

The role of hypoxia-inducible factor
in systemic human physiology

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF OXFORD

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Trinity Term 2010

To Jesus

Acknowledgements

I would like to express my most sincere and profound gratitude to my family Fabio, Francesca, Maurizia and Pietro for their support during all these years I have lived away from home, which is not seen as a good thing in Italia.

Ivie's company, Nigerian stew, pineapple tart and support have brightened up so many days, and were greatly appreciated.

I would like to thank my supervisors Keith Dorrington and Peter Robbins for giving me the fantastic opportunity to study and work with them, for their patience, encouragement, consideration, and for their invaluable teaching. A brief note to thank Keith also for the stimulating discussions on Christianity, and for not managing to put forward sufficiently convincing arguments in favour of atheism.

My doctoral studies were partly funded by University College, Oxford and by the Department of Physiology, thanks to the persuasiveness of my supervisors.

My doctoral work has been very enjoyable thanks to present and past members of the Robbins', Clarke's and Frayn's groups. In particular I would like to thank Marzieh Fatemian, David O'Connor, Chun Liu, Jerome Brooks, Quentin Croft, Thomas Smith, Rosa Cardenas, Mari Herigstad, Nick Talbot, Annabel Nickol, Carly Wheeler, Koji Ishida, Mary Slingo, Nayia Petousi, Peter Ratcliffe, Keith Frayn, Fredrik Karpe, Jane Cheeseman, Louise Dennis, Sandy Humphreys, Yaso Emmanuel, Lindsay Edwards and Kieran Clarke for their company and help with my work. I would also like to thank all the haematologists and physicians who helped recruiting patients for the studies. Professor Paul Greenhaff's help with the analysis of muscle biopsies is also acknowledged.

Most importantly, I am very grateful to patients and participants who gave their time and (part of their) bodies to take part in my experiments.

An important acknowledgement to VHL patients, for showing me that after all, in life, happiness is a choice.

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The role of hypoxia-inducible factor in systemic human physiology

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Thesis submitted for the degree of doctor of philosophy at the University of Oxford

Trinity Term 2010

Abstract

This thesis summarizes a research programme on the role of hypoxia-inducible factor (HIF) and its 2alpha subunit in systemic human physiology. Experiments were performed to assess the role of HIF in the regulation of skeletal muscle metabolism, cardiac anatomy, function and energy metabolism, and in cardiopulmonary physiology. Patients with different genetic mutations affecting the HIF pathway were recruited for each main study.

Chapter 1 presents an overview of human physiological responses to hypoxia in a historical perspective, with particular attention to the areas of human physiology that are relevant for the studies presented in the experimental chapters. Chapter 1 also presents a summary of the HIF pathway and the novel findings presented in this thesis.

Chapter 2 illustrates the methods used to perform the experiments.

Chapter 3 investigates skeletal muscle metabolism, cardiac anatomy, function and energy metabolism in patients with Chuvash polycythaemia, who have mildly elevated levels of HIF, associated with a mutation in von Hippel-Lindau gene, at whole body level. Chapter 3 shows major abnormalities associated with HIF pathway alterations in skeletal muscle energy metabolism, especially in conditions of metabolic stress such as during exercise and digestion of a meal.

Chapter 4 shows that patients with Chuvash polycythaemia also have small hearts and reduced cardiac energy levels.

Chapter 5 explores cardiopulmonary abnormalities in patients with gain-of-function mutations specifically in HIF-2alpha subunit; these patients are polycythaemic like patients with Chuvash polycythaemia. Observed abnormalities include pulmonary hypertension, elevated heart rate, cardiac output, ventilation, and the increment in pulmonary blood pressure in response to moderate hypoxia.

Chapter 6 presents results from experiments in patients with classic von Hippel-Lindau disease, who are not usually polycythaemic. However, some degree of haploinsufficiency was observed in their neutrophils, suggesting a pseudo-hypoxic phenotype. Chapter 6 shows that von Hippel-Lindau disease is not associated with major cardiopulmonary abnormalities.

Overall, the research reported in this thesis presents original experimental evidence for the effects of alterations in the HIF pathway on human physiology.

