. R., Soderblom, E. J., Bryan, M., Moseley, M. A., Muoio, D. M. and Andrews, N. C. (2015) Metabolic catastrophe in mice lacking transferrin receptor in muscle. *EBioMedicine* 2(11):1705-1717

Barron, B. A., Hoyer, J. D. and Tefferi, A. (2001) A bone marrow report of absent stainable iron is not diagnostic of iron deficiency. *Ann Hematol* 80(3):166-169

Bassett, D. R., Jr. and Howley, E. T. (2000) Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Med Sci Sports Exerc* 32(1):70-84

Beall, C. M., Cavalleri, G. L., Deng, L., Elston, R. C., Gao, Y., Knight, J., Li, C., Li, J. C., Liang, Y., McCormack, M., Montgomery, H. E., Pan, H., Robbins, P. A., Shianna, K. V., Tam, S. C., Tsering, N., Veeramah, K. R., Wang, W., Wangdui, P., Weale, M. E., Xu, Y., Xu, Z., Yang, L., Zaman, M. J., Zeng, C., Zhang, L., Zhang, X., Zhaxi, P. and Zheng, Y. T. (2010) Natural selection on EPAS1 (HIF2alpha) associated with low hemoglobin concentration in Tibetan highlanders. *Proc Natl Acad Sci U S A* 107(25):11459-11464

Beaver, W. L., Wasserman, K. and Whipp, B. J. (1985) Improved detection of lactate threshold during exercise using a log-log transformation. *J Appl Physiol (1985)* 59(6):1936-1940

Beck, I., Ramirez, S., Weinmann, R. and Caro, J. (1991) Enhancer element at the 3'-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. *J Biol Chem* 266(24):15563-15566

Belardinelli, R., Lacalaprice, F., Carle, F., Minnucci, A., Cianci, G., Perna, G. and D'Eusanio, G. (2003) Exercise-induced myocardial ischaemia detected by cardiopulmonary exercise testing. *Eur Heart J* 24(14):1304-1313

Berendsen, R. R., Lindeman, R. C., Boom, M., Aarts, L. P., van Dorp, E. L. and Teppema, L. J. (2016) Erythropoietin does not have effects on the ventilatory and pulmonary vascular response to acute hypoxia in men and women. *Exp Physiol* 101(9):1230-1240

Berg, J. M., Tymoczko, J. L. and Stryer, L. (2012). *Biochemistry VIIth edition.* WH Freeman and Company, New York.

Berger, M., Haimowitz, A., Van Tosh, A., Berdoff, R. L. and Goldberg, E. (1985) Quantitative assessment of pulmonary hypertension in patients with tricuspid regurgitation using continuous wave Doppler ultrasound. *J Am Coll Cardiol* 6(2):359-365

Bergofsky, E. H., Haas, F. and Porcelli, R. (1968) Determination of the sensitive vascular sites from which hypoxia and hypercapnia elicit rises in pulmonary arterial pressure. *Fed Proc* 27(6):1420-1425

Berk, L., Burchenal, J. H. and Castle, W. B. (1949) Erythropoietic effect of cobalt in patients with or without anemia. *N Engl J Med* 240(19):754-761

Bernhardt, W. M., Wiesener, M. S., Scigalla, P., Chou, J., Schmieder, R. E., Gunzler, V. and Eckardt, K. U. (2010) Inhibition of prolyl hydroxylases increases erythropoietin production in ESRD. *J Am Soc Nephrol* 21(12):2151-2156

Bert, P. (1878). *La pression barométrique.* Paris, G. Masson [French].

Beutner, C. A. (1850). Über die Strom-und Druckkräfte des Blutes in der arteria pulmonalis: Inauguraldissertation, Zürcher u. Furrer [German].

Beyne, J. (1942) Influence de l'anoxémie sur la grande circulation et sur la circulation pulmonaire. *CR Seances Soc Biol* 136:399-400 [French].

Biernacki, W., Gould, G. A., Whyte, K. F. and Flenley, D. C. (1989) Pulmonary hemodynamics, gas exchange, and the severity of emphysema as assessed by quantitative CT scan in chronic bronchitis and emphysema. *Am Rev Respir Dis* 139(6):1509-1515

Bishop, T., Talbot, N. P., Turner, P. J., Nicholls, L. G., Pascual, A., Hodson, E. J., Douglas, G., Fielding, J. W., Smith, T. G., Demetriades, M., Schofield, C. J., Robbins, P. A., Pugh, C. W., Buckler, K. J. and Ratcliffe, P. J. (2013) Carotid body hyperplasia and enhanced ventilatory responses to hypoxia in mice with heterozygous deficiency of PHD2. *J Physiol* 591(14):3565-3577

Blomstrand, E., Radegran, G. and Saltin, B. (1997) Maximum rate of oxygen uptake by human skeletal muscle in relation to maximal activities of enzymes in the Krebs cycle. *J Physiol* 501 (Pt 2):455-460

Bolger, A. P., Bartlett, F. R., Penston, H. S., O'Leary, J., Pollock, N., Kaprielian, R. and Chapman, C. M. (2006) Intravenous iron alone for the treatment of anemia in patients with chronic heart failure. *J Am Coll Cardiol* 48(6):1225-1227

Bonda, T., Kaminski, K. A., Kozuch, M., Kozieradzka, A., Wojtkowska, I., Dobrzycki, S., Kralisz, P., Nowak, K., Prokopczuk, P. and Musial, W. J. (2010) Circadian variations of interleukin 6 in coronary circulations of patients with myocardial infarction. *Cytokine* 50(2):204-209

Bonnet, S., Michelakis, E. D., Porter, C. J., Andrade-Navarro, M. A., Thebaud, B., Bonnet, S., Haromy, A., Harry, G., Moudgil, R., McMurtry, M. S., Weir, E. K. and Archer, S. L. (2006) An abnormal mitochondrial-hypoxia inducible factor-1alpha-Kv channel pathway disrupts oxygen sensing and triggers pulmonary arterial hypertension in fawn hooded rats: similarities to human pulmonary arterial hypertension. *Circulation* 113(22):2630-2641

Bonsdorff, E. V. A. and Jalavisto, E. (1948) A humoral mechanism in anoxic erythrocytosis. *Acta Physiol Scand* 16(2-3):150-170

Borg, G. (1970) Perceived exertion as an indicator of somatic stress. *Scand J Rehabil Med* 2(2):92-98

Bossone, E., Rubenfire, M., Bach, D. S., Ricciardi, M. and Armstrong, W. F. (1999) Range of tricuspid regurgitation velocity at rest and during exercise in normal adult men: implications for the diagnosis of pulmonary hypertension. *J Am Coll Cardiol* 33(6):1662-1666

Bothwell, T. H., Pirzio-Biroli, G. and Finch, C. A. (1958) Iron absorption. I. Factors influencing absorption. *J Lab Clin Med* 51(1):24-36

Boushy, S. F. and North, L. B. (1977) Hemodynamic changes in chronic obstructive pulmonary disease. *Chest* 72(5):565-570

Boutou, A. K., Karrar, S., Hopkinson, N. S. and Polkey, M. I. (2013) Anemia and survival in chronic obstructive pulmonary disease: a dichotomous rather than a continuous predictor. *Respiration* 85(2):126-131

Bradford, J. R. and Dean, H. P. (1894) The pulmonary circulation. *J Physiol* 16(1-2):34-158.125

Bradley, C. A., Fleetham, J. A. and Anthonisen, N. R. (1979) Ventilatory control in patients with hypoxemia due to obstructive lung disease. *Am Rev Respir Dis* 120(1):21-30

Breuer, W., Ermers, M. J., Pootrakul, P., Abramov, A., Hershko, C. and Cabantchik, Z. I. (2001) Desferrioxamine-chelatable iron, a component of serum non-transferrin-bound iron, used for assessing chelation therapy. *Blood* 97(3):792-798

Brimioulle, S., LeJeune, P. and Naeije, R. (1996) Effects of hypoxic pulmonary vasoconstriction on pulmonary gas exchange. *J Appl Physiol (1985)* 81(4):1535-1543

Briscoe, W. A. and Nash, E. S. (1965) The slow space in chronic obstructive pulmonary diseases. *Ann N Y Acad Sci* 121:706-722

Brodovsky, D., Macdonell, J. A. and Cherniack, R. M. (1960) The respiratory response to carbon dioxide in health and in emphysema. *J Clin Invest* 39:724-729

Broekhuizen, R., Wouters, E. F., Creutzberg, E. C. and Schols, A. M. (2006) Raised CRP levels mark metabolic and functional impairment in advanced COPD. *Thorax* 61(1):17-22

Bruick, R. K. and McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294(5545):1337-1340

Brusselmans, K., Compernolle, V., Tjwa, M., Wiesener, M. S., Maxwell, P. H., Collen, D. and Carmeliet, P. (2003) Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *J Clin Invest* 111(10):1519-1527

Buckler, K. J. (1997) A novel oxygen-sensitive potassium current in rat carotid body type I cells. *J Physiol* 498 (Pt 3):649-662

Buckler, K. J. and Turner, P. J. (2013) Oxygen sensitivity of mitochondrial function in rat arterial chemoreceptor cells. *J Physiol* 591(14):3549-3563

Buckler, K. J. and Turner, P. J. (2015) Functional properties of mitochondria in the type-1 cell and their role in oxygen sensing. *Adv Exp Med Biol* 860:69-80

Buckler, K. J. and Vaughan-Jones, R. D. (1994) Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type I cells. *J Physiol* 476(3):423-428

Bull, T. M., Golpon, H., Hebbel, R. P., Solovey, A., Cool, C. D., Tuder, R. M., Geraci, M. W. and Voelkel, N. F. (2003) Circulating endothelial cells in pulmonary hypertension. *Thromb Haemost* 90(4):698-703

Bunn, H. F. (2013) Erythropoietin. *Cold Spring Harb Perspect Med* 3(3):a011619

Burden, R. J., Morton, K., Richards, T., Whyte, G. P. and Pedlar, C. R. (2015a) Is iron treatment beneficial in, iron-deficient but non-anaemic (IDNA) endurance athletes? A systematic review and meta-analysis. *Br J Sports Med* 49(21):1389-1397

Burden, R. J., Pollock, N., Whyte, G. P., Richards, T., Moore, B., Busbridge, M., Srai, S. K., Otto, J. and Pedlar, C. R. (2015b) Effect of intravenous iron on aerobic capacity and iron metabolism in elite athletes. *Med Sci Sports Exerc* 47(7):1399-1407

Burrowes, K. S. and Tawhai, M. H. (2006) Computational predictions of pulmonary blood flow gradients: gravity versus structure. *Respir Physiol Neurobiol* 154(3):515-523

Burrows, B., Kettel, L. J., Niden, A. H., Rabinowitz, M. and Diener, C. F. (1972) Patterns of cardiovascular dysfunction in chronic obstructive lung disease. *N Engl J Med* 286(17):912-918

Burton, G. J., Charnock-Jones, D. S. and Jauniaux, E. (2009) Regulation of vascular growth and function in the human placenta. *Reproduction* 138(6):895-902

Burton, K. and Krebs, H. A. (1953) The free-energy changes associated with the individual steps of the tricarboxylic acid cycle, glycolysis and alcoholic fermentation and with the hydrolysis of the pyrophosphate groups of adenosinetriphosphate. *Biochem J* 54(1):94-107

Bushuev, V. I., Miasnikova, G. Y., Sergueeva, A. I., Polyakova, L. A., Okhotin, D., Gaskin, P. R., Debebe, Z., Nekhai, S., Castro, O. L., Prchal, J. T. and Gordeuk, V. R. (2006) Endothelin-1, vascular endothelial growth factor and systolic pulmonary artery pressure in patients with Chuvash polycythemia. *Haematologica* 91(6):744-749

Cahill, G. F., Jr. (1970) Starvation in man. *N Engl J Med* 282(12):668-675

Camacho, J., Arnalich, F., Zamorano, A. F. and Vazquez, J. J. (1991) Serum erythropoietin levels in the anaemia of chronic disorders. *J Intern Med* 229(1):49-54

Camaschella, C. (2005) Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. *Blood* 106(12):3710-3717

Camaschella, C. (2015) Iron-deficiency anemia. *N Engl J Med* 372(19):1832-1843

Carnot, P. and Deflandre, C. (1906) Sur l'activité hémopoiétique du sérum au cours de la régénération du sang. *CR Searces Acad Sci* 143:432-435 [French].

Casey, J. L., Koeller, D. M., Ramin, V. C., Klausner, R. D. and Harford, J. B. (1989) Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3' untranslated region of the mRNA. *EMBO J* 8(12):3693-3699

Cavill, I. (2002) Erythropoiesis and iron. *Best Pract Res Clin Haematol* 15(2):399-409

Celsing, F., Blomstrand, E., Werner, B., Pihlstedt, P. and Ekblom, B. (1986) Effects of iron deficiency on endurance and muscle enzyme activity in man. *Med Sci Sports Exerc* 18(2):156-161

Celsing, F., Ekblom, B., Sylven, C., Everett, J. and Astrand, P. O. (1988) Effects of chronic iron deficiency anaemia on myoglobin content, enzyme activity, and capillary density in the human skeletal muscle. *Acta Med Scand* 223(5):451-457

CGPM (1954) Résolution 4: Définition de l'atmosphère normale. *Comptes Rendus des Séances de la Dixième Conference Generale des Poids et Mesures*: www.bipm.org [Accessed 08/11/2016]

Chan, K. L., Currie, P. J., Seward, J. B., Hagler, D. J., Mair, D. D. and Tajik, A. J. (1987) Comparison of three Doppler ultrasound methods in the prediction of pulmonary artery pressure. *J Am Coll Cardiol* 9(3):549-554

Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M. and Schumacker, P. T. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *J Biol Chem* 275(33):25130-25138

Chandel, N. S. and Schumacker, P. T. (2000) Cellular oxygen sensing by mitochondria: old questions, new insight. *J Appl Physiol (1985)* 88(5):1880-1889

Chaouat, A., Bugnet, A. S., Kadaoui, N., Schott, R., Enache, I., Ducolone, A., Ehrhart, M., Kessler, R. and Weitzenblum, E. (2005) Severe pulmonary hypertension and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 172(2):189-194

Chaouat, A., Naeije, R. and Weitzenblum, E. (2008) Pulmonary hypertension in COPD. *Eur Respir J* 32(5):1371-1385

Chaouat, A., Savale, L., Chouaid, C., Tu, L., Sztrymf, B., Canuet, M., Maitre, B., Housset, B., Brandt, C., Le Corvoisier, P., Weitzenblum, E., Eddahibi, S. and Adnot, S. (2009) Role for interleukin-6 in COPD-related pulmonary hypertension. *Chest* 136(3):678-687

Chappell, S. L., Daly, L., Lotya, J., Alsaegh, A., Guetta-Baranes, T., Roca, J., Rabinovich, R., Morgan, K., Millar, A. B., Donnelly, S. C., Keatings, V., MacNee, W., Stolk, J., Hiemstra, P. S., Miniati, M., Monti, S., O'Connor, C. M. and Kalsheker, N. (2011) The role of IREB2 and transforming growth factor beta-1 genetic variants in COPD: a replication case-control study. *BMC Med Genet* 12:24

Chaudhry, S., Arena, R., Wasserman, K., Hansen, J. E., Lewis, G. D., Myers, J., Chronos, N. and Boden, W. E. (2009) Exercise-induced myocardial ischemia detected by cardiopulmonary exercise testing. *Am J Cardiol* 103(5):615-619

Chen, W., Huang, F. W., de Renshaw, T. B. and Andrews, N. C. (2011) Skeletal muscle hemojuvelin is dispensable for systemic iron homeostasis. *Blood* 117(23):6319-6325

Cheng, H.-Y. (2015). Right ventricular outflow limitation and capacity for exertion associated with age and iron status. DPhil Thesis, University of Oxford.

Christensen, C. C., Ryg, M. S., Edvardsen, A. and Skjonsberg, O. H. (2004) Relationship between exercise desaturation and pulmonary haemodynamics in COPD patients. *Eur Respir J* 24(4):580-586

Clanton, T. L., Hogan, M. C. and Gladden, L. B. (2013) Regulation of cellular gas exchange, oxygen sensing, and metabolic control. *Compr Physiol* 3(3):1135-1190

Clifton, I. J., McDonough, M. A., Ehrismann, D., Kershaw, N. J., Granatino, N. and Schofield, C. J. (2006) Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta-helix fold proteins. *J Inorg Biochem* 100(4):644-669

Cloonan, S. M., Glass, K., Laucho-Contreras, M. E., Bhashyam, A. R., Cervo, M., Pabon, M. A., Konrad, C., Polverino, F., Siempos, II, Perez, E., Mizumura, K., Ghosh, M. C., Parameswaran, H., Williams, N. C., Rooney, K. T., Chen, Z. H., Goldklang, M. P., Yuan, G. C., Moore, S. C., Demeo, D. L., Rouault, T. A., D'Armiento, J. M., Schon, E. A., Manfredi, G., Quackenbush, J., Mahmood, A., Silverman, E. K., Owen, C. A. and Choi, A. M. (2016) Mitochondrial iron chelation ameliorates cigarette smoke-induced bronchitis and emphysema in mice. *Nat Med* 22(2):163-174

Cohen, L. A., Gutierrez, L., Weiss, A., Leichtmann-Bardoogo, Y., Zhang, D. L., Crooks, D. R., Sougrat, R., Morgenstern, A., Galy, B., Hentze, M. W., Lazaro, F. J., Rouault, T. A. and Meyron-Holtz, E. G. (2010)

Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway. *Blood* 116(9):1574-1584

Connett, R. J., Gayeski, T. E. and Honig, C. R. (1984) Lactate accumulation in fully aerobic, working, dog gracilis muscle. *Am J Physiol* 246(1 Pt 2):H120-128

Cook, J. D. (1982) Clinical evaluation of iron deficiency. *Semin Hematol* 19(1):6-18

Cook, J. D., Finch, C. A. and Smith, N. J. (1976) Evaluation of the iron status of a population. *Blood* 48(3):449-455

Cook, J. D., Lipschitz, D. A., Miles, L. E. and Finch, C. A. (1974) Serum ferritin as a measure of iron stores in normal subjects. *Am J Clin Nutr* 27(7):681-687

Cooke, K. S., Hinkle, B., Salimi-Moosavi, H., Foltz, I., King, C., Rathanaswami, P., Winters, A., Steavenson, S., Begley, C. G., Molineux, G. and Sasu, B. J. (2013) A fully human anti-hepcidin antibody modulates iron metabolism in both mice and nonhuman primates. *Blood* 122(17):3054-3061

Cooperman, S. S., Meyron-Holtz, E. G., Olivierre-Wilson, H., Ghosh, M. C., McConnell, J. P. and Rouault, T. A. (2005) Microcytic anemia, erythropoietic protoporphyria, and neurodegeneration in mice with targeted deletion of iron-regulatory protein 2. *Blood* 106(3):1084-1091

Copur, A. S., Fulambarker, A., Molnar, J., Nadeem, R., McCormack, C., Ganesh, A., Kheir, F. and Hamon, S. (2015) Role of anemia in home oxygen therapy in chronic obstructive pulmonary disease patients. *Am J Ther* 22(5):361-366

Corcoran, S. E. and O'Neill, L. A. (2016) HIF1alpha and metabolic reprogramming in inflammation. *J Clin Invest* 126(10):3699-3707

Costill, D. L. (1972) Physiology of marathon running. *JAMA* 221(9):1024-1029

Costill, D. L., Daniels, J., Evans, W., Fink, W., Krahenbuhl, G. and Saltin, B. (1976) Skeletal muscle enzymes and fiber composition in male and female track athletes. *J Appl Physiol* 40(2):149-154

Cote, C., Zilberberg, M. D., Mody, S. H., Dordelly, L. J. and Celli, B. (2007) Haemoglobin level and its clinical impact in a cohort of patients with COPD. *Eur Respir J* 29(5):923-929