Abbreviations used in this thesis

3-OHB, 3-hydroxybutyrate
A, aortic cross sectional area
ATP, adenosine triphosphate
CO, cardiac output
CP, Chuvash polycythaemia
CS, citrate synthase
DBP, diastolic blood pressure
ECG, electrocardiography
FID, free induction decay
GluDH, glutamate dehydrogenase
GlyPDH, glyceraldehyde 3 phosphate dehydrogenase
HAD, beta-hydroxy acyl-CoA dehydrogenase
HIF, hypoxia-inducible factor
HR, heart rate
LDHA, lactate dehydrogenase A
LV EDV, left ventricle end-diastolic volume
LV EF, left ventricle ejection fraction
LV mass, left ventricle mass
M1-PKM, M1 isoform of muscle pyruvate kinase
M2-PKM, M2 isoform of muscle pyruvate kinase
MHC, myosin heavy chain
MLC, myosin light chain
MRI, magnetic resonance imaging
MRS, magnetic resonance spectroscopy
NEFA, non-esterified fatty acids
NRM, nuclear magnetic resonance
NOE, nuclear Overhauser enhancement
PASP, pulmonary arterial systolic pressure
 P_i , inorganic phosphate
ppm, parts per million
 PCO_2 , end-tidal pressure of carbon dioxide
PCr, phosphocreatine
PDCt, pyruvate dehydrogenase complex total activity

PDK, pyruvate dehydrogenase kinase

PDK1, PDK2, PDK3 and PDK4, pyruvate dehydrogenase kinase isoform 1, 2, 3 and 4 respectively

PDP, pyruvate dehydrogenase phosphatase

PDP1 and PDP2, pyruvate dehydrogenase phosphatase isoform 1 and 2 respectively

PHD, prolyl hydroxylases

PFKM, muscle phosphofructokinase

PKM, muscle pyruvate kinase

RT-PCR, reverse transcription polymerase chain reaction

RV EDV, right ventricle end-diastolic volume

RV EF, right ventricle ejection fraction

RV mass, right ventricle mass

SBP, systolic blood pressure

VE, minute ventilation

VHL, von Hippel-Lindau

VTI, velocity time integral

Δ , increment.

Chapter 1. Introduction

1.1 Overview

Animal life on earth does not necessarily depend on oxygen. Some very small animals living in deep basins without oxygen in the Mediterranean Sea have recently been described (Danovaro et al. 2010). Several other creatures need very little oxygen to survive far under the sea surface, but like those currently on display at the Natural History Museum in London say: “If you lived down here, you’d look weird too” (Figure 1).



Figure 1. A creature that lives with very little oxygen deep in the sea.

Source: www.mbandf.com

These forms of life are very intriguing, but I find other more evolved organisms even more fascinating, probably because I get in contact with them more frequently. Such forms of life do depend on oxygen, and have done so for billions of years. Oxygen became abundant on our planet as a waste product of photosynthesis, a process in which sunlight contributes towards the conversion of carbon dioxide and water into

glucose. This fundamental step in the history of our planet determined the possibility for oxygen dependent life to evolve. Glycolysis is an oxygen-independent metabolic process that converts glucose into pyruvate in the cytoplasm; through glycolysis one molecule of glucose can release two molecules of adenosine triphosphate (ATP), a cellular energy source. A substantial evolutionary step into the development of eukaryote and complex multicellular organisms was the appearance of mitochondria, the cellular power stations where in the presence of oxygen at physiological levels the metabolic process of aerobic respiration can release more than 30 molecules of ATP per molecule of glucose utilized. In organisms that rely on this energy production mechanism, limited oxygen availability (hypoxia) can result in reduced ATP production due to substrate limitation. However, excessive oxygen concentrations (hyperoxia) are not beneficial for cellular life either, as they determine reactions that lead to cell death. Cells in our body have evolved in a way that is compatible with a narrow range of oxygen concentrations, and when limited oxygen becomes available, a number of processes take place with the aim of maintaining cellular oxygen homeostasis. Several studies showed that the hypoxia-inducible factor (HIF) family of transcription factors is the master regulator of cellular response to hypoxia in cells and in animal models. With the studies presented in this thesis, I tried to investigate the role of HIF in the functioning of the human body at the level of the whole organism.

1.2 Human physiological response to hypoxia

The discovery of oxygen happened only towards the end of the 18th century. Towards the end of the 19th century, Angelo Mosso was one of the first physiologists to be interested in investigating human respiration at altitudes where the concentration of oxygen is markedly lower than at sea level (Mosso 1884). For his respiratory

investigations, he founded a scientific laboratory on top of the Monte Rosa (Italy), at an altitude of 4,559 m above sea level. The research laboratory named after Queen Margherita, who was an enthusiastic mountaineer and supporter of the laboratory, is still in use in 2010. Some early, mostly anecdotal evidence on the process of gradual acclimatization to altitude in humans was reported following the Duke of the Abruzzi's expedition to the Himalaya (De Filippi 1909). At the beginning of the 20th century, scientists such as J.S. Haldane, C.G. Douglas and M. P. FitzGerald at the Oxford Laboratory of Physiology contributed to several discoveries that are fundamental for our current understanding of human physiology in hypoxia. One of their most famous research expeditions took them to Colorado for more than a month in 1911: it was called the Anglo-American Pikes Peak (4,300 m) expedition, where their colleagues Y. Henderson and E.C Schneider, respectively from Yale University and Colorado College, joined them in a number of experiments (Douglas et al. 1913). The group is shown in Figure 2.