Cotroneo, E., Ashek, A., Wang, L., Wharton, J., Dubois, O., Bozorgi, S., Busbridge, M., Alavian, K. N., Wilkins, M. R. and Zhao, L. (2015) Iron homeostasis and pulmonary hypertension: iron deficiency leads to pulmonary vascular remodeling in the rat. *Circ Res* 116(10):1680-1690

Croft, Q. P., Formenti, F., Talbot, N. P., Lunn, D., Robbins, P. A. and Dorrington, K. L. (2013) Variations in alveolar partial pressure for carbon dioxide and oxygen have additive not synergistic acute effects on human pulmonary vasoconstriction. *PLoS One* 8(7):e67886

Cummins, E. P., Berra, E., Comerford, K. M., Ginouves, A., Fitzgerald, K. T., Seeballuck, F., Godson, C., Nielsen, J. E., Moynagh, P., Pouyssegur, J. and Taylor, C. T. (2006) Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. *Proc Natl Acad Sci U S A* 103(48):18154-18159

Currie, P. J., Seward, J. B., Chan, K. L., Fyfe, D. A., Hagler, D. J., Mair, D. D., Reeder, G. S., Nishimura, R. A. and Tajik, A. J. (1985) Continuous wave Doppler determination of right ventricular pressure: a simultaneous Doppler-catheterization study in 127 patients. *J Am Coll Cardiol* 6(4):750-756

Cuttica, M. J., Kalhan, R., Shlobin, O. A., Ahmad, S., Gladwin, M., Machado, R. F., Barnett, S. D. and Nathan, S. D. (2010) Categorization and impact of pulmonary hypertension in patients with advanced COPD. *Respir Med* 104(12):1877-1882

D'Alessio, F., Hentze, M. W. and Muckenthaler, M. U. (2012) The hemochromatosis proteins HFE, TfR2, and HJV form a membrane-associated protein complex for hepcidin regulation. *J Hepatol* 57(5):1052-1060

D'Alonzo, G. E., Barst, R. J., Ayres, S. M., Bergofsky, E. H., Brundage, B. H., Detre, K. M., Fishman, A. P., Goldring, R. M., Groves, B. M., Kernis, J. T. and et al. (1991) Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med* 115(5):343-349

Dai, Z., Li, M., Wharton, J., Zhu, M. M. and Zhao, Y. Y. (2016) Prolyl-4 hydroxylase 2 (PHD2) deficiency in endothelial cells and hematopoietic cells induces obliterative vascular remodeling and severe pulmonary arterial hypertension in mice and humans through hypoxia-inducible factor-2alpha. *Circulation* 133(24):2447-2458

Dale, J. C., Burritt, M. F. and Zinsmeister, A. R. (2002) Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels. *Am J Clin Pathol* 117(5):802-808

Davies, K. J., Donovan, C. M., Refino, C. J., Brooks, G. A., Packer, L. and Dallman, P. R. (1984) Distinguishing effects of anemia and muscle iron deficiency on exercise bioenergetics in the rat. *Am J Physiol* 246(6 Pt 1):E535-543

Davies, K. J., Maguire, J. J., Brooks, G. A., Dallman, P. R. and Packer, L. (1982) Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion. *Am J Physiol* 242(6):E418-427

Davis, M. R., Shawron, K. M., Rendina, E., Peterson, S. K., Lucas, E. A., Smith, B. J. and Clarke, S. L. (2011) Hypoxia inducible factor-2 alpha is translationally repressed in response to dietary iron deficiency in Sprague-Dawley rats. *J Nutr* 141(9):1590-1596

de Klerk, G., Rosengarten, P. C., Vet, R. J. and Goudsmit, R. (1981) Serum erythropoietin (EST) titers in anemia. *Blood* 58(6):1164-1170

de Torres, J. P., Cordoba-Lanus, E., Lopez-Aguilar, C., Muros de Fuentes, M., Montejo de Garcini, A., Aguirre-Jaime, A., Celli, B. R. and Casanova, C. (2006) C-reactive protein levels and clinically important predictive outcomes in stable COPD patients. *Eur Respir J* 27(5):902-907

Deck, K. M., Vasanthakumar, A., Anderson, S. A., Goforth, J. B., Kennedy, M. C., Antholine, W. E. and Eisenstein, R. S. (2009) Evidence that phosphorylation of iron regulatory protein 1 at Serine 138 destabilizes the [4Fe-4S] cluster in cytosolic aconitase by enhancing 4Fe-3Fe cycling. *J Biol Chem* 284(19):12701-12709

Decramer, M., Rennard, S., Troosters, T., Mapel, D. W., Giardino, N., Mannino, D., Wouters, E., Sethi, S. and Cooper, C. B. (2008) COPD as a lung disease with systemic consequences - clinical impact, mechanisms, and potential for early intervention. *COPD* 5(4):235-256

Deem, S., Swenson, E. R., Alberts, M. K., Hedges, R. G. and Bishop, M. J. (1998) Red-blood-cell augmentation of hypoxic pulmonary vasoconstriction: hematocrit dependence and the importance of nitric oxide. *Am J Respir Crit Care Med* 157(4 Pt 1):1181-1186

Degens, H., Gayan-Ramirez, G. and van Hees, H. W. (2015) Smoking-induced skeletal muscle dysfunction: from evidence to mechanisms. *Am J Respir Crit Care Med* 191(6):620-625

DeMeo, D. L., Mariani, T., Bhattacharya, S., Srisuma, S., Lange, C., Litonjua, A., Bueno, R., Pillai, S. G., Lomas, D. A., Sparrow, D., Shapiro, S. D., Criner, G. J., Kim, H. P., Chen, Z., Choi, A. M., Reilly, J. and Silverman, E. K. (2009) Integration of genomic and genetic approaches implicates IREB2 as a COPD susceptibility gene. *Am J Hum Genet* 85(4):493-502

Detry, M. A. and Ma, Y. (2016) Analyzing repeated measurements using mixed models. *JAMA* 315(4):407-408

Dipp, M. and Evans, A. M. (2001) Cyclic ADP-ribose is the primary trigger for hypoxic pulmonary vasoconstriction in the rat lung in situ. *Circ Res* 89(1):77-83

Doherty, D. E. (2008) A review of the role of FEV1 in the COPD paradigm. *COPD* 5(5):310-318

Doi, M., Nakano, K., Hiramoto, T. and Kohno, N. (2003) Significance of pulmonary artery pressure in emphysema patients with mild-to-moderate hypoxemia. *Respir Med* 97(8):915-920

Dorrington, K. L., Balanos, G. M., Talbot, N. P. and Robbins, P. A. (2010) Extent to which pulmonary vascular responses to PCO2 and PO2 play a functional role within the healthy human lung. *J Appl Physiol (1985)* 108(5):1084-1096

Dorrington, K. L., Clar, C., Young, J. D., Jonas, M., Tansley, J. G. and Robbins, P. A. (1997) Time course of the human pulmonary vascular response to 8 hours of isocapnic hypoxia. *Am J Physiol* 273(3 Pt 2):H1126-1134

Dorrington, K. L. and Talbot, N. P. (2004) Human pulmonary vascular responses to hypoxia and hypercapnia. *Pflugers Arch* 449(1):1-15

Drakesmith, H. and Prentice, A. M. (2012) Hepcidin and the iron-infection axis. *Science* 338(6108):768-772

Dull, W. L., Polu, J. M. and Sadoul, P. (1983) The pulmonary haemodynamic effects of almitrine infusion in men with chronic hypercapnia. *Clin Sci (Lond)* 64(1):25-31

Duranti, R., Misuri, G., Gorini, M., Goti, P., Gigliotti, F. and Scano, G. (1995) Mechanical loading and control of breathing in patients with severe chronic obstructive pulmonary disease. *Thorax* 50(2):127-133

Duru, S., Bilgin, E. and Ardic, S. (2012) Hepcidin: A useful marker in chronic obstructive pulmonary disease. *Ann Thorac Med* 7(1):31-35

Duteil, S., Bourrilhon, C., Raynaud, J. S., Wary, C., Richardson, R. S., Leroy-Willig, A., Jouanin, J. C., Guezennec, C. Y. and Carlier, P. G. (2004) Metabolic and vascular support for the role of myoglobin in humans: a multiparametric NMR study. *Am J Physiol Regul Integr Comp Physiol* 287(6):R1441-1449

Earle, R. H., Perkins, J. F., Jr. and Adams, W. E. (1958) Carbon dioxide retention during hypoventilation in experimental animals. *Anesthesiology* 19(2):153-164

Eckard, J., Dai, J., Wu, J., Jian, J., Yang, Q., Chen, H., Costa, M., Frenkel, K. and Huang, X. (2010) Effects of cellular iron deficiency on the formation of vascular endothelial growth factor and angiogenesis. Iron deficiency and angiogenesis. *Cancer Cell Int* 10:28

Eckardt, K. U., Boutellier, U., Kurtz, A., Schopen, M., Koller, E. A. and Bauer, C. (1989a) Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol (1985)* 66(4):1785-1788

Eckardt, K. U., Kurtz, A. and Bauer, C. (1989b) Regulation of erythropoietin production is related to proximal tubular function. *Am J Physiol* 256(5 Pt 2):F942-947

Edwards, L. M., Tyler, D. J., Kemp, G. J., Dwyer, R. M., Johnson, A., Holloway, C. J., Nevill, A. M. and Clarke, K. (2012) The reproducibility of 31-phosphorus MRS measures of muscle energetics at 3 Tesla in trained men. *PLoS One* 7(6):e37237

Ehrismann, D., Flashman, E., Genn, D. N., Mathioudakis, N., Hewitson, K. S., Ratcliffe, P. J. and Schofield, C. J. (2007) Studies on the activity of the hypoxia-inducible-factor hydroxylases using an oxygen consumption assay. *Biochem J* 401(1):227-234

Elbehairy, A. F., Ciavaglia, C. E., Webb, K. A., Guenette, J. A., Jensen, D., Mourad, S. M., Neder, J. A., O'Donnell, D. E. and Canadian Respiratory Research, N. (2015) Pulmonary gas exchange abnormalities in mild chronic obstructive pulmonary disease. Implications for dyspnea and exercise intolerance. *Am J Respir Crit Care Med* 191(12):1384-1394

Eltzschig, H. K., Bratton, D. L. and Colgan, S. P. (2014) Targeting hypoxia signalling for the treatment of ischaemic and inflammatory diseases. *Nat Rev Drug Discov* 13(11):852-869

Eltzschig, H. K. and Carmeliet, P. (2011) Hypoxia and inflammation. *N Engl J Med* 364(7):656-665

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J. and Ratcliffe, P. J. (2001) C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107(1):43-54

Ericsson, P. (1970) The effect of iron supplementation on the physical work capacity in the elderly. *Acta Med Scand* 188(5):361-374

Erslev, A. (1953) Humoral regulation of red cell production. *Blood* 8(4):349-357

Erslev, A. J. and Caro, J. (1987) Erythropoietin titers in response to anemia or hypoxia. *Blood Cells* 13(1-2):207-216

Erslev, A. J., Caro, J. and Besarab, A. (1985) Why the kidney? *Nephron* 41(3):213-216

Evans, A. M., Mustard, K. J., Wyatt, C. N., Peers, C., Dipp, M., Kumar, P., Kinnear, N. P. and Hardie, D. G. (2005) Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O2-sensing cells? *J Biol Chem* 280(50):41504-41511

Evstatiev, R., Bukaty, A., Jimenez, K., Kulnigg-Dabsch, S., Surman, L., Schmid, W., Eferl, R., Lippert, K., Scheiber-Mojdehkar, B., Kvasnicka, H. M., Khare, V. and Gasche, C. (2014) Iron deficiency alters megakaryopoiesis and platelet phenotype independent of thrombopoietin. *Am J Hematol* 89(5):524-529

Evstatiev, R. and Gasche, C. (2012) Iron sensing and signalling. *Gut* 61(6):933-952

Fabbri, L. M., Luppi, F., Beghe, B. and Rabe, K. F. (2008) Complex chronic comorbidities of COPD. *Eur Respir J* 31(1):204-212

Farmer, C. G. (1999) Evolution of the vertebrate cardio-pulmonary system. *Annu Rev Physiol* 61:573-592

Farrell, P. A., Wilmore, J. H., Coyle, E. F., Billing, J. E. and Costill, D. L. (1979) Plasma lactate accumulation and distance running performance. *Med Sci Sports* 11(4):338-344

Fatemian, M., Herigstad, M., Croft, Q. P., Formenti, F., Cardenas, R., Wheeler, C., Smith, T. G., Friedmannova, M., Dorrington, K. L. and Robbins, P. A. (2016) Determinants of ventilation and pulmonary artery pressure during early acclimatization to hypoxia in humans. *J Physiol* 594(5):1197-1213

Fatemian, M., Kim, D. Y., Poulin, M. J. and Robbins, P. A. (2001) Very mild exposure to hypoxia for 8 h can induce ventilatory acclimatization in humans. *Pflugers Arch* 441(6):840-843

Faura, J., Ramos, J., Reynafarje, C., English, E., Finne, P. and Finch, C. A. (1969) Effect of altitude on erythropoiesis. *Blood* 33(5):668-676

Fenn, W. O., Rahn, H. and Otis, A. B. (1946) A theoretical study of the composition of the alveolar air at altitude. *Am J Physiol* 146:637-653

Ferguson, B. J., Skikne, B. S., Simpson, K. M., Baynes, R. D. and Cook, J. D. (1992) Serum transferrin receptor distinguishes the anemia of chronic disease from iron deficiency anemia. *J Lab Clin Med* 119(4):385-390

Ferrari, M., Manea, L., Anton, K., Bruzzone, P., Meneghello, M., Zamboni, F., Purgato, L., Cazzoletti, L., Ferrari, P. and Testi, R. (2015) Anemia and hemoglobin serum levels are associated with exercise capacity and quality of life in chronic obstructive pulmonary disease. *BMC Pulm Med* 15:58

Fielding, J. and Zorab, P. A. (1964) Polycythaemia and iron deficiency in pulmonary "emphysema". *Lancet* 284(7354):284-286

Fijalkowska, I., Xu, W., Comhair, S. A., Janocha, A. J., Mavrakis, L. A., Krishnamachary, B., Zhen, L., Mao, T., Richter, A., Erzurum, S. C. and Tuder, R. M. (2010) Hypoxia inducible-factor1alpha regulates the metabolic shift of pulmonary hypertensive endothelial cells. *Am J Pathol* 176(3):1130-1138

Finberg, K. E. (2013) Regulation of systemic iron homeostasis. *Curr Opin Hematol* 20(3):208-214

Finch, C. A. (1959) Body iron exchange in man. *J Clin Invest* 38(2):392-396

Finch, C. A. (1994) Regulators of iron balance in humans. *Blood* 84(6):1697-1702

Finch, C. A., Bellotti, V., Stray, S., Lipschitz, D. A., Cook, J. D., Pippard, M. J. and Huebers, H. A. (1986) Plasma ferritin determination as a diagnostic tool. *West J Med* 145(5):657-663

Finch, C. A., Gollnick, P. D., Hlastala, M. P., Miller, L. R., Dillmann, E. and Mackler, B. (1979) Lactic acidosis as a result of iron deficiency. *J Clin Invest* 64(1):129-137

Finch, C. A. and Huebers, H. (1982) Perspectives in iron metabolism. *N Engl J Med* 306(25):1520-1528

Finch, C. A., Miller, L. R., Inamdar, A. R., Person, R., Seiler, K. and Mackler, B. (1976) Iron deficiency in the rat. Physiological and biochemical studies of muscle dysfunction. *J Clin Invest* 58(2):447-453

Fischer, R., Simmerlein, R., Huber, R. M., Schiffl, H. and Lang, S. M. (2007) Lung disease severity, chronic inflammation, iron deficiency, and erythropoietin response in adults with cystic fibrosis. *Pediatr Pulmonol* 42(12):1193-1197

Fishman, A. P. (1976a) Hypoxia on the pulmonary circulation. How and where it acts. *Circ Res* 38(4):221-231

Fishman, A. P. (1976b) State of the art: chronic cor pulmonale. *Am Rev Respir Dis* 114(4):775-794

FitzGerald, M. P. (1913) The changes in the breathing and the blood at various high altitudes. *Philos Trans R Soc Lond B Biol Sci* 203(294-302):351-371

Flamme, I., Oehme, F., Ellinghaus, P., Jeske, M., Keldenich, J. and Thuss, U. (2014) Mimicking hypoxia to treat anemia: HIF-stabilizer BAY 85-3934 (Molidustat) stimulates erythropoietin production without hypertensive effects. *PLoS One* 9(11):e111838

Fleming, R. E. and Ponka, P. (2012) Iron overload in human disease. *N Engl J Med* 366(4):348-359

Flenley, D. C., Franklin, D. H. and Millar, J. S. (1970) The hypoxic drive to breathing in chronic bronchitis and emphysema. *Clin Sci* 38(4):503-518

Fletcher, C. M., Gilson, J. G., Hugh-Jones, P. and Scadding, J. G. (1959) Terminology, definitions, and classification of chronic pulmonary emphysema and related conditions. A report of the conclusions of a CIBA guest symposium. *Thorax* 14(4):286-299

Formenti, F., Beer, P. A., Croft, Q. P., Dorrington, K. L., Gale, D. P., Lappin, T. R., Lucas, G. S., Maher, E. R., Maxwell, P. H., McMullin, M. F., O'Connor, D. F., Percy, M. J., Pugh, C. W., Ratcliffe, P. J., Smith, T. G., Talbot, N. P. and Robbins, P. A. (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 J* 25(6):2001-2011

Formenti, F., Constantin-Teodosiu, D., Emmanuel, Y., Cheeseman, J., Dorrington, K. L., Edwards, L. M., Humphreys, S. M., Lappin, T. R., McMullin, M. F., McNamara, C. J., Mills, W., Murphy, J. A., O'Connor, D. F., Percy, M. J., Ratcliffe, P. J., Smith, T. G., Treacy, M., Frayn, K. N., Greenhaff, P. L., Karpe, F., Clarke, K. and Robbins, P. A. (2010) Regulation of human metabolism by hypoxia-inducible factor. *Proc Natl Acad Sci U S A* 107(28):12722-12727

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 16(9):4604-4613

Franssen, F. M., O'Donnell, D. E., Goossens, G. H., Blaak, E. E. and Schols, A. M. (2008) Obesity and the lung: 5. Obesity and COPD. *Thorax* 63(12):1110-1117

Gaitanos, G. C., Williams, C., Boobis, L. H. and Brooks, S. (1993) Human muscle metabolism during intermittent maximal exercise. *J Appl Physiol (1985)* 75(2):712-719

Gallo, R. C., Fraimow, W., Cathcart, R. T. and Erslev, A. J. (1964) Erythropoietic response in chronic pulmonary disease. *Arch Intern Med* 113:559-568

Galvin, I., Drummond, G. B. and Nirmalan, M. (2007) Distribution of blood flow and ventilation in the lung: gravity is not the only factor. *Br J Anaesth* 98(4):420-428

Galy, B., Ferring, D., Minana, B., Bell, O., Janser, H. G., Muckenthaler, M., Schumann, K. and Hentze, M. W. (2005) Altered body iron distribution and microcytosis in mice deficient in iron regulatory protein 2 (IRP2). *Blood* 106(7):2580-2589

Gammella, E., Diaz, V., Recalcati, S., Buratti, P., Samaja, M., Dey, S., Noguchi, C. T., Gassmann, M. and Cairo, G. (2015) Erythropoietin's inhibiting impact on hepcidin expression occurs indirectly. *Am J Physiol Regul Integr Comp Physiol* 308(4):R330-335

Ganz, T. (2013) Systemic iron homeostasis. *Physiol Rev* 93(4):1721-1741

Ganz, T. and Nemeth, E. (2009) Iron sequestration and anemia of inflammation. *Semin Hematol* 46(4):387-393

Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Bassel-Duby, R. and Williams, R. S. (1998) Mice without myoglobin. *Nature* 395(6705):905-908

Gassmann, M. and Muckenthaler, M. U. (2015) Adaptation of iron requirement to hypoxic conditions at high altitude. *J Appl Physiol (1985)* 119(12):1432-1440

Ge, R. L., Chen, Q. H., Wang, L. H., Gen, D., Yang, P., Kubo, K., Fujimoto, K., Matsuzawa, Y., Yoshimura, K., Takeoka, M. and et al. (1994) Higher exercise performance and lower VO2max in Tibetan than Han residents at 4,700 m altitude. *J Appl Physiol (1985)* 77(2):684-691

Gea, J., Agusti, A. and Roca, J. (2013) Pathophysiology of muscle dysfunction in COPD. *J Appl Physiol (1985)* 114(9):1222-1234

Geisser, P. (2009) The pharmacology and safety profile of ferric carboxymaltose (Ferinject®: structure/ reactivity relationships of iron preparations. *Port J Nephrol Hypert* 23(1):11-36

Geisser, P. and Burckhardt, S. (2011) The pharmacokinetics and pharmacodynamics of iron preparations. *Pharmaceutics* 3(1):12-33

Gelb, A. F., Klein, E., Schiffman, P., Lugliani, R. and Aronstam, P. (1977) Ventilatory response and drive in acute and chronic obstructive pulmonary disease. *Am Rev Respir Dis* 116(1):9-16

Ghosh, M. C., Zhang, D. L., Jeong, S. Y., Kovtunovych, G., Ollivierre-Wilson, H., Noguchi, A., Tu, T., Senecal, T., Robinson, G., Crooks, D. R., Tong, W. H., Ramaswamy, K., Singh, A., Graham, B. B., Tuder, R. M., Yu, Z. X., Eckhaus, M., Lee, J., Springer, D. A. and Rouault, T. A. (2013) Deletion of iron regulatory protein 1 causes polycythemia and pulmonary hypertension in mice through translational derepression of HIF2alpha. *Cell Metab* 17(2):271-281

Gkouvatsos, K., Papanikolaou, G. and Pantopoulos, K. (2012) Regulation of iron transport and the role of transferrin. *Biochim Biophys Acta* 1820(3):188-202

Gleadle, J. M., Ebert, B. L., Firth, J. D. and Ratcliffe, P. J. (1995) Regulation of angiogenic growth factor expression by hypoxia, transition metals, and chelating agents. *Am J Physiol* 268(6 Pt 1):C1362-1368

Glenny, R. W., Bernard, S., Robertson, H. T. and Hlastala, M. P. (1999) Gravity is an important but secondary determinant of regional pulmonary blood flow in upright primates. *J Appl Physiol (1985)* 86(2):623-632

Goddard, A. F., James, M. W., McIntyre, A. S. and Scott, B. B. (2011) Guidelines for the management of iron deficiency anaemia. *Gut* 60(10):1309-1316

Goetze, O., Schmitt, J., Spliethoff, K., Theurl, I., Weiss, G., Swinkels, D. W., Tjalsma, H., Maggiorini, M., Krayenbuhl, P., Rau, M., Fruehauf, H., Wojtal, K. A., Mullhaupt, B., Fried, M., Gassmann, M., Lutz, T. and Geier, A. (2013) Adaptation of iron transport and metabolism to acute high-altitude hypoxia in mountaineers. *Hepatology* 58(6):2153-2162

Goldberg, M. A., Dunning, S. P. and Bunn, H. F. (1988) Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* 242(4884):1412-1415

Goldberg, M. A., Glass, G. A., Cunningham, J. M. and Bunn, H. F. (1987) The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc Natl Acad Sci U S A* 84(22):7972-7976

Golding, E. M., Teague, W. E., Jr. and Dobson, G. P. (1995) Adjustment of K' to varying pH and pMg for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria permitting quantitative bioenergetic assessment. *J Exp Biol* 198(Pt 8):1775-1782

Goldwasser, E., Jacobson, L. O., Fried, W. and Plzak, L. (1957) Mechanism of the erythropoietic effect of cobalt. *Science* 125(3257):1085-1086

Goldwasser, E., Jacobson, L. O., Fried, W. and Plzak, L. F. (1958) Studies on erythropoiesis. V. The effect of cobalt on the production of erythropoietin. *Blood* 13(1):55-60

Gonzalez, N. C. and Wood, J. G. (2010) Alveolar hypoxia-induced systemic inflammation: what low PO(2) does and does not do. *Adv Exp Med Biol* 662:27-32

Goodnough, L. T., Nemeth, E. and Ganz, T. (2010) Detection, evaluation, and management of iron-restricted erythropoiesis. *Blood* 116(23):4754-4761

Goodwin, M. L., Harris, J. E., Hernandez, A. and Gladden, L. B. (2007) Blood lactate measurements and analysis during exercise: a guide for clinicians. *J Diabetes Sci Technol* 1(4):558-569

Govus, A. D., Abbiss, C. R., Garvican-Lewis, L. A., Swinkels, D. W., Laarakkers, C. M., Gore, C. J. and Peeling, P. (2014) Acute hypoxic exercise does not alter post-exercise iron metabolism in moderately trained endurance athletes. *Eur J Appl Physiol* 114(10):2183-2191

Gowanlock, Z., Sriram, S., Martin, A., Xenocostas, A. and Lazo-Langner, A. (2016) Erythropoietin levels in elderly patients with anemia of unknown etiology. *PLoS One* 11(6):e0157279

Green, R., Charlton, R., Seftel, H., Bothwell, T., Mayet, F., Adams, B., Finch, C. and Layrisse, M. (1968) Body iron excretion in man: a collaborative study. *Am J Med* 45(3):336-353

Groves, B. M., Reeves, J. T., Sutton, J. R., Wagner, P. D., Cymerman, A., Malconian, M. K., Rock, P. B., Young, P. M. and Houston, C. S. (1987) Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *J Appl Physiol (1985)* 63(2):521-530

Gruber, M., Hu, C. J., Johnson, R. S., Brown, E. J., Keith, B. and Simon, M. C. (2007) Acute postnatal ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci U S A* 104(7):2301-2306

Gueorguieva, R. and Krystal, J. H. (2004) Move over ANOVA: progress in analyzing repeated-measures data and its reflection in papers published in the Archives of General Psychiatry. *Arch Gen Psychiatry* 61(3):310-317

Gunshin, H., Allerson, C. R., Polycarpou-Schwarz, M., Rofts, A., Rogers, J. T., Kishi, F., Hentze, M. W., Rouault, T. A., Andrews, N. C. and Hediger, M. A. (2001) Iron-dependent regulation of the divalent metal ion transporter. *FEBS Lett* 509(2):309-316

Gunshin, H., Mackenzie, B., Berger, U. V., Gunshin, Y., Romero, M. F., Boron, W. F., Nussberger, S., Gollan, J. L. and Hediger, M. A. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 388(6641):482-488

Guo, B., Phillips, J. D., Yu, Y. and Leibold, E. A. (1995) Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J Biol Chem* 270(37):21645-21651

Guo, J., Zheng, C., Xiao, Q., Gong, S., Zhao, Q., Wang, L., He, J., Yang, W., Shi, X., Sun, X. and Liu, J. (2015) Impact of anaemia on lung function and exercise capacity in patients with stable severe chronic obstructive pulmonary disease. *BMJ Open* 5(10):e008295

Guyatt, G. H. (1992) Transferrin as a predictor of iron deficiency. *Am J Med* 92(4):453

Guyatt, G. H., Oxman, A. D., Ali, M., Willan, A., McIlroy, W. and Patterson, C. (1992) Laboratory diagnosis of iron-deficiency anemia: an overview. *J Gen Intern Med* 7(2):145-153

Haack, M., Kraus, T., Schuld, A., Dalal, M., Koethe, D. and Pollmacher, T. (2002) Diurnal variations of interleukin-6 plasma levels are confounded by blood drawing procedures. *Psychoneuroendocrinology* 27(8):921-931

Hakim, T. S., Michel, R. P. and Chang, H. K. (1982) Partitioning of pulmonary vascular resistance in dogs by arterial and venous occlusion. *J Appl Physiol Respir Environ Exerc Physiol* 52(3):710-715

Haldane, J. S. (1922). *Respiration.* New Haven, CT, Yale University Press.

Halpin, D. M., Decramer, M., Celli, B., Kesten, S., Liu, D. and Tashkin, D. P. (2012) Exacerbation frequency and course of COPD. *Int J Chron Obstruct Pulmon Dis* 7:653-661

Hammond, H. K., White, F. C., Bhargava, V. and Shabetai, R. (1992) Heart size and maximal cardiac output are limited by the pericardium. *Am J Physiol* 263(6 Pt 2):H1675-1681

Hampl, V., Bibova, J., Stranak, Z., Wu, X., Michelakis, E. D., Hashimoto, K. and Archer, S. L. (2002) Hypoxic fetoplacental vasoconstriction in humans is mediated by potassium channel inhibition. *Am J Physiol Heart Circ Physiol* 283(6):H2440-2449

Hanson, E. S., Foot, L. M. and Leibold, E. A. (1999) Hypoxia post-translationally activates iron-regulatory protein 2. *J Biol Chem* 274(8):5047-5052

Harrison, P. M. and Arosio, P. (1996) The ferritins: molecular properties, iron storage function and cellular regulation. *Biochim Biophys Acta* 1275(3):161-203

Hauge, A. and Melmon, K. L. (1968) Role of histamine in hypoxic pulmonary hypertension in the rat. II. Depletion of histamine, serotonin, and catecholamines. *Circ Res* 22(3):385-392

Hawkins, R. C. (2007) Total iron binding capacity or transferrin concentration alone outperforms iron and saturation indices in predicting iron deficiency. *Clin Chim Acta* 380(1-2):203-207

Heistad, D. D., Armstrong, M. L. and Amundsen, S. (1986) Blood flow through vasa vasorum in arteries and veins: effects of luminal PO2. *Am J Physiol* 250(3 Pt 2):H434-442

Hentze, M. W., Caughman, S. W., Rouault, T. A., Barriocanal, J. G., Dancis, A., Harford, J. B. and Klausner, R. D. (1987) Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. *Science* 238(4833):1570-1573

Hentze, M. W., Muckenthaler, M. U., Galy, B. and Camaschella, C. (2010) Two to tango: regulation of Mammalian iron metabolism. *Cell* 142(1):24-38

Herigstad, M. and Robbins, P. A. (2009) Pulmonary vascular response to air-breathing exercise in humans following an 8-h exposure to hypoxia. *Respir Physiol Neurobiol* 169(1):11-15

Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W. and Schofield, C. J. (2002) Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* 277(29):26351-26355

Hickey, M. M., Richardson, T., Wang, T., Mosqueira, M., Arguiri, E., Yu, H., Yu, Q. C., Solomides, C. C., Morrisey, E. E., Khurana, T. S., Christofidou-Solomidou, M. and Simon, M. C. (2010) The von Hippel-Lindau Chuvash mutation promotes pulmonary hypertension and fibrosis in mice. *J Clin Invest* 120(3):827-839

Hilde, J. M., Skjorten, I., Hansteen, V., Melsom, M. N., Hisdal, J., Humerfelt, S. and Steine, K. (2013) Haemodynamic responses to exercise in patients with COPD. *Eur Respir J* 41(5):1031-1041

Hill, A. V., Long, C. N. H. and Lupton, H. (1924a) Muscular exercise, lactic Acid, and the supply and utilisation of oxygen - Parts I-III. *Proc R Soc Lond B Biol Sci* 96(679):438-475

Hill, A. V., Long, C. N. H. and Lupton, H. (1924b) Muscular exercise, lactic acid, and the supply and utilisation of oxygen - Parts IV - VI. *Proc R Soc Lond B Biol Sci* 97(681):84-138

Hinkle, P. C., Kumar, M. A., Resetar, A. and Harris, D. L. (1991) Mechanistic stoichiometry of mitochondrial oxidative phosphorylation. *Biochemistry* 30(14):3576-3582

Hodson, E. J., Nicholls, L. G., Turner, P. J., Llyr, R., Fielding, J. W., Douglas, G., Ratnayaka, I., Robbins, P. A., Pugh, C. W., Buckler, K. J., Ratcliffe, P. J. and Bishop, T. (2016) Regulation of ventilatory sensitivity and carotid body proliferation in hypoxia by the PHD2/HIF-2 pathway. *J Physiol* 594(5):1179-1195

Holt, J. H. and Branscomb, B. V. (1965) Hemodynamic responses to controlled 100% oxygen breathing in emphysema. *J Appl Physiol* 20(2):215-220

Hoo, Z. H. and Wildman, M. J. (2012) Intravenous iron among cystic fibrosis patients. *J Cyst Fibros* 11(6):560-562

Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R. and Cerretelli, P. (1990) Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int J Sports Med* 11 Suppl 1:S3-9

Hoppeler, H., Luthi, P., Claassen, H., Weibel, E. R. and Howald, H. (1973) The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. *Pflugers Arch* 344(3):217-232

Howard, L. S., Barson, R. A., Howse, B. P., McGill, T. R., McIntyre, M. E., O'Connor, D. F. and Robbins, P. A. (1995) Chamber for controlling end-tidal gas tensions over sustained periods in humans. *J Appl Physiol (1985)* 78(3):1088-1091

Howard, L. S., Watson, G. M., Wharton, J., Rhodes, C. J., Chan, K., Khengar, R., Robbins, P. A., Kiely, D. G., Condliffe, R., Elliott, C. A., Pepke-Zaba, J., Sheares, K., Morrell, N. W., Davies, R., Ashby, D., Gibbs, J. S. and Wilkins, M. R. (2013) Supplementation of iron in pulmonary hypertension: Rationale and design of a phase II clinical trial in idiopathic pulmonary arterial hypertension. *Pulm Circ* 3(1):100-107

Hsia, C. C., Hyde, D. M. and Weibel, E. R. (2016) Lung structure and the intrinsic challenges of gas exchange. *Compr Physiol* 6(2):827-895

Hsu, A. R., Barnholt, K. E., Grundmann, N. K., Lin, J. H., McCallum, S. W. and Friedlander, A. L. (2006) Sildenafil improves cardiac output and exercise performance during acute hypoxia, but not normoxia. *J Appl Physiol (1985)* 100(6):2031-2040

Hu, J., Discher, D. J., Bishopric, N. H. and Webster, K. A. (1998) Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. *Biochem Biophys Res Commun* 245(3):894-899

Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 271(50):32253-32259

Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 95(14):7987-7992

Hughes, D. A., Stuart-Smith, S. E. and Bain, B. J. (2004) How should stainable iron in bone marrow films be assessed? *J Clin Pathol* 57(10):1038-1040

Hunt, J. M., Copland, J., McDonald, C. F., Barter, C. E., Rochford, P. D. and Pierce, R. J. (1989) Cardiopulmonary response to oxygen therapy in hypoxaemic chronic airflow obstruction. *Thorax* 44(11):930-936

Hurst, J. R., Vestbo, J., Anzueto, A., Locantore, N., Mullerova, H., Tal-Singer, R., Miller, B., Lomas, D. A., Agusti, A., Macnee, W., Calverley, P., Rennard, S., Wouters, E. F. and Wedzicha, J. A. (2010) Susceptibility to exacerbation in chronic obstructive pulmonary disease. *N Engl J Med* 363(12):1128-1138

Hurtado, A., Merino, C. and Delgado, E. (1945) Influence of anoxemia on the hemopoietic activity. *Arch Intern Med (Chic)* 75(5):284-323

Hutton, J. J., Jr., Trappel, A. L. and Udenfriend, S. (1966) Requirements for alpha-ketoglutarate, ferrous ion and ascorbate by collagen proline hydroxylase. *Biochem Biophys Res Commun* 24(2):179-184

Infusino, I., Braga, F., Dolci, A. and Panteghini, M. (2012) Soluble transferrin receptor (sTfR) and sTfR/log ferritin index for the diagnosis of iron-deficiency anemia. A meta-analysis. *Am J Clin Pathol* 138(5):642-649

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 292(5516):464-468

Iwai, K., Klausner, R. D. and Rouault, T. A. (1995) Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. *EMBO J* 14(21):5350-5357

Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 292(5516):468-472

Jacobs, A., Miller, F., Worwood, M., Beamish, M. R. and Wardrop, C. A. (1972) Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. *Br Med J* 4(5834):206-208

Jacobs, A., Waters, W. E., Campbell, H. and Barrow, A. (1969) A random sample from Wales. 3. Serum iron, iron binding capacity and transferrin saturation. *Br J Haematol* 17(6):581-587

Jacobson, L. O., Goldwasser, E., Fried, W. and Plzak, L. (1957) Role of the kidney in erythropoiesis. *Nature* 179(4560):633-634

Jafri, S., Sivasothy, P., Wells, F. and Morrell, N. W. (2011) Clinical demonstration of efficiency and reversibility of hypoxic pulmonary vasoconstriction in a patient presenting with unilateral incomplete bronchial occlusion. *Pulm Circ* 1(1):119-121

Jakobsson, P. and Jorfeldt, L. (1995) Long-term oxygen therapy may improve skeletal muscle metabolism in advanced chronic obstructive pulmonary disease patients with chronic hypoxaemia. *Respir Med* 89(7):471-476

Jankowska, E. A., Kasztura, M., Sokolski, M., Bronisz, M., Nawrocka, S., Oleskowska-Florek, W., Zymlinski, R., Biegus, J., Siwolowski, P., Banasiak, W., Anker, S. D., Filippatos, G., Cleland, J. G. and 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. *Eur Heart J* 35(36):2468-2476

Jasmin, G. and Solymoss, B. (1975) Polycythemia induced in rats by intrarenal injection of nickel sulfide Ni3S2. *Proc Soc Exp Biol Med* 148(3):774-776

Jeneson, J. A., Wiseman, R. W. and Kushmerick, M. J. (1997) Non-invasive quantitative 31P MRS assay of mitochondrial function in skeletal muscle in situ. *Mol Cell Biochem* 174(1-2):17-22

Jeneson, J. A., Wiseman, R. W., Westerhoff, H. V. and Kushmerick, M. J. (1996) The signal transduction function for oxidative phosphorylation is at least second order in ADP. *J Biol Chem* 271(45):27995-27998

Jensen, F. B. (2004) Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O2 and CO2 transport. *Acta Physiol Scand* 182(3):215-227

Jernudd-Wilhelmsson, Y., Hornblad, Y. and Hedenstierna, G. (1986) Ventilation-perfusion relationships in interstitial lung disease. *Eur J Respir Dis* 68(1):39-49

Jiang, B. H., Rue, E., Wang, G. L., Roe, R. and Semenza, G. L. (1996a) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* 271(30):17771-17778

Jiang, B. H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996b) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am J Physiol* 271(4 Pt 1):C1172-1180

Jobic, Y., Slama, M., Tribouilloy, C., Lan Cheong Wah, L., Choquet, D., Boschat, J., Penther, P. and Lesbre, J. P. (1993) Doppler echocardiographic evaluation of valve regurgitation in healthy volunteers. *Br Heart J* 69(2):109-113

Johns, M. W. (1991) A new method for measuring daytime sleepiness: the Epworth sleepiness scale. *Sleep* 14(6):540-545

Johnson, R. L., Jr., Spicer, W. S., Bishop, J. M. and Forster, R. E. (1960) Pulmonary capillary blood volume, flow and diffusing capacity during exercise. *J Appl Physiol* 15:893-902

Jomova, K. and Valko, M. (2011) Advances in metal-induced oxidative stress and human disease. *Toxicology* 283(2-3):65-87

Jones, P. W., Quirk, F. H., Baveystock, C. M. and Littlejohns, P. (1992) A self-complete measure of health status for chronic airflow limitation. The St. George's Respiratory Questionnaire. *Am Rev Respir Dis* 145(6):1321-1327

Jourdanet, D. (1863). *De l'anemie des altitudes et de l'anemie en general dans les rapports avec la pression de l'atmosphere.* Paris, JB Balliere et fils [French].