Figure 2. A photograph of the Pikes Peak expedition members

From the left: Haldane, FitzGerald, Schneider, Henderson and Douglas at Pikes Peak, Colorado, 1911. At the beginning of the 20th century, hats were very popular amongst westerners.

Compared with the research laboratory on Monte Rosa, Pikes Peak could easily and rapidly be reached by railway, so it provided several logistical advantages for the research, and offered the opportunity to gather information on responses to acute exposure to hypoxia. According to the group's report, "the cuisine was excellent, with an ample supply of fresh meat, vegetables and fruit; and the discomforts or hardships usually associated with a stay on the summit of a high mountain in other parts of the world were entirely absent" (Douglas et al. 1913). Nevertheless, because of the rapid ascent, initially the group showed symptoms of acute mountain sickness: Haldane "on evening of arrival suffered from nausea, could not take food and had some diarrhoea and abdominal pain, but slept well and was quite fit next day, though he still had some diarrhoea and could not eat much." "The hyperpnoea on exertion seemed to diminish after the first day or two, but remained excessive. The colour of lips soon became very good, and better than usual". Most of these and similar symptoms disappeared after a few days. FitzGerald was the only female member of the group. In agreement with the Oxford University philosophy of the time, where women were not admitted to membership of the University until 1920, FitzGerald was not allowed to enjoy much of the 'excellent cuisine'. In order to understand differences between the effects of acute exposure to hypoxia at Pikes Peak and the effects of chronic exposure to hypoxia in residents at moderate altitudes, she was sent around mining camps in Colorado measuring alveolar partial pressure of carbon dioxide and haemoglobin concentrations. The numerous findings of this expedition were published in a 134-page research article in the Philosophical Transactions of the Royal Society of London (Douglas et al.

1913). A wealth of experimental evidence was collected on the process of human acclimatization to hypoxia, including changes in alveolar partial pressure of carbon dioxide, haemoglobin concentration, heart rate, blood pressure, respiration at rest and during exercise (Figure 3).



Figure 3. A photograph taken during one of the experiments at Pikes Peak.

From the left: Schneider, Haldane timing the experiment and Douglas, with his famous bag in which expired gases are collected.

These early observations led to some understanding of the differences between acute exposure to hypoxia, acclimatization, and adaptation in subsequent generations of populations chronically exposed to hypoxia, such as on the African plateau, in Tibet and on the Andes.

Human physiological responses to hypoxia clearly depend on the severity of hypoxia, its duration and the frequency of exposure. Several changes can be observed in acute settings; some of them such as the increase in ventilation (volume of air breathed per minute), heart rate and cardiac output (the amount of blood that is pumped by the heart

in one minute) take place in less than a minute. Acclimatization to hypoxia is the process by which individuals gradually adjust to hypoxia over a period of hours to months. In contrast, adaptation to a hypoxic environment usually describes changes that occur over generations and potentially confer advantages for life at altitude.

Acute exposure to hypoxia is associated with marked respiratory and cardiovascular responses, and several other physiological changes that combine to restore adequate oxygenation. A rapid increase in breathing (hyperventilation, i.e. increased frequency and depth of breathing) that aims at taking in the amount of oxygen necessary to survive can be observed within a minute, stimulated by peripheral chemoreceptors (Robbins 2007). Hyperventilation determines the increase in the ratio between the elimination of carbon dioxide and its production. This increase reduces the partial pressure of carbon dioxide in arterial blood (hypocapnia), which results in alkalosis, an increase in arterial pH. Subsequent renal compensation occurs to stabilize arterial pH; in particular, a marked increase in bicarbonate excretion is achieved through diuresis, with concomitant, temporary reduction of plasma volume. Hypoxic ventilatory decline is observed over 15-30 minutes, possibly controlled centrally in the medulla or peripherally at the carotid bodies (Robbins 1995).