Julius, D. and Nathans, J. (2012) Signaling by sensory receptors. *Cold Spring Harb Perspect Biol* 4(1):a005991

Kaelin, W. G., Jr. and Ratcliffe, P. J. (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell* 30(4):393-402

Kapitsinou, P. P., Liu, Q., Unger, T. L., Rha, J., Davidoff, O., Keith, B., Epstein, J. A., Moores, S. L., Erickson-Miller, C. L. and Haase, V. H. (2010) Hepatic HIF-2 regulates erythropoietic responses to hypoxia in renal anemia. *Blood* 116(16):3039-3048

Kapitsinou, P. P., Rajendran, G., Astleford, L., Michael, M., Schonfeld, M. P., Fields, T., Shay, S., French, J. L., West, J. and Haase, V. H. (2016) The endothelial prolyl-4-hydroxylase domain 2/hypoxia-inducible factor 2 axis regulates pulmonary artery pressure in mice. *Mol Cell Biol* 36(10):1584-1594

Karadag, F., Cildag, O., Altinisik, M., Kozaci, L. D., Kiter, G. and Altun, C. (2004) Trace elements as a component of oxidative stress in COPD. *Respirology* 9(1):33-37

Karul, A. B., Karadag, F., Yensel, N., Altinisik, M., Altun, C. and Cildag, O. (2003) Should chronic obstructive pulmonary disease outpatients be routinely evaluated for trace elements? *Biol Trace Elem Res* 94(1):41-48

Kato, M. and Staub, N. C. (1966) Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. *Circ Res* 19(2):426-440

Kautz, L., Jung, G., Valore, E. V., Rivella, S., Nemeth, E. and Ganz, T. (2014) Identification of erythroferrone as an erythroid regulator of iron metabolism. *Nat Genet* 46(7):678-684

Kawabata, H., Yang, R., Hirama, T., Vuong, P. T., Kawano, S., Gombart, A. F. and Koeffler, H. P. (1999) Molecular cloning of transferrin receptor 2. A new member of the transferrin receptor-like family. *J Biol Chem* 274(30):20826-20832

Kawakami, Y., Kishi, F., Yamamoto, H. and Miyamoto, K. (1983) Relation of oxygen delivery, mixed venous oxygenation, and pulmonary hemodynamics to prognosis in chronic obstructive pulmonary disease. *N Engl J Med* 308(18):1045-1049

Kell, D. B. (2009) Iron behaving badly: inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. *BMC Med Genomics* 2:2

Kemna, E. H., Kartikasari, A. E., van Tits, L. J., Pickkers, P., Tjalsma, H. and Swinkels, D. W. (2008) Regulation of hepcidin: insights from biochemical analyses on human serum samples. *Blood Cells Mol Dis* 40(3):339-346

Kemp, G. J., Ahmad, R. E., Nicolay, K. and Prompers, J. J. (2015) Quantification of skeletal muscle mitochondrial function by 31P magnetic resonance spectroscopy techniques: a quantitative review. *Acta Physiol (Oxf)* 213(1):107-144

Kemp, G. J., Meyerspeer, M. and Moser, E. (2007) Absolute quantification of phosphorus metabolite concentrations in human muscle in vivo by 31P MRS: a quantitative review. *NMR Biomed* 20(6):555-565

Kemp, G. J. and Radda, G. K. (1994) Quantitative interpretation of bioenergetic data from 31P and 1H magnetic resonance spectroscopic studies of skeletal muscle: an analytical review. *Magn Reson Q* 10(1):43-63

Kent, B. D., Mitchell, P. D. and McNicholas, W. T. (2011) Hypoxemia in patients with COPD: cause, effects, and disease progression. *Int J Chron Obstruct Pulmon Dis* 6:199-208

Kerbaul, F., Van der Linden, P., Pierre, S., Rondelet, B., Melot, C., Brimioulle, S. and Naeije, R. (2004) Prevention of hemodilution-induced inhibition of hypoxic pulmonary vasoconstriction by N-acetylcysteine in dogs. *Anesth Analg* 99(2):547-551

Khalid, S., McGrowder, D., Kemp, M. and Johnson, P. (2007) The use of soluble transferrin receptor to assess iron deficiency in adults with cystic fibrosis. *Clin Chim Acta* 378(1-2):194-200

Khushu, S., Rana, P., Sekhri, T., Sripathy, G. and Tripathi, R. P. (2010) Bio-energetic impairment in human calf muscle in thyroid disorders: a 31P MRS study. *Magn Reson Imaging* 28(5):683-689

Kim, J. W., Tchernyshyov, I., Semenza, G. L. and Dang, C. V. (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 3(3):177-185

Kjaergaard, J., Akkan, D., Iversen, K. K., Kjoller, E., Kober, L., Torp-Pedersen, C. and Hassager, C. (2007) Prognostic importance of pulmonary hypertension in patients with heart failure. *Am J Cardiol* 99(8):1146-1150

Kling, P. J., Dragsten, P. R., Roberts, R. A., Dos Santos, B., Brooks, D. J., Hedlund, B. E. and Taetle, R. (1996) Iron deprivation increases erythropoietin production in vitro, in normal subjects and patients with malignancy. *Br J Haematol* 95(2):241-248

Klip, I. T., Comin-Colet, J., Voors, A. A., Ponikowski, P., Enjuanes, C., Banasiak, W., Lok, D. J., Rosentryt, P., Torrens, A., Polonski, L., van Veldhuisen, D. J., van der Meer, P. and Jankowska, E. A. (2013) Iron deficiency in chronic heart failure: an international pooled analysis. *Am Heart J* 165(4):575-582

Knovich, M. A., Storey, J. A., Coffman, L. G., Torti, S. V. and Torti, F. M. (2009) Ferritin for the clinician. *Blood Rev* 23(3):95-104

Knowles, H. J., Mole, D. R., Ratcliffe, P. J. and Harris, A. L. (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 Res* 66(5):2600-2607

Koulaouzidis, A., Said, E., Cottier, R. and Saeed, A. A. (2009) Soluble transferrin receptors and iron deficiency, a step beyond ferritin. A systematic review. *J Gastrointestin Liver Dis* 18(3):345-352

Krause, A., Neitz, S., Magert, H. J., Schulz, A., Forssmann, W. G., Schulz-Knappe, P. and Adermann, K. (2000) LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Lett* 480(2-3):147-150

Krause, J. R. and Stolc, V. (1979) Serum ferritin and bone marrow iron stores. I. Correlation with absence of iron in biopsy specimens. *Am J Clin Pathol* 72(5):817-820

Kroot, J. J., Hendriks, J. C., Laarakkers, C. M., Klaver, S. M., Kemna, E. H., Tjalsma, H. and Swinkels, D. W. (2009) (Pre)analytical imprecision, between-subject variability, and daily variations in serum and urine hepcidin: implications for clinical studies. *Anal Biochem* 389(2):124-129

Krumdieck, N. (1943) Erythropoietic substance in the serum of anemic animals. *Exp Biol Med* 54(1):14-17

Kumar, H. and Choi, D. K. (2015) Hypoxia inducible factor pathway and physiological adaptation: a cell survival pathway? *Mediators Inflamm* 2015:584758

Kumar, P. and Prabhakar, N. R. (2012) Peripheral chemoreceptors: function and plasticity of the carotid body. *Compr Physiol* 2(1):141-219

Kurtz, A., Jelkmann, W., Sinowatz, F. and Bauer, C. (1983) Renal mesangial cell cultures as a model for study of erythropoietin production. *Proc Natl Acad Sci U S A* 80(13):4008-4011

Kushmerick, M. J. (1995) Skeletal muscle: a paradigm for testing principles of bioenergetics. *J Bioenerg Biomembr* 27(6):555-569

Ladroue, C., Carcenac, R., Leporrier, M., Gad, S., Le Hello, C., Galateau-Salle, F., Feunteun, J., Pouyssegur, J., Richard, S. and Gardie, B. (2008) PHD2 mutation and congenital erythrocytosis with paraganglioma. *N Engl J Med* 359(25):2685-2692

Lam, C. S., Borlaug, B. A., Kane, G. C., Enders, F. T., Rodeheffer, R. J. and Redfield, M. M. (2009) Age-associated increases in pulmonary artery systolic pressure in the general population. *Circulation* 119(20):2663-2670

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002a) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* 16(12):1466-1471

Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L. (2002b) Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295(5556):858-861

Lane, D. J. and Howell, J. B. (1970) Relationship between sensitivity to carbon dioxide and clinical features in patients with chronic airways obstruction. *Thorax* 25(2):150-159

Lanikova, L., Lorenzo, F., Yang, C., Vankayalapati, H., Drachtman, R., Divoky, V. and Prchal, J. T. (2013) Novel homozygous VHL mutation in exon 2 is associated with congenital polycythemia but not with cancer. *Blood* 121(19):3918-3924

Laufberger, V. (1937) Sur la cristallisation de la ferritine. *Bull Soc chim biol* 19:1575-1582 [French].

Lee, A. S., Patterson, R. W. and Kaufman, R. D. (1987) Relationships among ventilation-perfusion distribution, multiple inert gas methodology and metabolic blood-gas tensions. *Br J Anaesth* 59(12):1579-1598

Lee, F. S. and Percy, M. J. (2011) The HIF pathway and erythrocytosis. *Annu Rev Pathol* 6:165-192

Lee, J. H., Cho, M. H., Hersh, C. P., McDonald, M. L., Wells, J. M., Dransfield, M. T., Bowler, R. P., Lynch, D. A., Lomas, D. A., Crapo, J. D., Silverman, E. K., Copdgene and Investigators, E. (2015) IREB2 and GALC are associated with pulmonary artery enlargement in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 52(3):365-376

Lee, J. H., Oh, Y. M., Seo, J. B., Lee, Y. K., Kim, W. J., Sheen, S. S., Kim, T. H., Lee, J. H., Kim, E. K., Lee, J. S., Huh, J. W., Lim, S. Y., Yoon, H. I., Shin, T. R., Lee, S. M., Lee, S. Y. and Lee, S. D. (2011) Pulmonary artery pressure in chronic obstructive pulmonary disease without resting hypoxaemia. *Int J Tuberc Lung Dis* 15(6):830-837

Lemon, W. S. (1929) A study of the effect of chronic pulmonary diseases on the volume and composition of the blood. *Ann Intern Med* 3(5):430-446

Leverve, X. M. (1999) Energy metabolism in critically ill patients: lactate is a major oxidizable substrate. *Curr Opin Clin Nutr Metab Care* 2(2):165-169

Leverve, X. M. (2007) Mitochondrial function and substrate availability. *Crit Care Med* 35(9 Suppl):S454-460

Levett, D. Z. and Grocott, M. P. (2015) Cardiopulmonary exercise testing for risk prediction in major abdominal surgery. *Anesthesiol Clin* 33(1):1-16

Levine, B. E., Bigelow, D. B., Hamstra, R. D., Beckwitt, H. J., Mitchell, R. S., Nett, L. M., Stephen, T. A. and Petty, T. L. (1967) The role of long-term continuous oxygen administration in patients with chronic airway obstruction with hypoxemia. *Ann Intern Med* 66(4):639-650

Liem, K. F. (1988) Form and function of lungs - the evolution of air breathing mechanisms. *Am Zool* 28(2):739-759

Lin, L., Valore, E. V., Nemeth, E., Goodnough, J. B., Gabayan, V. and Ganz, T. (2007) Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. *Blood* 110(6):2182-2189

Lindholm, M. E., Fischer, H., Poellinger, L., Johnson, R. S., Gustafsson, T., Sundberg, C. J. and Rundqvist, H. (2014) Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 307(3):R248-255

Lipschitz, D. A., Cook, J. D. and Finch, C. A. (1974) A clinical evaluation of serum ferritin as an index of iron stores. *N Engl J Med* 290(22):1213-1216

Liu, Q., Davidoff, O., Niss, K. and Haase, V. H. (2012) Hypoxia-inducible factor regulates hepcidin via erythropoietin-induced erythropoiesis. *J Clin Invest* 122(12):4635-4644

Liu, Q., Sham, J. S., Shimoda, L. A. and Sylvester, J. T. (2001) Hypoxic constriction of porcine distal pulmonary arteries: endothelium and endothelin dependence. *Am J Physiol Lung Cell Mol Physiol* 280(5):L856-865

Liu, Y., Yu, Z., Hua, D., Chen, Y., Zheng, S. and Wang, L. (2015) Association of serum hepcidin levels with the presence and severity of obstructive sleep apnea syndrome. *Med Sci Monit* 21:27-31

Loenarz, C. and Schofield, C. J. (2008) Expanding chemical biology of 2-oxoglutarate oxygenases. *Nat Chem Biol* 4(3):152-156

Lopez-Barneo, J., Lopez-Lopez, J. R., Urena, J. and Gonzalez, C. (1988) Chemotransduction in the carotid body: K+ current modulated by PO2 in type I chemoreceptor cells. *Science* 241(4865):580-582

Lorenzo, F. R., Huff, C., Myllymaki, M., Olenchock, B., Swierczek, S., Tashi, T., Gordeuk, V., Wuren, T., Ri-Li, G., McClain, D. A., Khan, T. M., Koul, P. A., Guchhait, P., Salama, M. E., Xing, J., Semenza, G. L., Liberzon, E., Wilson, A., Simonson, T. S., Jorde, L. B., Kaelin, W. G., Jr., Koivunen, P. and Prchal, J. T. (2014) A genetic mechanism for Tibetan high-altitude adaptation. *Nat Genet* 46(9):951-956

Lorenzo, F. R., Yang, C., Ng Tang Fui, M., Vankayalapati, H., Zhuang, Z., Huynh, T., Grossmann, M., Pacak, K. and Prchal, J. T. (2013) A novel EPAS1/HIF2A germline mutation in a congenital polycythemia with paraganglioma. *J Mol Med (Berl)* 91(4):507-512

Lumb, A. B. and Slinger, P. (2015) Hypoxic pulmonary vasoconstriction: physiology and anesthetic implications. *Anesthesiology* 122(4):932-946

Lundby, A. K., Keiser, S., Siebenmann, C., Schaffer, L. and Lundby, C. (2014) Kidney-synthesized erythropoietin is the main source for the hypoxia-induced increase in plasma erythropoietin in adult humans. *Eur J Appl Physiol* 114(6):1107-1111

Lundgrin, E. L., Janocha, A. J., Koch, C. D., Gebremedhin, A., Di Rienzo, A., Alkorta-Aranburu, G., Brittenham, G. M., Erzurum, S. C. and Beall, C. M. (2013) Plasma hepcidin of Ethiopian highlanders with steady-state hypoxia. *Blood* 122(11):1989-1991

Lymboussaki, A., Pignatti, E., Montosi, G., Garuti, C., Haile, D. J. and Pietrangelo, A. (2003) The role of the iron responsive element in the control of ferroportin1/IREG1/MTP1 gene expression. *J Hepatol* 39(5):710-715

Lynch, D. A., Austin, J. H., Hogg, J. C., Grenier, P. A., Kauczor, H. U., Bankier, A. A., Barr, R. G., Colby, T. V., Galvin, J. R., Gevenois, P. A., Coxson, H. O., Hoffman, E. A., Newell, J. D., Jr., Pistolesi, M., Silverman, E. K. and Crapo, J. D. (2015) CT-definable subtypes of chronic obstructive pulmonary disease: a statement of the Fleischner Society. *Radiology* 277(1):192-205

Lyseng-Williamson, K. A. and Keating, G. M. (2009) Ferric carboxymaltose: a review of its use in iron-deficiency anaemia. *Drugs* 69(6):739-756

Ma, Y., Mazumdar, M. and Memtsoudis, S. G. (2012) Beyond repeated-measures analysis of variance: advanced statistical methods for the analysis of longitudinal data in anesthesia research. *Reg Anesth Pain Med* 37(1):99-105

Macdougall, I. C., Hutton, R. D., Cavill, I., Coles, G. A. and Williams, J. D. (1989) Poor response to treatment of renal anaemia with erythropoietin corrected by iron given intravenously. *BMJ* 299(6692):157-158

Madden, J. A., Vadula, M. S. and Kurup, V. P. (1992) Effects of hypoxia and other vasoactive agents on pulmonary and cerebral artery smooth muscle cells. *Am J Physiol* 263(3 Pt 1):L384-393

Mahon, P. C., Hirota, K. and Semenza, G. L. (2001) FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* 15(20):2675-2686

Maltais, F., Decramer, M., Casaburi, R., Barreiro, E., Burelle, Y., Debigare, R., Dekhuijzen, P. N., Franssen, F., Gayan-Ramirez, G., Gea, J., Gosker, H. R., Gosselink, R., Hayot, M., Hussain, S. N., Janssens, W., Polkey, M. I., Roca, J., Saey, D., Schols, A. M., Spruit, M. A., Steiner, M., Taivassalo, T., Troosters, T., Vogiatzis, I. and Wagner, P. D. (2014) An official American Thoracic Society/European Respiratory Society statement: update on limb muscle dysfunction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 189(9):e15-62

Manier, G., Guenard, H., Castaing, Y., Varene, N. and Vargas, E. (1988) Pulmonary gas exchange in Andean natives with excessive polycythemia - effect of hemodilution. *J Appl Physiol (1985)* 65(5):2107-2117

Mansfield, K. D., Guzy, R. D., Pan, Y., Young, R. M., Cash, T. P., Schumacker, P. T. and Simon, M. C. (2005) Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. *Cell Metab* 1(6):393-399

Margaria, R., Cerretelli, P. and Mangili, F. (1964) Balance and kinetics of anaerobic energy release during strenuous exercise in Man. *J Appl Physiol* 19:623-628

Markolovic, S., Wilkins, S. E. and Schofield, C. J. (2015) Protein Hydroxylation Catalyzed by 2-Oxoglutarate-dependent Oxygenases. *J Biol Chem* 290(34):20712-20722

Marshall, B. E., Clarke, W. R., Costarino, A. T., Chen, L., Miller, F. and Marshall, C. (1994a) The dose-response relationship for hypoxic pulmonary vasoconstriction. *Respir Physiol* 96(2-3):231-247

Marshall, B. E., Hanson, C. W., Frasch, F. and Marshall, C. (1994b) Role of hypoxic pulmonary vasoconstriction in pulmonary gas exchange and blood flow distribution. 2. Pathophysiology. *Intensive Care Med* 20(5):379-389

Marshall, B. E. and Marshall, C. (1988) A model for hypoxic constriction of the pulmonary circulation. *J Appl Physiol (1985)* 64(1):68-77

Marshall, B. E., Marshall, C., Frasch, F. and Hanson, C. W. (1994c) Role of hypoxic pulmonary vasoconstriction in pulmonary gas exchange and blood flow distribution. 1. Physiologic concepts. *Intensive Care Med* 20(4):291-297

Marshall, B. E., Marshall, C., Magno, M., Lilagan, P. and Pietra, G. G. (1991) Influence of bronchial arterial PO2 on pulmonary vascular resistance. *J Appl Physiol (1985)* 70(1):405-415

Marshall, C. and Marshall, B. (1983) Site and sensitivity for stimulation of hypoxic pulmonary vasoconstriction. *J Appl Physiol Respir Environ Exerc Physiol* 55(3):711-716

Martinez-Rivera, C., Portillo, K., Munoz-Ferrer, A., Martinez-Ortiz, M. L., Molins, E., Serra, P., Ruiz-Manzano, J. and Morera, J. (2012) Anemia is a mortality predictor in hospitalized patients for COPD exacerbation. *COPD* 9(3):243-250

Martinez, F. J., Foster, G., Curtis, J. L., Criner, G., Weinmann, G., Fishman, A., DeCamp, M. M., Benditt, J., Sciurba, F., Make, B., Mohsenifar, Z., Diaz, P., Hoffman, E. and Wise, R. (2006) Predictors of mortality in patients with emphysema and severe airflow obstruction. *Am J Respir Crit Care Med* 173(12):1326-1334