The oxygen content of arterial blood is determined by haemoglobin oxygen-carrying capacity, saturation and dissolved oxygen. Haemoglobin concentration in the blood increases during acclimatization to hypoxia (Viault 1890). This change is associated with an improved oxygen carrying capacity of the blood. A few hours of exposure to hypoxia are sufficient to stimulate the production of a hormone called erythropoietin (EPO), which increases the production of new red blood cells and raises haematocrit

(i.e. the concentration of red blood cells in whole blood). The increase in the concentrations of haemoglobin and haematocrit is in part apparent because of the decrease in plasma volume, as mentioned earlier. Overall, the augmented haemoglobin, blood haematocrit and associated amount of oxygen transported to body tissues allow cardiac output to decrease towards normoxic values over a period of weeks.

The oxygen supply to tissues in the body depends on the levels of oxygen in arterial blood and cardiac output. Acute exposure to hypoxia is associated with reduced oxygen saturation in the blood. One compensatory mechanism to maintain oxygen supply to the tissues is through the increase of cardiac output (Grollman 1930). This increase is achieved primarily through an increase in heart rate driven by sympathetic activity, while the amount of blood ejected by the heart at each beat (stroke volume) does not necessarily appear to increase significantly above resting levels (Fukuda et al. 2010; Thomson et al. 2006; Vogel & Harris 1967).

In hypoxia, the blood vessels in the lungs constrict, a phenomenon known as hypoxic pulmonary vasoconstriction. This response is not advantageous for acclimatization and, at a first sight appears counterintuitive. However, in different circumstances, such response could be beneficial: for example, in a dysfunctional part of a lung local pulmonary gas exchange becomes less efficient, such as in the setting of pneumonia. Blood vessels constrict in this region and as a consequence a greater blood flow is directed to better ventilated, oxygen-rich regions in the lungs. The first observation of hypoxic pulmonary vasoconstriction was performed in cat (Euler & Liljestrand 1946; Liljestrand 1948). Soon after, experimental evidence was presented for the same

phenomenon in unanaesthetized humans breathing spontaneously (Motley et al. 1947). In more recent years, some major advances have improved our understanding of the complex phenomenon of hypoxic pulmonary vasoconstriction, but significant controversy remains on many of the fundamental underlying mechanisms (Ward & McMurtry 2009; Weir & Archer 1995). Hypoxic pulmonary vasoconstriction develops in two stages: an initial vasoconstriction takes place within 5 minutes, and a second can be observed after about 40 minutes of exposure (Talbot et al. 2005). It should also be noted that individuals' response to hypoxia is variable (Dorrington et al. 1997).

Differently, the vasculature in some organs (e.g. brain, heart) dilates in response to local hypoxia in order to increase blood flow and rapidly restore oxygen homeostasis. This phenomenon of local vasodilation is particularly evident at the level of the microvasculature, and is often referred to as autoregulation. Depending on the organ and circumstances considered, vasodilation can be determined directly by the local reduction in oxygen availability (Krogh 1919; Ross et al. 1962), or indirectly through the action of metabolites (Berne 1963; Duling & Berne 1970; Tune et al. 2000). Larger blood vessels upstream of arterioles are subject to increased sympathetic drive, which can lead to elevation of systolic and diastolic arterial blood pressure (Calbet 2003).

Another strategy to facilitate oxygen delivery to the tissues in the body in hypoxia is to reduce the distance between muscle fibres and blood vessels, hence reducing oxygen diffusion distance. In theory this can be achieved by increasing the density of capillaries (number of capillaries per volume), or by reducing the size of the tissue fibres, or both. The phenomenon of increased capillary density in skeletal muscle in response to hypoxia is rather controversial, as is the concomitant reduction in muscle

fibre size, which would per se increase the capillary density (Adair et al. 1990; Banchero 1987). Early studies of acclimatization suggested that several new capillaries can grow in the tissues in response to hypoxia, particularly in active tissues such as the myocardium (Renkin 1967). Several studies have showed a decrease in skeletal muscle fibre size, which contributes to the reduction in oxygen diffusion distance (Banchero 1975; Cassin et al. 1971; Hoppeler et al. 1990; MacDougall et al. 1991). Studies that took reduction in muscle fibre size into consideration did not necessarily find an increase in the number of capillaries per cross sectional area (Banchero 1982; Mizuno et al. 2008; Sillau et al. 1980; Sillau & Banchero 1977; Snyder et al. 1985). Other more recent studies have not found that limited exposure to hypoxia changes either muscle size or capillary density (Lundby et al. 2004), or suggest that such changes can be observed only in some muscles (Deveci et al. 2001). It should also be kept in mind that since at altitude physical work is harder than at sea level, levels of physical activity tend to decrease, a factor that would per se induce muscle atrophy.