Martinez, J. A., Guerra, C. C., Nery, L. E. and Jardim, J. R. (1997) Iron stores and coagulation parameters in patients with hypoxemic polycythemia secondary to chronic obstructive pulmonary disease: the effect of phlebotomies. *Sao Paulo Med J* 115(2):1395-1402

Martinez, S. and Hausinger, R. P. (2015) Catalytic Mechanisms of Fe(II)- and 2-Oxoglutarate-dependent Oxygenases. *J Biol Chem* 290(34):20702-20711

Mason, S. D., Howlett, R. A., Kim, M. J., Olfert, I. M., Hogan, M. C., McNulty, W., Hickey, R. P., Wagner, P. D., Kahn, C. R., Giordano, F. J. and Johnson, R. S. (2004) Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. *PLoS Biol* 2(10):e288

Mason, S. D., Rundqvist, H., Papandreou, I., Duh, R., McNulty, W. J., Howlett, R. A., Olfert, I. M., Sundberg, C. J., Denko, N. C., Poellinger, L. and Johnson, R. S. (2007) HIF-1alpha in endurance training: suppression of oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 293(5):R2059-2069

Mast, A. E., Blinder, M. A., Gronowski, A. M., Chumley, C. and Scott, M. G. (1998) Clinical utility of the soluble transferrin receptor and comparison with serum ferritin in several populations. *Clin Chem* 44(1):45-51

Maxwell, P. and Salnikow, K. (2004) HIF-1: an oxygen and metal responsive transcription factor. *Cancer Biol Ther* 3(1):29-35

Maxwell, P. H., Osmond, M. K., Pugh, C. W., Heryet, A., Nicholls, L. G., Tan, C. C., Doe, B. G., Ferguson, D. J., Johnson, M. H. and Ratcliffe, P. J. (1993a) Identification of the renal erythropoietin-producing cells using transgenic mice. *Kidney Int* 44(5):1149-1162

Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (1993b) Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. *Proc Natl Acad Sci U S A* 90(6):2423-2427

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399(6733):271-275

Mayr, R., Janecke, A. R., Schranz, M., Griffiths, W. J., Vogel, W., Pietrangelo, A. and Zoller, H. (2010) Ferroportin disease: a systematic meta-analysis of clinical and molecular findings. *J Hepatol* 53(5):941-949

McClain, D. A., Abuelgasim, K. A., Nouraie, M., Salomon-Andonie, J., Niu, X., Miasnikova, G., Polyakova, L. A., Sergueeva, A., Okhotin, D. J., Cherqaoui, R., Okhotin, D., Cox, J. E., Swierczek, S., Song, J., Simon, M. C., Huang, J., Simcox, J. A., Yoon, D., Prchal, J. T. and Gordeuk, V. R. (2013) Decreased serum glucose and glycosylated hemoglobin levels in patients with Chuvash polycythemia: a role for HIF in glucose metabolism. *J Mol Med (Berl)* 91(1):59-67

McDonough, M. A., Li, V., Flashman, E., Chowdhury, R., Mohr, C., Lienard, B. M., Zondlo, J., Oldham, N. J., Clifton, I. J., Lewis, J., McNeill, L. A., Kurzeja, R. J., Hewitson, K. S., Yang, E., Jordan, S., Syed, R. S. and Schofield, C. J. (2006) Cellular oxygen sensing: Crystal structure of hypoxia-inducible factor prolyl hydroxylase (PHD2). *Proc Natl Acad Sci U S A* 103(26):9814-9819

McFarlane, D. B., Pinkerton, P. H., Dagg, J. H. and Goldberg, A. (1967) Incidence of iron deficiency, with and without anaemia, in women in general practice. *Br J Haematol* 13(5):790-796

McGowan, J. P. and Crichton, A. (1923) On the effect of deficiency of iron in the diet of pigs: preliminary communication. *Biochem J* 17(2):204-207

McIntyre, R. C., Jr., Moore, F. A., Moore, E. E., Piedalue, F., Haenel, J. S. and Fullerton, D. A. (1995) Inhaled nitric oxide variably improves oxygenation and pulmonary hypertension in patients with acute respiratory distress syndrome. *J Trauma* 39(3):418-425

McKay, R. H., Higuchi, D. A., Winder, W. W., Fell, R. D. and Brown, E. B. (1983) Tissue effects of iron deficiency in the rat. *Biochim Biophys Acta* 757(3):352-358

McKeown, S. R. (2014) Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. *Br J Radiol* 87(1035):20130676

McLane, J. A., Fell, R. D., McKay, R. H., Winder, W. W., Brown, E. B. and Holloszy, J. O. (1981) Physiological and biochemical effects of iron deficiency on rat skeletal muscle. *Am J Physiol* 241(1):C47-54

McLaughlin, V. V., Archer, S. L., Badesch, D. B., Barst, R. J., Farber, H. W., Lindner, J. R., Mathier, M. A., McGoon, M. D., Park, M. H., Rosenson, R. S., Rubin, L. J., Tapson, V. F., Varga, J., Harrington, R. A., Anderson, J. L., Bates, E. R., Bridges, C. R., Eisenberg, M. J., Ferrari, V. A., Grines, C. L., Hlatky, M. A., Jacobs, A. K., Kaul, S., Lichtenberg, R. C., Lindner, J. R., Moliterno, D. J., Mukherjee, D., Pohost, G. M., Rosenson, R. S., Schofield, R. S., Shubrooks, S. J., Stein, J. H., Tracy, C. M., Weitz, H. H. and Wesley, D. J. (2009) ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. *Circulation* 119(16):2250-2294

McMullin, M. F. (2010) HIF pathway mutations and erythrocytosis. *Expert Rev Hematol* 3(1):93-101

McNeill, L. A., Flashman, E., Buck, M. R., Hewitson, K. S., Clifton, I. J., Jeschke, G., Claridge, T. D., Ehrismann, D., Oldham, N. J. and Schofield, C. J. (2005) Hypoxia-inducible factor prolyl hydroxylase 2 has a high affinity for ferrous iron and 2-oxoglutarate. *Mol Biosyst* 1(4):321-324

McNeill, L. A., Hewitson, K. S., Gleadle, J. M., Horsfall, L. E., Oldham, N. J., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J. and Schofield, C. J. (2002) The use of dioxygen by HIF prolyl hydroxylase (PHD1). *Bioorg Med Chem Lett* 12(12):1547-1550

McQuillan, B. M., Picard, M. H., Leavitt, M. and Weyman, A. E. (2001) Clinical correlates and reference intervals for pulmonary artery systolic pressure among echocardiographically normal subjects. *Circulation* 104(23):2797-2802

Medical Research Council (1981) Long term domiciliary oxygen therapy in chronic hypoxic cor pulmonale complicating chronic bronchitis and emphysema. Report of the Medical Research Council Working Party. *Lancet* 317(8222):681-686

Melot, C., Lejeune, P., Leeman, M., Moraine, J. J. and Naeije, R. (1989) Prostaglandin E1 in the adult respiratory distress syndrome. Benefit for pulmonary hypertension and cost for pulmonary gas exchange. *Am Rev Respir Dis* 139(1):106-110

Melot, C., Naeije, R., Mols, P., Hallemans, R., Lejeune, P. and Jaspar, N. (1987) Pulmonary vascular tone improves pulmonary gas exchange in the adult respiratory distress syndrome. *Am Rev Respir Dis* 136(5):1232-1236

Meyer, A., Zoll, J., Charles, A. L., Charloux, A., de Blay, F., Diemunsch, P., Sibilia, J., Piquard, F. and Geny, B. (2013) Skeletal muscle mitochondrial dysfunction during chronic obstructive pulmonary disease: central actor and therapeutic target. *Exp Physiol* 98(6):1063-1078

Meyer, R. A. (1988) A linear model of muscle respiration explains monoexponential phosphocreatine changes. *Am J Physiol* 254(4 Pt 1):C548-553

Meyron-Holtz, E. G., Ghosh, M. C., Iwai, K., LaVaute, T., Brazzolotto, X., Berger, U. V., Land, W., Ollivierre-Wilson, H., Grinberg, A., Love, P. and Rouault, T. A. (2004) Genetic ablations of iron regulatory proteins 1 and 2 reveal why iron regulatory protein 2 dominates iron homeostasis. *EMBO J* 23(2):386-395

Michelakis, E., Rebeyka, I., Bateson, J., Olley, P., Puttagunta, L. and Archer, S. (2000) Voltage-gated potassium channels in human ductus arteriosus. *Lancet* 356(9224):134-137

Michelakis, E. D., Rebeyka, I., Wu, X., Nsair, A., Thebaud, B., Hashimoto, K., Dyck, J. R., Haromy, A., Harry, G., Barr, A. and Archer, S. L. (2002) O2 sensing in the human ductus arteriosus: regulation of voltage-gated K+ channels in smooth muscle cells by a mitochondrial redox sensor. *Circ Res* 91(6):478-486

Mik, E. G. (2013) Special article: measuring mitochondrial oxygen tension: from basic principles to application in humans. *Anesth Analg* 117(4):834-846

Miles, M. P., Andring, J. M., Pearson, S. D., Gordon, L. K., Kasper, C., Depner, C. M. and Kidd, J. R. (2008) Diurnal variation, response to eccentric exercise, and association of inflammatory mediators with muscle damage variables. *J Appl Physiol (1985)* 104(2):451-458

Milledge, J. S. and Cotes, P. M. (1985) Serum erythropoietin in humans at high altitude and its relation to plasma renin. *J Appl Physiol (1985)* 59(2):360-364

Millet, G. P., Faiss, R. and Pialoux, V. (2012) Point: Hypobaric hypoxia induces different physiological responses from normobaric hypoxia. *J Appl Physiol (1985)* 112(10):1783-1784

Millikan, G. A. (1939) Muscle hemoglobin. *Physiol Rev* 19(4):503-523

Minai, O. A., Chaouat, A. and Adnot, S. (2010) Pulmonary hypertension in COPD: epidemiology, significance, and management: pulmonary vascular disease: the global perspective. *Chest* 137(6 Suppl):39S-51S

Minamishima, Y. A., Moslehi, J., Bardeesy, N., Cullen, D., Bronson, R. T. and Kaelin, W. G., Jr. (2008) Somatic inactivation of the PHD2 prolyl hydroxylase causes polycythemia and congestive heart failure. *Blood* 111(6):3236-3244

Minchenko, A. and Caro, J. (2000) Regulation of endothelin-1 gene expression in human microvascular endothelial cells by hypoxia and cobalt: role of hypoxia responsive element. *Mol Cell Biochem* 208(1-2):53-62

Miyake, T., Kung, C. K. and Goldwasser, E. (1977) Purification of human erythropoietin. *J Biol Chem* 252(15):5558-5564

Moore, R., Berlowitz, D., Pretto, J., Brazzale, D., Denehy, L., Jackson, B. and McDonald, C. (2009) Acute effects of hyperoxia on resting pattern of ventilation and dyspnoea in COPD. *Respirology* 14(4):545-550

Moore, R. A., Gaskell, H., Rose, P. and Allan, J. (2011) Meta-analysis of efficacy and safety of intravenous ferric carboxymaltose (Ferinject) from clinical trial reports and published trial data. *BMC Blood Disord* 11:4

Moretti, D., Goede, J. S., Zeder, C., Jiskra, M., Chatzinakou, V., Tjalsma, H., Melse-Boonstra, A., Brittenham, G., Swinkels, D. W. and Zimmermann, M. B. (2015) Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women. *Blood* 126(17):1981-1989

Morrell, N. W., Nijran, K. S., Biggs, T. and Seed, W. A. (1995) Magnitude and time course of acute hypoxic pulmonary vasoconstriction in man. *Respir Physiol* 100(3):271-281

Morse, E. E., Lee, T. Y., Reiss, R. F. and Sunderman, F. W., Jr. (1977) Dose-response and time-response study of erythrocytosis in rats after intrarenal injection of nickel subsulfide. *Ann Clin Lab Sci* 7(1):17-24

Moslehi, J., Minamishima, Y. A., Shi, J., Neuberg, D., Charytan, D. M., Padera, R. F., Signoretti, S., Liao, R. and Kaelin, W. G., Jr. (2010) Loss of hypoxia-inducible factor prolyl hydroxylase activity in cardiomyocytes phenocopies ischemic cardiomyopathy. *Circulation* 122(10):1004-1016

Motley, H. L., Cournand, A., Werko, L., Himmelstein, A. and Dresdale, D. (1947) The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. *Am J Physiol* 150(2):315-320

Murray-Kolb, L. E. (2011) Iron status and neuropsychological consequences in women of reproductive age: what do we know and where are we headed? *J Nutr* 141(4):747S-755S

Murray, T. R., Chen, L., Marshall, B. E. and Macarak, E. J. (1990) Hypoxic contraction of cultured pulmonary vascular smooth muscle cells. *Am J Respir Cell Mol Biol* 3(5):457-465

Myers, J. and Ashley, E. (1997) Dangerous curves. A perspective on exercise, lactate, and the anaerobic threshold. *Chest* 111(3):787-795

Naeije, R. and Brimioulle, S. (2001) Physiology in medicine: importance of hypoxic pulmonary vasoconstriction in maintaining arterial oxygenation during acute respiratory failure. *Crit Care* 5(2):67-71

Naeije, R. and Chesler, N. (2012) Pulmonary circulation at exercise. *Compr Physiol* 2(1):711-741

Naeije, R. and Dedobbeleer, C. (2013) Pulmonary hypertension and the right ventricle in hypoxia. *Exp Physiol* 98(8):1247-1256

Naeije, R., Huez, S., Lamotte, M., Retailleau, K., Neupane, S., Abramowicz, D. and Faoro, V. (2010) Pulmonary artery pressure limits exercise capacity at high altitude. *Eur Respir J* 36(5):1049-1055

Naeije, R. and Vanderpool, R. (2013) Pulmonary hypertension and chronic mountain sickness. *High Alt Med Biol* 14(2):117-125

Nanas, J. N., Matsouka, C., Karageorgopoulos, D., Leonti, A., Tsolakis, E., Drakos, S. G., Tsagalou, E. P., Maroulidis, G. D., Alexopoulos, G. P., Kanakakis, J. E. and Anastasiou-Nana, M. I. (2006) Etiology of anemia in patients with advanced heart failure. *J Am Coll Cardiol* 48(12):2485-2489

Nandal, A., Ruiz, J. C., Subramanian, P., Ghimire-Rijal, S., Sinnamon, R. A., Stemmler, T. L., Bruick, R. K. and Philpott, C. C. (2011) Activation of the HIF prolyl hydroxylase by the iron chaperones PCBP1 and PCBP2. *Cell Metab* 14(5):647-657

Nathan, D. G., Schupak, E., Stohlman, F., Jr. and Merrill, J. P. (1964) Erythropoiesis in anephric Man. *J Clin Invest* 43:2158-2165

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B. K. and Ganz, T. (2004a) IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 113(9):1271-1276

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T. and Kaplan, J. (2004b) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 306(5704):2090-2093

Newman, J. H., Holt, T. N., Cogan, J. D., Womack, B., Phillips, J. A., 3rd, Li, C., Kendall, Z., Stenmark, K. R., Thomas, M. G., Brown, R. D., Riddle, S. R., West, J. D. and Hamid, R. (2015) Increased prevalence of EPAS1 variant in cattle with high-altitude pulmonary hypertension. *Nat Commun* 6:6863

Noctural Oxygen Therapy Trial Group (1980) Continuous or nocturnal oxygen therapy in hypoxemic chronic obstructive lung disease: a clinical trial. *Ann Intern Med* 93(3):391-398

Nordrehaug, J. E., Bjorkhaug, A., Danielsen, R. and Vik-Mo, H. (1991) Arterial and venous measurement in resting forearm of metabolic indicators during rest and leg exercise. *Clin Physiol* 11(5):469-476

Nunomiya, A., Shin, J., Kitajima, Y., Dan, T., Miyata, T. and Nagatomi, R. (2016) Activation of the hypoxia-inducible factor pathway induced by prolyl hydroxylase domain 2 deficiency enhances the effect of running training in mice. *Acta Physiol (Oxf)* [Epub 09/07/2016]

O'Hagan, K. A., Cocchiglia, S., Zhdanov, A. V., Tambuwala, M. M., Cummins, E. P., Monfared, M., Agbor, T. A., Garvey, J. F., Papkovsky, D. B., Taylor, C. T. and Allan, B. B. (2009) PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *Proc Natl Acad Sci U S A* 106(7):2188-2193

Ohira, Y., Edgerton, V. R., Gardner, G. W., Senewiratne, B., Barnard, R. J. and Simpson, D. R. (1979) Work capacity, heart rate and blood lactate responses to iron treatment. *Br J Haematol* 41(3):365-372

Okonko, D. O., Grzeslo, A., Witkowski, T., Mandal, A. K., Slater, R. M., Roughton, M., Foldes, G., Thum, T., Majda, J., Banasiak, W., Missouris, C. G., Poole-Wilson, P. A., Anker, S. D. and Ponikowski, P. (2008) Effect of intravenous iron sucrose on exercise tolerance in anemic and nonanemic patients with symptomatic chronic heart failure and iron deficiency FERRIC-HF: a randomized, controlled, observer-blinded trial. *J Am Coll Cardiol* 51(2):103-112

Okura, H., Takada, Y., Yamabe, A., Ozaki, T., Yamagishi, H., Toda, I., Yoshiyama, M., Yoshikawa, J. and Yoshida, K. (2011) Prevalence and Correlates of Physiological Valvular Regurgitation in Healthy Subjects. *Circulation Journal* 75(11):2699-2704

Oliva, G., Chiuini, F. and Tramontana, C. (1949) On the humoral regulation of the normoerythropoiesis. *Acta Med Scand* 133(1):27-30

Olschewski, A., Hong, Z., Nelson, D. P. and Weir, E. K. (2002) Graded response of K+ current, membrane potential, and [Ca2+]i to hypoxia in pulmonary arterial smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 283(5):L1143-1150

Olszowka, A. J. (1975) Can VA/Q distributions in the lung be recovered from inert gas retention data? *Respir Physiol* 25(2):191-198

Oswald-Mammosser, M., Apprill, M., Bachez, P., Ehrhart, M. and Weitzenblum, E. (1991) Pulmonary hemodynamics in chronic obstructive pulmonary disease of the emphysematous type. *Respiration* 58(5-6):304-310

Oswald-Mammosser, M., Weitzenblum, E., Quoix, E., Moser, G., Chaouat, A., Charpentier, C. and Kessler, R. (1995) Prognostic factors in COPD patients receiving long-term oxygen therapy. Importance of pulmonary artery pressure. *Chest* 107(5):1193-1198

Pak, M., Lopez, M. A., Gabayan, V., Ganz, T. and Rivera, S. (2006) Suppression of hepcidin during anemia requires erythropoietic activity. *Blood* 108(12):3730-3735

Pan, Y., Mansfield, K. D., Bertozzi, C. C., Rudenko, V., Chan, D. A., Giaccia, A. J. and Simon, M. C. (2007) Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. *Mol Cell Biol* 27(3):912-925

Park, C. H., Valore, E. V., Waring, A. J. and Ganz, T. (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem* 276(11):7806-7810

Pauwels, R. A., Buist, A. S., Calverley, P. M., Jenkins, C. R. and Hurd, S. S. (2001) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 163(5):1256-1276

Peacock, A. J., Challenor, V. and Sutherland, G. (1990) Estimation of pulmonary artery pressure by Doppler echocardiography in normal subjects made hypoxic. *Respir Med* 84(4):335-337

Peeling, P., Sim, M., Badenhorst, C. E., Dawson, B., Govus, A. D., Abbiss, C. R., Swinkels, D. W. and Trinder, D. (2014) Iron status and the acute post-exercise hepcidin response in athletes. *PLoS One* 9(3):e93002

Percy, M. J., Chung, Y. J., Harrison, C., Mercieca, J., Hoffbrand, A. V., Dinardo, C. L., Santos, P. C., Fonseca, G. H., Gualandro, S. F., Pereira, A. C., Lappin, T. R., McMullin, M. F. and Lee, F. S. (2012) Two new mutations in the HIF2A gene associated with erythrocytosis. *Am J Hematol* 87(4):439-442

Percy, M. J., Furlow, P. W., Lucas, G. S., Li, X., Lappin, T. R., McMullin, M. F. and Lee, F. S. (2008) A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med* 358(2):162-168

Percy, M. J., Zhao, Q., Flores, A., Harrison, C., Lappin, T. R., Maxwell, P. H., McMullin, M. F. and Lee, F. S. (2006) A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci U S A* 103(3):654-659

Perrotta, S., Stiehl, D. P., Punzo, F., Scianguetta, S., Borriello, A., Bencivenga, D., Casale, M., Nobili, B., Fasoli, S., Balduzzi, A., Cro, L., Nytko, K. J., Wenger, R. H. and Della Ragione, F. (2013) Congenital erythrocytosis associated with gain-of-function HIF2A gene mutations and erythropoietin levels in the normal range. *Haematologica* 98(10):1624-1632

Perry, S. F., Wilson, R. J. A., Straus, C., Harris, M. B. and Remmers, J. E. (2001) Which came first, the lung or the breath? *Comp Biochem Phys A* 129(1):37-47

Peters, J. P. and Van Slyke, D. D. (1931). *Quantitative Clinical Chemistry: Methods.* Baltimore, Williams & Wilkins Co.

Petousi, N., Croft, Q. P., Cavalleri, G. L., Cheng, H. Y., Formenti, F., Ishida, K., Lunn, D., McCormack, M., Shianna, K. V., Talbot, N. P., Ratcliffe, P. J. and Robbins, P. A. (2014) Tibetans living at sea level have a hyporesponsive hypoxia-inducible factor system and blunted physiological responses to hypoxia. *J Appl Physiol (1985)* 116(7):893-904

Petousi, N. and Robbins, P. A. (2014) Human adaptation to the hypoxia of high altitude: the Tibetan paradigm from the pregenomic to the postgenomic era. *J Appl Physiol (1985)* 116(7):875-884

Petty, T. L. (2006) The history of COPD. *Int J Chron Obstruct Pulmon Dis* 1(1):3-14

Philippot, Q., Deslee, G., Adair-Kirk, T. L., Woods, J. C., Byers, D., Conradi, S., Dury, S., Perotin, J. M., Lebargy, F., Cassan, C., Le Naour, R., Holtzman, M. J. and Pierce, R. A. (2014) Increased iron sequestration in alveolar macrophages in chronic obstructive pulmonary disease. *PLoS One* 9(5):e96285

Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brissot, P. and Loreal, O. (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *J Biol Chem* 276(11):7811-7819

Piperno, A., Galimberti, S., Mariani, R., Pelucchi, S., Ravasi, G., Lombardi, C., Bilo, G., Revera, M., Giuliano, A., Faini, A., Mainini, V., Westerman, M., Ganz, T., Valsecchi, M. G., Mancia, G. and Parati, G. (2011) Modulation of hepcidin production during hypoxia-induced erythropoiesis in humans in vivo: data from the HIGHCARE project. *Blood* 117(10):2953-2959

Pirzio-Biroli, G., Bothwell, T. H. and Finch, C. A. (1958) Iron absorption. II. The absorption of radioiron administered with a standard meal in man. *J Lab Clin Med* 51(1):37-48

Plesner, L. L., Schoos, M. M., Dalsgaard, M., Goetze, J. P., Kjoller, E., Vestbo, J. and Iversen, K. (2016) Iron Deficiency in COPD Associates with Increased Pulmonary Artery Pressure Estimated by Echocardiography. *Heart Lung Circ* [Epub 25/05/2016]

Pollycove, M. and Mortimer, R. (1961) The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J Clin Invest* 40:753-782

Polonifi, A., Politou, M., Kalotychou, V., Xiromeritis, K., Tsironi, M., Berdoukas, V., Vaiopoulos, G. and Aessopos, A. (2010) Iron metabolism gene expression in human skeletal muscle. *Blood Cells Mol Dis* 45(3):233-237

Pond, M. N., Morton, A. M. and Conway, S. P. (1996) Functional iron deficiency in adults with cystic fibrosis. *Respir Med* 90(7):409-413

Ponikowski, P., van Veldhuisen, D. J., 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, S. D. and the CONFIRM-HF Investigators (2015) Beneficial effects of long-term intravenous iron therapy with ferric carboxymaltose in patients with symptomatic heart failure and iron deficiency. *Eur Heart J* 36(11):657-668

Ponka, P., Beaumont, C. and Richardson, D. R. (1998) Function and regulation of transferrin and ferritin. *Semin Hematol* 35(1):35-54

Ponka, P. and Lok, C. N. (1999) The transferrin receptor: role in health and disease. *Int J Biochem Cell Biol* 31(10):1111-1137

Portillo, K., Martinez-Rivera, C. and Ruiz-Manzano, J. (2013) Anaemia in chronic obstructive pulmonary disease. Does it really matter? *Int J Clin Pract* 67(6):558-565

Post, J. M., Hume, J. R., Archer, S. L. and Weir, E. K. (1992) Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am J Physiol* 262(4 Pt 1):C882-890

Prabhakar, N. R. and Peers, C. (2014) Gasotransmitter regulation of ion channels: a key step in O2 sensing by the carotid body. *Physiology (Bethesda)* 29(1):49-57

Prabhakar, N. R. and Semenza, G. L. (2015) Oxygen sensing and homeostasis. *Physiology (Bethesda)* 30(5):340-348

Price, D. C., Forsyth, E. M., Cohn, S. H. and Cronkite, E. P. (1964) The study of menstrual and other blood loss, and consequent iron deficiency, by Fe59 whole-body counting. *Can Med Assoc J* 90(2):51-54

Prime, F. J. and Westlake, E. K. (1954) The respiratory response to CO2 in emphysema. *Clin Sci (Lond)* 13(3):321-332

Priyadarshi, A., Periyasamy, S., Burke, T. J., Britton, S. L., Malhotra, D. and Shapiro, J. I. (2002) Effects of reduction of renal mass on renal oxygen tension and erythropoietin production in the rat. *Kidney Int* 61(2):542-546

Provenzano, R., Besarab, A., Sun, C. H., Diamond, S. A., Durham, J. H., Cangiano, J. L., Aiello, J. R., Novak, J. E., Lee, T., Leong, R., Roberts, B. K., Saikali, K. G., Hemmerich, S., Szczech, L. A., Yu, K. H. and Neff, T. B. (2016) Oral hypoxia-inducible factor prolyl hydroxylase inhibitor Roxadustat (FG-4592) for the treatment of anemia in patients with CKD. *Clin J Am Soc Nephrol* 11(6):982-991

Puente-Maestu, L., Lazaro, A. and Humanes, B. (2013) Metabolic derangements in COPD muscle dysfunction. *J Appl Physiol (1985)* 114(9):1282-1290

Pugh, C. W., Tan, C. C., Jones, R. W. and Ratcliffe, P. J. (1991) Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene. *Proc Natl Acad Sci U S A* 88(23):10553-10557

Pyne, D. B., Boston, T., Martin, D. T. and Logan, A. (2000) Evaluation of the Lactate Pro blood lactate analyser. *Eur J Appl Physiol* 82(1-2):112-116

Qi, X., Cui, C., Peng, Y., Zhang, X., Yang, Z., Zhong, H., Zhang, H., Xiang, K., Cao, X., Wang, Y., Ouzhuluobu, Basang, Ciwangsangbu, Bianba, Gonggalanzi, Wu, T., Chen, H., Shi, H. and Su, B. (2013) Genetic evidence of paleolithic colonization and neolithic expansion of modern humans on the tibetan plateau. *Mol Biol Evol* 30(8):1761-1778

Rabe, K. F., Hurd, S., Anzueto, A., Barnes, P. J., Buist, S. A., Calverley, P., Fukuchi, Y., Jenkins, C., Rodriguez-Roisin, R., van Weel, C. and Zielinski, J. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 176(6):532-555

Racker, E. (1974) History of the Pasteur effect and its pathobiology. *Mol Cell Biochem* 5(1-2):17-23

Radermacher, P., Santak, B., Becker, H. and Falke, K. J. (1989) Prostaglandin E1 and nitroglycerin reduce pulmonary capillary pressure but worsen ventilation-perfusion distributions in patients with adult respiratory distress syndrome. *Anesthesiology* 70(4):601-606

Rankin, E. B., Biju, M. P., Liu, Q., Unger, T. L., Rha, J., Johnson, R. S., Simon, M. C., Keith, B. and Haase, V. H. (2007) Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. *J Clin Invest* 117(4):1068-1077

Rapoport, B. I. (2010) Metabolic factors limiting performance in marathon runners. *PLoS Comput Biol* 6(10):e1000960

Ravasi, G., Pelucchi, S., Greni, F., Mariani, R., Giuliano, A., Parati, G., Silvestri, L. and Piperno, A. (2014) Circulating factors are involved in hypoxia-induced hepcidin suppression. *Blood Cells Mol Dis* 53(4):204-210

Reeves, J. T., Groves, B. M., Cymerman, A., Sutton, J. R., Wagner, P. D., Turkevich, D. and Houston, C. S. (1990) Operation Everest II: cardiac filling pressures during cycle exercise at sea level. *Respir Physiol* 80(2-3):147-154

Reid, D. W., Withers, N. J., Francis, L., Wilson, J. W. and Kotsimbos, T. C. (2002) Iron deficiency in cystic fibrosis: relationship to lung disease severity and chronic Pseudomonas aeruginosa infection. *Chest* 121(1):48-54

Reinhardt, R. (1912) Über das Verhältnis von CO2-Ausscheidung zur Atemgrösse beim Lungenemphysem. *Dtsch Arch Klin Med* 109:192 [German].

Reissmann, K. R. (1950) Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. *Blood* 5(4):372-380

Reissmann, K. R. and Dietrich, M. R. (1956) On the presence of ferritin in the peripheral blood of patients with hepatocellular disease. *J Clin Invest* 35(6):588-595

Remels, A. H., Gosker, H. R., Verhees, K. J., Langen, R. C. and Schols, A. M. (2015) TNF-alpha-induced NF-kappaB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1alpha. *Endocrinology* 156(5):1770-1781

Ren, X., Dorrington, K. L., Maxwell, P. H. and Robbins, P. A. (2000) Effects of desferrioxamine on serum erythropoietin and ventilatory sensitivity to hypoxia in humans. *J Appl Physiol (1985)* 89(2):680-686

Rhodes, C. J., Howard, L. S., Busbridge, M., Ashby, D., Kondili, E., Gibbs, J. S., Wharton, J. and Wilkins, M. R. (2011) Iron deficiency and raised hepcidin in idiopathic pulmonary arterial hypertension: clinical prevalence, outcomes, and mechanistic insights. *J Am Coll Cardiol* 58(3):300-309

Richalet, J. P., Souberbielle, J. C., Antezana, A. M., Dechaux, M., Le Trong, J. L., Bienvenu, A., Daniel, F., Blanchot, C. and Zittoun, J. (1994) Control of erythropoiesis in humans during prolonged exposure to the altitude of 6,542 m. *Am J Physiol* 266(3 Pt 2):R756-764

Richardson, R. S., Grassi, B., Gavin, T. P., Haseler, L. J., Tagore, K., Roca, J. and Wagner, P. D. (1999) Evidence of O2 supply-dependent VO2 max in the exercise-trained human quadriceps. *J Appl Physiol (1985)* 86(3):1048-1053

Ringbaek, T., Martinez, G., Brondum, E., Thogersen, J., Morgan, M. and Lange, P. (2010) Shuttle walking test as predictor of survival in chronic obstructive pulmonary disease patients enrolled in a rehabilitation program. *J Cardiopulm Rehabil Prev* 30(6):409-414

Rishi, G., Crampton, E. M., Wallace, D. F. and Subramaniam, V. N. (2013) In situ proximity ligation assays indicate that hemochromatosis proteins Hfe and transferrin receptor 2 (Tfr2) do not interact. *PLoS One* 8(10):e77267

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., Johnson, R. S., Haddad, G. G. and Karin, M. (2008) NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature* 453(7196):807-811

Robach, P., Cairo, G., Gelfi, C., Bernuzzi, F., Pilegaard, H., Vigano, A., Santambrogio, P., Cerretelli, P., Calbet, J. A., Moutereau, S. and Lundby, C. (2007) Strong iron demand during hypoxia-induced erythropoiesis is associated with down-regulation of iron-related proteins and myoglobin in human skeletal muscle. *Blood* 109(11):4724-4731

Robbins, P. A., Swanson, G. D. and Howson, M. G. (1982) A prediction-correction scheme for forcing alveolar gases along certain time courses. *J Appl Physiol Respir Environ Exerc Physiol* 52(5):1353-1357

Robertson, T. P., Aaronson, P. I. and Ward, J. P. (2003) Ca2+ sensitization during sustained hypoxic pulmonary vasoconstriction is endothelium dependent. *Am J Physiol Lung Cell Mol Physiol* 284(6):L1121-1126

Robinson, J. C., Graham, B. B., Rouault, T. C. and Tuder, R. M. (2014) The crossroads of iron with hypoxia and cellular metabolism. Implications in the pathobiology of pulmonary hypertension. *Am J Respir Cell Mol Biol* 51(6):721-729

Roe, M. A., Spinks, C., Heath, A. L., Harvey, L. J., Foxall, R., Wimperis, J., Wolf, C. and Fairweather-Tait, S. J. (2007) Serum prohepcidin concentration: no association with iron absorption in healthy men; and no relationship with iron status in men carrying HFE mutations, hereditary haemochromatosis patients undergoing phlebotomy treatment, or pregnant women. *Br J Nutr* 97(3):544-549

Rogers, J. T., Bridges, K. R., Durmowicz, G. P., Glass, J., Auron, P. E. and Munro, H. N. (1990) Translational control during the acute phase response. Ferritin synthesis in response to interleukin-1. *J Biol Chem* 265(24):14572-14578

Rosenberger, C., Mandriota, S., Jurgensen, J. S., Wiesener, M. S., Horstrup, J. H., Frei, U., Ratcliffe, P. J., Maxwell, P. H., Bachmann, S. and Eckardt, K. U. (2002) Expression of hypoxia-inducible factor-1alpha and -2alpha in hypoxic and ischemic rat kidneys. *J Am Soc Nephrol* 13(7):1721-1732

Rossaint, R., Falke, K. J., Lopez, F., Slama, K., Pison, U. and Zapol, W. M. (1993) Inhaled nitric oxide for the adult respiratory distress syndrome. *N Engl J Med* 328(6):399-405

Roughton, F. J. W. (1945) The average time spent by the blood in the human lung capillary and its relation to the rates of CO uptake and elimination in Man. *Am J Physiol* 143(4):621-633

Ruchala, P. and Nemeth, E. (2014) The pathophysiology and pharmacology of hepcidin. *Trends Pharmacol Sci* 35(3):155-161

Ruhenstroth-Bauer, G. (1950) Versuche zum Nachweis eines spezifischen erythropetischen. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 211(1):32-56 [German].

Ruiter, G., Lankhorst, S., Boonstra, A., Postmus, P. E., Zweegman, S., Westerhof, N., van der Laarse, W. J. and Vonk-Noordegraaf, A. (2011) Iron deficiency is common in idiopathic pulmonary arterial hypertension. *Eur Respir J* 37(6):1386-1391

Ruiter, G., Lanser, I. J., de Man, F. S., van der Laarse, W. J., Wharton, J., Wilkins, M. R., Howard, L. S., Vonk-Noordegraaf, A. and Voskuyl, A. E. (2014) Iron deficiency in systemic sclerosis patients with and without pulmonary hypertension. *Rheumatology (Oxford)* 53(2):285-292

Ruiter, G., Manders, E., Happe, C. M., Schalij, I., Groepenhoff, H., Howard, L. S., Wilkins, M. R., Bogaard, H. J., Westerhof, N., van der Laarse, W. J., de Man, F. S. and Vonk-Noordegraaf, A. (2015) Intravenous iron therapy in patients with idiopathic pulmonary arterial hypertension and iron deficiency. *Pulm Circ* 5(3):466-472

Russell, R. C., Sufan, R. I., Zhou, B., Heir, P., Bunda, S., Sybingco, S. S., Greer, S. N., Roche, O., Heathcote, S. A., Chow, V. W., Boba, L. M., Richmond, T. D., Hickey, M. M., Barber, D. L., Cheresh, D. A., Simon, M. C., Irwin, M. S., Kim, W. Y. and Ohh, M. (2011) Loss of JAK2 regulation via a heterodimeric VHL-SOCS1 E3 ubiquitin ligase underlies Chuvash polycythemia. *Nat Med* 17(7):845-853

Sabit, R., Thomas, P., Shale, D. J., Collins, P. and Linnane, S. J. (2010) The effects of hypoxia on markers of coagulation and systemic inflammation in patients with COPD. *Chest* 138(1):47-51

Sable, C. A., Aliyu, Z. Y., Dham, N., Nouraie, M., Sachdev, V., Sidenko, S., Miasnikova, G. Y., Polyakova, L. A., Sergueeva, A. I., Okhotin, D. J., Bushuev, V., Remaley, A. T., Niu, X., Castro, O. L., Gladwin, M. T., Kato, G. J., Prchal, J. T. and Gordeuk, V. R. (2011) Pulmonary artery pressure and iron deficiency in patients with upregulation of hypoxia sensing due to homozygous VHLR200W mutation (Chuvash polycythemia). *Haematologica* 97(2):193-200

Salceda, S. and 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. *J Biol Chem* 272(36):22642-22647

Saltin, B. and Calbet, J. A. (2006) Point: in health and in a normoxic environment, VO2 max is limited primarily by cardiac output and locomotor muscle blood flow. *J Appl Physiol (1985)* 100(2):744-745

Sanchez, M., Galy, B., Muckenthaler, M. U. and Hentze, M. W. (2007) Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nat Struct Mol Biol* 14(5):420-426

Sandek, K., Bratel, T., Hellstrom, G. and Lagerstrand, L. (2001a) Ventilation-perfusion inequality and carbon dioxide sensitivity in hypoxaemic chronic obstructive pulmonary disease (COPD) and effects of 6 months of long-term oxygen treatment (LTOT). *Clin Physiol* 21(5):584-593

Sandek, K., Bratel, T. and Lagerstrand, L. (2001b) Effects on diffusing capacity and ventilation--perfusion relationships of budesonide inhalations for 2 months in chronic obstructive pulmonary disease (COPD). *Respir Med* 95(8):676-684

Sandek, K., Bratel, T., Lagerstrand, L. and Rosell, H. (2002) Relationship between lung function, ventilation-perfusion inequality and extent of emphysema as assessed by high-resolution computed tomography. *Respir Med* 96(11):934-943

Scadding, J. G. (1963) Meaning of diagnostic terms in broncho-pulmonary disease. *Br Med J* 2(5370):1425-1430

Schaap, C. C., Hendriks, J. C., Kortman, G. A., Klaver, S. M., Kroot, J. J., Laarakkers, C. M., Wiegerinck, E. T., Tjalsma, H., Janssen, M. C. and Swinkels, D. W. (2013) Diurnal rhythm rather than dietary iron mediates daily hepcidin variations. *Clin Chem* 59(3):527-535

Scharf, S. M., Iqbal, M., Keller, C., Criner, G., Lee, S. and Fessler, H. E. (2002) Hemodynamic characterization of patients with severe emphysema. *Am J Respir Crit Care Med* 166(3):314-322

Schloesser, L. L., Kipp, M. A. and Wenzel, F. J. (1965) Thrombocytosis in iron-deficiency anemia. *J Lab Clin Med* 66:107-114

Schmidt, P. J., Toran, P. T., Giannetti, A. M., Bjorkman, P. J. and Andrews, N. C. (2008) The transferrin receptor modulates Hfe-dependent regulation of hepcidin expression. *Cell Metab* 7(3):205-214

Schneckenpointner, R., Jorres, R. A., Meidenbauer, N., Kollert, F., Pfeifer, M. and Budweiser, S. (2014) The clinical significance of anaemia and disturbed iron homeostasis in chronic respiratory failure. *Int J Clin Pract* 68(1):130-138

Schoene, R. B., Escourrou, P., Robertson, H. T., Nilson, K. L., Parsons, J. R. and Smith, N. J. (1983) Iron repletion decreases maximal exercise lactate concentrations in female athletes with minimal iron-deficiency anemia. *J Lab Clin Med* 102(2):306-312

Schofield, C. J. and Ratcliffe, P. J. (2004) Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 5(5):343-354

Schrijen, F., Uffholtz, H., Polu, J. M. and Poincelot, F. (1978) Pulmonary and systemic hemodynamic evolution in chronic bronchitis. *Am Rev Respir Dis* 117(1):25-31

Scott, D. E. and Pritchard, J. A. (1967) Iron deficiency in healthy young college women. *JAMA* 199(12):897-900

Scott, R. W. (1920) Observations on the pathologic physiology of chronic pulmonary emphysema. *Arch Intern Med (Chic)* 26(5):544

Seeger, W., Adir, Y., Barbera, J. A., Champion, H., Coghlan, J. G., Cottin, V., De Marco, T., Galie, N., Ghio, S., Gibbs, S., Martinez, F. J., Semigran, M. J., Simonneau, G., Wells, A. U. and Vachiery, J. L. (2013) Pulmonary hypertension in chronic lung diseases. *J Am Coll Cardiol* 62(25 Suppl):D109-116

Seldin, M. M., Peterson, J. M., Byerly, M. S., Wei, Z. and Wong, G. W. (2012) Myonectin (CTRP15), a novel myokine that links skeletal muscle to systemic lipid homeostasis. *J Biol Chem* 287(15):11968-11980

Selinger, S. R., Kennedy, T. P., Buescher, P., Terry, P., Parham, W., Gofreed, D., Medinger, A., Spagnolo, S. V. and Michael, J. R. (1987) Effects of removing oxygen from patients with chronic obstructive pulmonary disease. *Am Rev Respir Dis* 136(1):85-91

Semenza, G. L. (2011) Oxygen sensing, homeostasis, and disease. *N Engl J Med* 365(6):537-547

Semenza, G. L., Agani, F., Booth, G., Forsythe, J., Iyer, N., Jiang, B. H., Leung, S., Roe, R., Wiener, C. and Yu, A. (1997) Structural and functional analysis of hypoxia-inducible factor 1. *Kidney Int* 51(2):553-555

Semenza, G. L., Nejfelt, M. K., Chi, S. M. and Antonarakis, S. E. (1991) Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc Natl Acad Sci U S A* 88(13):5680-5684

Semenza, G. L. and Wang, G. L. (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. *Mol Cell Biol* 12(12):5447-5454

Severinghaus, J. W. (1966) Blood gas calculator. *J Appl Physiol* 21(3):1108-1116

Severinghaus, J. W. and Astrup, P. B. (1986) History of blood gas analysis. IV. Leland Clark's oxygen electrode. *J Clin Monit* 2(2):125-139

Sharma, R. K. and Chakrabarti, S. (2016) Anaemia secondary to erythropoietin resistance: important predictor of adverse outcomes in chronic obstructive pulmonary disease. *Postgrad Med J* 92:636-639

Sheftel, A. D., Mason, A. B. and Ponka, P. (2012) The long history of iron in the Universe and in health and disease. *Biochim Biophys Acta* 1820(3):161-187

Sheridan, D. J. and Gatenby, P. B. (1975) Urinary erythropoietin levels in normal, anaemic, polycythaemic, and uraemic subjects. *Ir J Med Sci* 144(2):67-71

Shimoda, L. A. and Laurie, S. S. (2014) HIF and pulmonary vascular responses to hypoxia. *J Appl Physiol (1985)* 116(7):867-874

Siebert, S., Williams, B. D., Henley, R., Ellis, R., Cavill, I. and Worwood, M. (2003) Single value of serum transferrin receptor is not diagnostic for the absence of iron stores in anaemic patients with rheumatoid arthritis. *Clin Lab Haematol* 25(3):155-160

Siedlinski, M., Tingley, D., Lipman, P. J., Cho, M. H., Litonjua, A. A., Sparrow, D., Bakke, P., Gulsvik, A., Lomas, D. A., Anderson, W., Kong, X., Rennard, S. I., Beaty, T. H., Hokanson, J. E., Crapo, J. D., Lange, C. and Silverman, E. K. (2013) Dissecting direct and indirect genetic effects on chronic obstructive pulmonary disease (COPD) susceptibility. *Hum Genet* 132(4):431-441

Siimes, M. A., Refino, C. and Dallman, P. R. (1980) Manifestation of iron deficiency at various levels of dietary iron intake. *Am J Clin Nutr* 33(3):570-574

Silverberg, D. S., Mor, R., Weu, M. T., Schwartz, D., Schwartz, I. F. and Chernin, G. (2014) Anemia and iron deficiency in COPD patients: prevalence and the effects of correction of the anemia with erythropoiesis stimulating agents and intravenous iron. *BMC Pulm Med* 14:24

Sime, F., Penaloza, D. and Ruiz, L. (1971) Bradycardia, increased cardiac output, and reversal of pulmonary hypertension in altitude natives living at sea level. *Br Heart J* 33(5):647-657

Simonson, T. S., Yang, Y., Huff, C. D., Yun, H., Qin, G., Witherspoon, D. J., Bai, Z., Lorenzo, F. R., Xing, J., Jorde, L. B., Prchal, J. T. and Ge, R. (2010) Genetic evidence for high-altitude adaptation in Tibet. *Science* 329(5987):72-75

Sims, M. W., Margolis, D. J., Localio, A. R., Panettieri, R. A., Kawut, S. M. and Christie, J. D. (2009) Impact of pulmonary artery pressure on exercise function in severe COPD. *Chest* 136(2):412-419

Singanayagam, A., Schembri, S. and Chalmers, J. D. (2013) Predictors of mortality in hospitalized adults with acute exacerbation of chronic obstructive pulmonary disease. *Ann Am Thorac Soc* 10(2):81-89

Singh, I., Ma, K. C. and Berlin, D. A. (2016) Pathophysiology of pulmonary hypertension in chronic parenchymal lung disease. *Am J Med* 129(4):366-371

Singh, S. J., Morgan, M. D., Scott, S., Walters, D. and Hardman, A. E. (1992) Development of a shuttle walking test of disability in patients with chronic airways obstruction. *Thorax* 47(12):1019-1024

Siri, W. E., Van Dyke, D. C., Winchell, H. S., Pollycove, M., Parker, H. G. and Cleveland, A. S. (1966) Early erythropoietin, blood, and physiological responses to severe hypoxia in man. *J Appl Physiol* 21(1):73-80

Skikne, B. S., Flowers, C. H. and Cook, J. D. (1990) Serum transferrin receptor: a quantitative measure of tissue iron deficiency. *Blood* 75(9):1870-1876

Skjaerpe, T. and Hatle, L. (1986) Noninvasive estimation of systolic pressure in the right ventricle in patients with tricuspid regurgitation. *Eur Heart J* 7(8):704-710

Slingo, M. E., Turner, P. J., Christian, H. C., Buckler, K. J. and Robbins, P. A. (2014) The von Hippel-Lindau Chuvash mutation in mice causes carotid-body hyperplasia and enhanced ventilatory sensitivity to hypoxia. *J Appl Physiol (1985)* 116(7):885-892

Smith, C. P. and Thevenod, F. (2009) Iron transport and the kidney. *Biochim Biophys Acta* 1790(7):724-730

Smith, G. A., Fisher, S. A., Doree, C., Di Angelantonio, E. and Roberts, D. J. (2014) Oral or parenteral iron supplementation to reduce deferral, iron deficiency and/or anaemia in blood donors. *Cochrane Database Syst Rev* 7:CD009532

Smith, T. G., Balanos, G. M., Croft, Q. P., Talbot, N. P., Dorrington, K. L., Ratcliffe, P. J. and Robbins, P. A. (2008a) The increase in pulmonary arterial pressure caused by hypoxia depends on iron status. *J Physiol* 586(24):5999-6005

Smith, T. G., Brooks, J. T., Balanos, G. M., Lappin, T. R., Layton, D. M., Leedham, D. L., Liu, C., Maxwell, P. H., McMullin, M. F., McNamara, C. J., Percy, M. J., Pugh, C. W., Ratcliffe, P. J., Talbot, N. P., Treacy, M. and Robbins, P. A. (2006) Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. *PLoS Med* 3(7):e290

Smith, T. G., Brooks, J. T., Balanos, G. M., Lappin, T. R., Layton, D. M., Leedham, D. L., Liu, C., Maxwell, P. H., McMullin, M. F., McNamara, C. J., Percy, M. J., Pugh, C. W., Ratcliffe, P. J., Talbot, N. P., Treacy, M. and Robbins, P. A. (2008b) Mutation of the von Hippel-Lindau gene alters human cardiopulmonary physiology. *Adv Exp Med Biol* 605:51-56

Smith, T. G., Robbins, P. A. and Ratcliffe, P. J. (2008c) The human side of hypoxia-inducible factor. *Br J Haematol* 141(3):325-334

Smith, T. G., Talbot, N. P., Chang, R. W., Wilkinson, E., Nickol, A. H., Newman, D. G., Robbins, P. A. and Dorrington, K. L. (2012) Pulmonary artery pressure increases during commercial air travel in healthy passengers. *Aviat Space Environ Med* 83(7):673-676

Smith, T. G., Talbot, N. P., Privat, C., Rivera-Ch, M., Nickol, A. H., Ratcliffe, P. J., Dorrington, K. L., Leon-Velarde, F. and Robbins, P. A. (2009) Effects of iron supplementation and depletion on hypoxic pulmonary hypertension: two randomized controlled trials. *JAMA* 302(13):1444-1450

Snider, G. L. (2003) Nosology for our day: its application to chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 167(5):678-683

Soler, X., Gaio, E., Powell, F. L., Ramsdell, J. W., Loredo, J. S., Malhotra, A. and Ries, A. L. (2015) High prevalence of obstructive sleep apnea in patients with moderate to severe chronic obstructive pulmonary disease. *Ann Am Thorac Soc* 12(8):1219-1225

Sonnweber, T., Nachbaur, D., Schroll, A., Nairz, M., Seifert, M., Demetz, E., Haschka, D., Mitterstiller, A. M., Kleinsasser, A., Burtscher, M., Trubsbach, S., Murphy, A. T., Wroblewski, V., Witcher, D. R., Mleczko-Sanecka, K., Vecchi, C., Muckenthaler, M. U., Pietrangelo, A., Theurl, I. and Weiss, G. (2014) Hypoxia induced downregulation of hepcidin is mediated by platelet derived growth factor BB. *Gut* 63(12):1951-1959

Soon, E., Treacy, C. M., Toshner, M. R., MacKenzie-Ross, R., Manglam, V., Busbridge, M., Sinclair-McGarvie, M., Arnold, J., Sheares, K. K., Morrell, N. W. and Pepke-Zaba, J. (2011) Unexplained iron deficiency in idiopathic and heritable pulmonary arterial hypertension. *Thorax* 66(4):326-332

Srinivas, V., Leshchinsky, I., Sang, N., King, M. P., Minchenko, A. and Caro, J. (2001) Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway. *J Biol Chem* 276(25):21995-21998

Staub, N. C., Bishop, J. M. and Forster, R. E. (1961) Velocity of O2 uptake by human red blood cells. *J Appl Physiol* 16(3):511-516

Steiner, M. K., Syrkina, O. L., Kolliputi, N., Mark, E. J., Hales, C. A. and Waxman, A. B. (2009) Interleukin-6 overexpression induces pulmonary hypertension. *Circ Res* 104(2):236-244

Stevenson, J. G. (1989) Comparison of several noninvasive methods for estimation of pulmonary artery pressure. *J Am Soc Echocardiogr* 2(3):157-171

Stone, A. C., Machan, J. T., Mazer, J., Casserly, B. and Klinger, J. R. (2011) Echocardiographic evidence of pulmonary hypertension is associated with increased 1-year mortality in patients admitted with chronic obstructive pulmonary disease. *Lung* 189(3):207-212

Storz, J. F. and Moriyama, H. (2008) Mechanisms of hemoglobin adaptation to high altitude hypoxia. *High Alt Med Biol* 9(2):148-157

Stray-Gundersen, J., Musch, T. I., Haidet, G. C., Swain, D. P., Ordway, G. A. and Mitchell, J. H. (1986) The effect of pericardiectomy on maximal oxygen consumption and maximal cardiac output in untrained dogs. *Circ Res* 58(4):523-530

Stringer, W., Casaburi, R. and Older, P. (2012) Cardiopulmonary exercise testing: does it improve perioperative care and outcome? *Curr Opin Anaesthesiol* 25(2):178-184

Stringer, W. W., Hansen, J. E. and Wasserman, K. (1997) Cardiac output estimated noninvasively from oxygen uptake during exercise. *J Appl Physiol (1985)* 82(3):908-912

Sullivan, M. J., Cobb, F. R. and Higginbotham, M. B. (1991) Stroke volume increases by similar mechanisms during upright exercise in normal men and women. *Am J Cardiol* 67(16):1405-1412

Sunderman, F. W., Jr., Hopfer, S. M., Reid, M. C., Shen, S. K. and Kevorkian, C. B. (1982) Erythropoietin-mediated erythrocytosis in rodents after intrarenal injection of nickel subsulfide. *Yale J Biol Med* 55(2):123-136

Swan, H. J., Ganz, W., Forrester, J., Marcus, H., Diamond, G. and Chonette, D. (1970) Catheterization of the heart in man with use of a flow-directed balloon-tipped catheter. *N Engl J Med* 283(9):447-451

Swenson, E. R. (2013) Hypoxic pulmonary vasoconstriction. *High Alt Med Biol* 14(2):101-110

Sylvester, J. T., Shimoda, L. A., Aaronson, P. I. and Ward, J. P. (2012) Hypoxic pulmonary vasoconstriction. *Physiol Rev* 92(1):367-520

Szoke, D. and Panteghini, M. (2012) Diagnostic value of transferrin. *Clin Chim Acta* 413(15-16):1184-1189

Talbot, N. P., Balanos, G. M., Dorrington, K. L. and Robbins, P. A. (2005) Two temporal components within the human pulmonary vascular response to approximately 2 h of isocapnic hypoxia. *J Appl Physiol (1985)* 98(3):1125-1139

Talbot, N. P., Croft, Q. P., Curtis, M. K., Turner, B. E., Dorrington, K. L., Robbins, P. A. and Smith, T. G. (2014) Contrasting effects of ascorbate and iron on the pulmonary vascular response to hypoxia in humans. *Physiol Rep* 2(12):e12220

Talbot, N. P., Crosby, A., Balanos, G. M., Dorrington, K. L. and Robbins, P. A. (2007) Iron chelation does not potentiate early acclimatisation to sustained hypoxia in humans. *FASEB J* 21(6):A925

Talbot, N. P., Lakhal, S., Smith, T. G., Privat, C., Nickol, A. H., Rivera-Ch, M., Leon-Velarde, F., Dorrington, K. L., Mole, D. R. and Robbins, P. A. (2012) Regulation of hepcidin expression at high altitude. *Blood* 119(3):857-860

Talbot, N. P., Smith, T. G., Lakhal-Littleton, S., Gulsever, C., Rivera-Ch, M., Dorrington, K. L., Mole, D. R. and Robbins, P. A. (2016) Suppression of plasma hepcidin by venesection during steady-state hypoxia. *Blood* 127(9):1206-1207

Talbott, J. H. (1936) Studies at high altitudes. II. Morphology and oxygen combining capacity of the blood. *Folia Haematologica* 55:23-36

Tan, Q., Kerestes, H., Percy, M. J., Pietrofesa, R., Chen, L., Khurana, T. S., Christofidou-Solomidou, M., Lappin, T. R. and Lee, F. S. (2013) Erythrocytosis and pulmonary hypertension in a mouse model of human HIF2A gain of function mutation. *J Biol Chem* 288(24):17134-17144

Tan, W. C., Sin, D. D., Bourbeau, J., Hernandez, P., Chapman, K. R., Cowie, R., FitzGerald, J. M., Marciniuk, D. D., Maltais, F., Buist, A. S., Road, J., Hogg, J. C., Kirby, M., Coxson, H., Hague, C., Leipsic, J., O'Donnell, D. E., Aaron, S. D. and Can, C. C. R. G. (2015) Characteristics of COPD in never-smokers and ever-smokers in the general population: results from the CanCOLD study. *Thorax* 70(9):822-829

Tanabe, N., Muro, S., Hirai, T., Oguma, T., Terada, K., Marumo, S., Kinose, D., Ogawa, E., Hoshino, Y. and Mishima, M. (2011) Impact of exacerbations on emphysema progression in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 183(12):1653-1659

Tandara, L., Grubisic, T. Z., Ivan, G., Jurisic, Z., Tandara, M., Gugo, K., Mladinov, S. and Salamunic, I. (2015) Systemic inflammation up-regulates serum hepcidin in exacerbations and stabile chronic obstructive pulmonary disease. *Clin Biochem* 48(18):1252-1257

Tanimoto, K., Makino, Y., Pereira, T. and Poellinger, L. (2000) Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J* 19(16):4298-4309

Tanner, R. K., Fuller, K. L. and Ross, M. L. (2010) Evaluation of three portable blood lactate analysers: Lactate Pro, Lactate Scout and Lactate Plus. *Eur J Appl Physiol* 109(3):551-559

Tashkin, D. P., Celli, B., Senn, S., Burkhart, D., Kesten, S., Menjoge, S. and Decramer, M. (2008) A 4-year trial of tiotropium in chronic obstructive pulmonary disease. *N Engl J Med* 359(15):1543-1554

Tenenbein, M., Kowalski, S., Sienko, A., Bowden, D. H. and Adamson, I. Y. (1992) Pulmonary toxic effects of continuous desferrioxamine administration in acute iron poisoning. *Lancet* 339(8795):699-701

Thomas, D. W., Hinchliffe, R. F., Briggs, C., Macdougall, I. C., Littlewood, T., Cavill, I. and British Committee for Standards in Haematology (2013) Guideline for the laboratory diagnosis of functional iron deficiency. *Br J Haematol* 161(5):639-648

Thompson, C. H., Green, Y. S., Ledingham, J. G., Radda, G. K. and Rajagopalan, B. (1993a) The effect of iron deficiency on skeletal muscle metabolism of the rat. *Acta Physiol Scand* 147(1):85-90

Thompson, C. H., Kemp, G. J., Taylor, D. J., Radda, G. K. and Rajagopalan, B. (1993b) No evidence of mitochondrial abnormality in skeletal muscle of patients with iron-deficient anaemia. *J Intern Med* 234(2):149-154

Thomsen, M., Ingebrigtsen, T. S., Marott, J. L., Dahl, M., Lange, P., Vestbo, J. and Nordestgaard, B. G. (2013) Inflammatory biomarkers and exacerbations in chronic obstructive pulmonary disease. *JAMA* 309(22):2353-2361

Timms, R. M., Khaja, F. U. and Williams, G. W. (1985) Hemodynamic response to oxygen therapy in chronic obstructive pulmonary disease. *Ann Intern Med* 102(1):29-36

Toblli, J. E., Di Gennaro, F. and Rivas, C. (2015) Changes in echocardiographic parameters in iron deficiency patients with heart failure and chronic kidney disease treated with intravenous iron. *Heart Lung Circ* 24(7):686-695

Tolkien, Z., Stecher, L., Mander, A. P., Pereira, D. I. and Powell, J. J. (2015) Ferrous sulfate supplementation causes significant gastrointestinal side-effects in adults: a systematic review and meta-analysis. *PLoS One* 10(2):e0117383

Traver, G. A., Cline, M. G. and Burrows, B. (1979) Predictors of mortality in chronic obstructive pulmonary disease. A 15-year follow-up study. *Am Rev Respir Dis* 119(6):895-902

Trenell, M. I., Sue, C. M., Kemp, G. J., Sachinwalla, T. and Thompson, C. H. (2006) Aerobic exercise and muscle metabolism in patients with mitochondrial myopathy. *Muscle Nerve* 33(4):524-531

Tschopp, J. M., Gabathuler, J., Righetti, A. and Junod, A. F. (1985) Comparative effects of acute O2 breathing and terbutaline in patients with chronic obstructive pulmonary disease. A combined hemodynamic and radionuclide study. *Eur J Respir Dis* 67(5):351-359

Tuder, R. M. and Petrache, I. (2012) Pathogenesis of chronic obstructive pulmonary disease. *J Clin Invest* 122(8):2749-2755

Turner, B. E., Hodkinson, P. D., Timperley, A. C. and Smith, T. G. (2015) Pulmonary artery pressure response to simulated air travel in a hypobaric chamber. *Aerosp Med Hum Perform* 86(6):529-534

Turner, P. J. and Buckler, K. J. (2013) Oxygen and mitochondrial inhibitors modulate both monomeric and heteromeric TASK-1 and TASK-3 channels in mouse carotid body type-1 cells. *J Physiol* 591(23):5977-5998

van der Vorm, L. N., Hendriks, J. C., Laarakkers, C. M., Klaver, S., Armitage, A. E., Bamberg, A., Geurts-Moespot, A. J., Girelli, D., Herkert, M., Itkonen, O., Konrad, R. J., Tomosugi, N., Westerman, M., Bansal, S. S., Campostrini, N., Drakesmith, H., Fillet, M., Olbina, G., Pasricha, S. R., Pitts, K. R., Sloan, J. H., Tagliaro, F., Weykamp, C. W. and Swinkels, D. W. (2016) Toward worldwide hepcidin assay harmonization: Identification of a commutable secondary reference material. *Clin Chem* 62(7):993-1001

van der Zwaard, S., de Ruiter, C. J., Noordhof, D. A., Sterrenburg, R., Bloemers, F. W., de Koning, J. J., Jaspers, R. T. and van der Laarse, W. J. (2016) Maximal oxygen uptake is proportional to muscle fiber oxidative capacity, from chronic heart failure patients to professional cyclists. *J Appl Physiol (1985)* 121(3):636-645

Van Thienen, R. and Hespel, P. (2016) Enhanced muscular oxygen extraction in athletes exaggerates hypoxemia during exercise in hypoxia. *J Appl Physiol (1985)* 120(3):351-361

Vander Heiden, M. G., Cantley, L. C. and Thompson, C. B. (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324(5930):1029-1033

Vanier, T., Dulfano, J., Wu, C. and Desforges, J. F. (1963) Emphysema, hypoxia and the polycthemic response. *N Engl J Med* 269(4):169-178

Vasquez, A. and Logomarsino, J. V. (2016) Anemia in chronic obstructive pulmonary disease and the potential role of iron deficiency. *COPD* 13(1):100-109

Vaux, E. C., Metzen, E., Yeates, K. M. and Ratcliffe, P. J. (2001) Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain. *Blood* 98(2):296-302

Veith, C., Schermuly, R. T., Brandes, R. P. and Weissmann, N. (2016) Molecular mechanisms of hypoxia-inducible factor-induced pulmonary arterial smooth muscle cell alterations in pulmonary hypertension. *J Physiol* 594(5):1167-1177

Vejlstrup, N. G. and Dorrington, K. L. (1993) Intense slow hypoxic pulmonary vasoconstriction in gas-filled and liquid-filled lungs: an in vivo study in the rabbit. *Acta Physiol Scand* 148(3):305-313

Verga Falzacappa, M. V., Vujic Spasic, M., Kessler, R., Stolte, J., Hentze, M. W. and Muckenthaler, M. U. (2007) STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood* 109(1):353-358

Vestbo, J., Hurd, S. S., Agusti, A. G., Jones, P. W., Vogelmeier, C., Anzueto, A., Barnes, P. J., Fabbri, L. M., Martinez, F. J., Nishimura, M., Stockley, R. A., Sin, D. D. and Rodriguez-Roisin, R. (2013) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 187(4):347-365

Viault, F. (1890) Sur l'augmentation considérable du nombre des globules rouges dans le sang chez les habitants des hauts plateaux de l'Amérique du Sud. *CR Acad Sci Paris* 111:917-918 [French].

Viethen, T., Gerhardt, F., Dumitrescu, D., Knoop-Busch, S., Ten Freyhaus, H., Rudolph, T. K., Baldus, S. and Rosenkranz, S. (2014) 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(2):233-239

Volke, M., Gale, D. P., Maegdefrau, U., Schley, G., Klanke, B., Bosserhoff, A. K., Maxwell, P. H., Eckardt, K. U. and Warnecke, C. (2009) Evidence for a lack of a direct transcriptional suppression of the iron regulatory peptide hepcidin by hypoxia-inducible factors. *PLoS One* 4(11):e7875

von Euler, U. S. and Liljestrand, G. (1946) Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol Scand* 12(4):301-320

Wagner, P. D. (1992) Ventilation-perfusion matching during exercise. *Chest* 101(5 Suppl):192s-198s

Wagner, P. D., Dantzker, D. R., Dueck, R., Clausen, J. L. and West, J. B. (1977) Ventilation-perfusion inequality in chronic obstructive pulmonary disease. *J Clin Invest* 59(2):203-216

Wagner, P. D., Laravuso, R. B., Uhl, R. R. and West, J. B. (1974a) Continuous distributions of ventilation-perfusion ratios in normal subjects breathing air and 100 per cent O2. *J Clin Invest* 54(1):54-68

Wagner, P. D., Naumann, P. F. and Laravuso, R. B. (1974b) Simultaneous measurement of eight foreign gases in blood by gas chromatography. *J Appl Physiol* 36(5):600-605

Wagner, P. D., Saltzman, H. A. and West, J. B. (1974c) Measurement of continuous distributions of ventilation-perfusion ratios: theory. *J Appl Physiol* 36(5):588-599

Wagner, P. D. and West, J. B. (1972) Effects of diffusion impairment on O2 and CO2 time courses in pulmonary capillaries. *J Appl Physiol* 33(1):62-71

Walters, G. O., Miller, F. M. and Worwood, M. (1973) Serum ferritin concentration and iron stores in normal subjects. *J Clin Pathol* 26(10):770-772

Waltner, K. and Waltner, K. (1929) Kobalt und blut. *Klinische Wochenschrift* 8(7):313 [German].

Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A* 92(12):5510-5514

Wang, G. L. and Semenza, G. L. (1993) Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* 82(12):3610-3615

Wang, G. L. and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* 270(3):1230-1237

Wang, J., Fu, X., Yang, K., Jiang, Q., Chen, Y., Jia, J., Duan, X., Wang, E. W., He, J., Ran, P., Zhong, N., Semenza, G. L. and Lu, W. (2015) Hypoxia inducible factor-1-dependent up-regulation of BMP4 mediates hypoxia-induced increase of TRPC expression in PASMCs. *Cardiovasc Res* 107(1):108-118

Wang, J., Weigand, L., Lu, W., Sylvester, J. T., Semenza, G. L. and Shimoda, L. A. (2006) Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca2+ in pulmonary arterial smooth muscle cells. *Circ Res* 98(12):1528-1537

Wang, W., Knovich, M. A., Coffman, L. G., Torti, F. M. and Torti, S. V. (2010) Serum ferritin: Past, present and future. *Biochim Biophys Acta* 1800(8):760-769

Warburg, O. (1924) Über den stoffwechsel der carcinomzelle. *Naturwissenschaften* 12(50):1131-1137 [German].

Ward, H. P., Kurnick, J. E. and Pisarczyk, M. J. (1971) Serum level of erythropoietin in anemias associated with chronic infection, malignancy, and primary hematopoietic disease. *J Clin Invest* 50(2):332-335

Watts, G. (2010) Obituary: Clement Alfred Finch. *Lancet* 376(9740):510

Waypa, G. B., Guzy, R., Mungai, P. T., Mack, M. M., Marks, J. D., Roe, M. W. and Schumacker, P. T. (2006) Increases in mitochondrial reactive oxygen species trigger hypoxia-induced calcium responses in pulmonary artery smooth muscle cells. *Circ Res* 99(9):970-978

Weibel, E. R. and Hoppeler, H. (2005) Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. *J Exp Biol* 208(Pt 9):1635-1644

Weinstein, D. A., Roy, C. N., Fleming, M. D., Loda, M. F., Wolfsdorf, J. I. and Andrews, N. C. (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood* 100(10):3776-3781

Weir, E. K., Lopez-Barneo, J., Buckler, K. J. and Archer, S. L. (2005) Acute oxygen-sensing mechanisms. *N Engl J Med* 353(19):2042-2055

Weisse, A. B., Moschos, C. B., Frank, M. J., Levinson, G. E., Cannilla, J. E. and Regan, T. J. (1975) Hemodynamic effects of staged hematocrit reduction in patients with stable cor pulmonale and severely elevated hematocrit levels. *Am J Med* 58(1):92-98

Weitzenblum, E., Hirth, C., Ducolone, A., Mirhom, R., Rasaholinjanahary, J. and Ehrhart, M. (1981) Prognostic value of pulmonary artery pressure in chronic obstructive pulmonary disease. *Thorax* 36(10):752-758

Weitzenblum, E., Hirth, C., Parini, J. P., Rasaholinjanahary, J. and Oudet, P. (1978) Clinical, functional and pulmonary hemodynamic course of patients with chronic obstructive pulmonary disease followed-up over 3 years. *Respiration* 36(1):1-9

Weitzenblum, E., Loiseau, A., Hirth, C., Mirhom, R. and Rasaholinjanahary, J. (1979) Course of pulmonary hemodynamics in patients with chronic obstructive pulmonary disease. *Chest* 75(6):656-662

Weitzenblum, E., Sautegeau, A., Ehrhart, M., Mammosser, M. and Pelletier, A. (1985) Long-term oxygen therapy can reverse the progression of pulmonary hypertension in patients with chronic obstructive pulmonary disease. *Am Rev Respir Dis* 131(4):493-498

Wenger, R. H. and Hoogewijs, D. (2010) Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. *Am J Physiol Renal Physiol* 298(6):F1287-1296

West, J. B. (1962) Regional differences in gas exchange in the lung of erect man. *J Appl Physiol* 17:893-898

West, J. B. (2011a) Causes of and compensations for hypoxemia and hypercapnia. *Compr Physiol* 1(3):1541-1553

West, J. B. (2011b) Comparative physiology of the pulmonary circulation. *Compr Physiol* 1(3):1525-1539

West, J. B. (2013) Torricelli and the ocean of air: the first measurement of barometric pressure. *Physiology (Bethesda)* 28(2):66-73

West, J. B., Dollery, C. T. and Naimark, A. (1964) Distribution of blood flow in isolated lung; relation to vascular and alveolar pressures. *J Appl Physiol* 19:713-724

West, J. B., Lahiri, S., Maret, K. H., Peters, R. M., Jr. and Pizzo, C. J. (1983) Barometric pressures at extreme altitudes on Mt. Everest: physiological significance. *J Appl Physiol Respir Environ Exerc Physiol* 54(5):1188-1194

West, J. B. and Wagner, P. D. (1980) Predicted gas exchange on the summit of Mt. Everest. *Respir Physiol* 42(1):1-16

West, J. B., Watson, R. R. and Fu, Z. (2007) The human lung: did evolution get it wrong? *Eur Respir J* 29(1):11-17

Westcott, R. N., Fowler, N. O., Scott, R. C., Hauenstein, V. D. and McGuire, J. (1951) Anoxia and human pulmonary vascular resistance. *J Clin Invest* 30(9):957-970

Whitman, E. M., Pisarcik, S., Luke, T., Fallon, M., Wang, J., Sylvester, J. T., Semenza, G. L. and Shimoda, L. A. (2008) Endothelin-1 mediates hypoxia-induced inhibition of voltage-gated K+ channel expression in pulmonary arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* 294(2):L309-318

Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J. and Maxwell, P. H. (1998) Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 92(7):2260-2268

Wilkinson, N. and Pantopoulos, K. (2013) IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood* 122(9):1658-1668

Wilkinson, N. and Pantopoulos, K. (2014) The IRP/IRE system in vivo: insights from mouse models. *Front Pharmacol* 5:176

Willis, W. T., Ganley, K. J. and Herman, R. M. (2005) Fuel oxidation during human walking. *Metabolism* 54(6):793-799

Winslow, R. M., Monge, C. C., Brown, E. G., Klein, H. G., Sarnquist, F., Winslow, N. J. and McKneally, S. S. (1985) Effects of hemodilution on O2 transport in high-altitude polycythemia. *J Appl Physiol (1985)* 59(5):1495-1502

Wittenberg, B. A. and Wittenberg, J. B. (1989) Transport of oxygen in muscle. *Annu Rev Physiol* 51:857-878

Woodruff, P. G., Agusti, A., Roche, N., Singh, D. and Martinez, F. J. (2015) Current concepts in targeting chronic obstructive pulmonary disease pharmacotherapy: making progress towards personalised management. *Lancet* 385(9979):1789-1798

World Health Organisation (2016) www.who.int/nutrition/topics/ida/en [Accessed 08/11/2016].

Wrighting, D. M. and Andrews, N. C. (2006) Interleukin-6 induces hepcidin expression through STAT3. *Blood* 108(9):3204-3209

Wu, X. G., Wang, Y., Wu, Q., Cheng, W. H., Liu, W., Zhao, Y., Mayeur, C., Schmidt, P. J., Yu, P. B., Wang, F. and Xia, Y. (2014) HFE interacts with the BMP type I receptor ALK3 to regulate hepcidin expression. *Blood* 124(8):1335-1343

Xiao, J. J., Krzyzanski, W., Wang, Y. M., Li, H., Rose, M. J., Ma, M., Wu, Y., Hinkle, B. and Perez-Ruixo, J. J. (2010) Pharmacokinetics of anti-hepcidin monoclonal antibody Ab 12B9m and hepcidin in cynomolgus monkeys. *AAPS J* 12(4):646-657

Xu, W., Barrientos, T., Mao, L., Rockman, H. A., Sauve, A. A. and Andrews, N. C. (2015) Lethal cardiomyopathy in mice lacking transferrin receptor in the heart. *Cell Rep* 13(3):533-545

Yamaguchi, K., Mori, M., Kawai, A., Takasugi, T., Oyamada, Y. and Koda, E. (1997) Inhomogeneities of ventilation and the diffusing capacity to perfusion in various chronic lung diseases. *Am J Respir Crit Care Med* 156(1):86-93

Yang, C., Sun, M. G., Matro, J., Huynh, T. T., Rahimpour, S., Prchal, J. T., Lechan, R., Lonser, R., Pacak, K. and Zhuang, Z. (2013) Novel HIF2A mutations disrupt oxygen sensing, leading to polycythemia, paragangliomas, and somatostatinomas. *Blood* 121(13):2563-2566

Yi, X., Liang, Y., Huerta-Sanchez, E., Jin, X., Cuo, Z. X., Pool, J. E., Xu, X., Jiang, H., Vinckenbosch, N., Korneliussen, T. S., Zheng, H., Liu, T., He, W., Li, K., Luo, R., Nie, X., Wu, H., Zhao, M., Cao, H., Zou, J., Shan, Y., Li, S., Yang, Q., Asan, Ni, P., Tian, G., Xu, J., Liu, X., Jiang, T., Wu, R., Zhou, G., Tang, M., Qin, J., Wang, T., Feng, S., Li, G., Huasang, Luosang, J., Wang, W., Chen, F., Wang, Y., Zheng, X., Li, Z., Bianba, Z., Yang, G., Wang, X., Tang, S., Gao, G., Chen, Y., Luo, Z., Gusang, L., Cao, Z., Zhang, Q., Ouyang, W., Ren, X., Liang, H., Zheng, H., Huang, Y., Li, J., Bolund, L., Kristiansen, K., Li, Y., Zhang, Y., Zhang, X., Li, R., Li, S., Yang, H., Nielsen, R., Wang, J. and Wang, J. (2010) Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* 329(5987):75-78

Yock, P. G. and Popp, R. L. (1984) Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. *Circulation* 70(4):657-662

Yoshida, K., Yoshikawa, J., Shakudo, M., Akasaka, T., Jyo, Y., Takao, S., Shiratori, K., Koizumi, K., Okumachi, F., Kato, H. and et al. (1988) Color Doppler evaluation of valvular regurgitation in normal subjects. *Circulation* 78(4):840-847

Young, I. H. and Bye, P. T. (2011) Gas exchange in disease: asthma, chronic obstructive pulmonary disease, cystic fibrosis, and interstitial lung disease. *Compr Physiol* 1(2):663-697

Young, S. P., Bomford, A. and Williams, R. (1984) The effect of the iron saturation of transferrin on its binding and uptake by rabbit reticulocytes. *Biochem J* 219(2):505-510

Young, T., Skatrud, J. and Peppard, P. E. (2004) Risk factors for obstructive sleep apnea in adults. *JAMA* 291(16):2013-2016

Youngson, C., Nurse, C., Yeger, H. and Cutz, E. (1993) Oxygen sensing in airway chemoreceptors. *Nature* 365(6442):153-155

Yu, A. Y., Shimoda, L. A., Iyer, N. V., Huso, D. L., Sun, X., McWilliams, R., Beaty, T., Sham, J. S., Wiener, C. M., Sylvester, J. T. and Semenza, G. L. (1999) Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *J Clin Invest* 103(5):691-696

Yuan, Y., Hilliard, G., Ferguson, T. and Millhorn, D. E. (2003) Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J Biol Chem* 278(18):15911-15916

Zakin, M. M. (1992) Regulation of transferrin gene expression. *FASEB J* 6(14):3253-3258

Zhang, D. L., Ghosh, M. C. and Rouault, T. A. (2014a) The physiological functions of iron regulatory proteins in iron homeostasis - an update. *Front Pharmacol* 5:124

Zhang, X., Zhang, W., Ma, S. F., Miasniakova, G., Sergueeva, A., Ammosova, T., Xu, M., Nekhai, S., Nourai, M., Wade, M. S., Prchal, J. T., Garcia, J. G., Machado, R. F. and Gordeuk, V. R. (2014b) Iron deficiency modifies gene expression variation induced by augmented hypoxia sensing. *Blood Cells Mol Dis* 52(1):35-45

Zhao, N., Zhang, A. S. and Enns, C. A. (2013) Iron regulation by hepcidin. *J Clin Invest* 123(6):2337-2343

Zhu, B. Z., Har-El, R., Kitrossky, N. and Chevion, M. (1998) New modes of action of desferrioxamine: scavenging of semiquinone radical and stimulation of hydrolysis of tetrachlorohydroquinone. *Free Radic Biol Med* 24(2):360-369

Zielinski, J., Tobiasz, M., Hawrylkiewicz, I., Sliwinski, P. and Palasiewicz, G. (1998) Effects of long-term oxygen therapy on pulmonary hemodynamics in COPD patients: a 6-year prospective study. *Chest* 113(1):65-70

Zigmond, A. S. and Snaith, R. P. (1983) The hospital anxiety and depression scale. *Acta Psychiatr Scand* 67(6):361-370

Zimmermann, M. B. (2008) Methods to assess iron and iodine status. *Br J Nutr* 99 Suppl 3:S2-9

Zimmermann, M. B. and Hurrell, R. F. (2007) Nutritional iron deficiency. *Lancet* 370(9586):511-520