A number of metabolic changes take place during acclimatization to hypoxia, particularly in skeletal muscle, where a trend towards a switch from aerobic to glycolytic metabolism can be observed. The process of cellular (aerobic) respiration involves the production of ATP by mitochondria and requires the physiological presence of oxygen. In hypoxia, glycolytic products are metabolized through anaerobic respiration, a process that does not depend on mitochondria. Accordingly, exposure to hypoxia is normally associated with a loss of mitochondria (Cerretelli et al. 2009; Hoppeler et al. 1990). Muscle oxidative capacity can be moderately reduced and an associated reduction in citrate synthase, succinate dehydrogenase and hexokinase activities can be observed (Green et al. 1989; Howald et al. 1990).

The capacity to exercise is reduced in hypoxia. This limitation is associated with lower oxygen availability at the muscular tissue level, and with smaller cardiac output, limited by the above mentioned pulmonary hypoxic vasoconstriction. Environmental hypoxia, and increased oxygen utilization of exercising muscle can lead to a marked hypoxic state in muscular tissue (Lundby et al. 2009). It could be predicted that limited oxygen availability may be associated with an early switch to anaerobic metabolism and a consequent increased production of lactate by the exercising muscle in hypoxia. Nevertheless, regulation of lactate production by exercising muscle in hypoxia has been the subject of intense debate for many years, and underlying mechanisms supporting the different theories are still far from clear (Dempsey et al. 1972; Grassi et al. 1996; Hochachka et al. 2002; Klausen et al. 1966; Lundby et al. 2009; Lundby et al. 2000; van Hall 2007; van Hall et al. 2009; West 2007).

In contrast, long term exposure to altitudes where oxygen availability is very limited imposes severe physiological stress to the human body, and may lead to natural selection: genetic mutations could be associated with physiological changes that facilitate reproduction and life at altitude. For example, earlier in 2010 studies showed that the lower haemoglobin in Tibetans is associated with specific genetic mutations in HIF-2alpha and in PHD2 (Beall et al. 2010; Bigham et al. 2010; Yi et al. 2010), particularly relevant for the implications of Chapter 5.

The study of human physiological responses to hypoxia is particularly important because of the several medical conditions in which the whole organism or parts of it

become hypoxic, for example in cardiovascular disease, respiratory disease or cancer. Improving our understanding of human physiological responses to hypoxia is a necessary basis for research investigating how to interfere with such responses when travelling to altitude and in clinical settings. Great advances have been made since the discovery of the hypoxia-inducible factor (HIF) family of transcription factors (Firth et al. 1994; Firth et al. 1995; Wang et al. 1995; Wang & Semenza 1995), and the description of human genetic mutations affecting the regulation of the HIF pathway locally or at the level of the whole organism (Ang et al. 2002; Latif et al. 1993). Such mutations are associated with alterations of oxygen sensing: for example early observations were reported for a condition called Chuvash polycythaemia (CP), where patients show elevated haematocrit (Polyakova 1974). The next paragraph describes the HIF pathway in more detail, and presents a short literature review on the involvement of HIF in the regulation of cellular, animal and human physiology.

1.3 Involvement of hypoxia-inducible factor (HIF) in response to hypoxia

The homeostatic drive underlying the systemic effects described above is similarly evident at the cellular level. In cells hypoxia stimulates a coordinated transcriptional response that seeks to restore oxygen homeostasis; the HIF family of transcription factors orchestrates such response. An early observation towards the discovery of HIF was that a nuclear factor induced by hypoxia binds to the human erythropoietin gene enhancer (Semenza & Wang 1992). Later studies revealed that the hypoxia-inducible factor was a heterodimer composed of two subunits: an oxygen dependent HIF-1alpha subunit, and a constitutively expressed HIF-1beta subunit (Wang et al. 1995; Wang & Semenza 1995). Similar oxygen dependent proteins that dimerise with HIF-1beta were also identified and named HIF-2alpha (Tian et al. 1997) and HIF-3alpha (Gu et al.

1998). HIF-1alpha and HIF-2alpha have been subject of intense investigation that provided evidence for their regulation, functions and target genes. Differently, the function and regulation of HIF-3alpha are not well defined.

The regulation of HIF-alpha subunits varies in different cells. Generally, under aerobic conditions, and in the presence of alpha-ketoglutarate and iron, HIF-alpha subunits are hydroxylated by prolyl hydroxylases (Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001), bind to the von Hippel-Lindau (VHL) protein (Maxwell et al. 1999; Ohh et al. 2000), and undergo rapid ubiquitination and proteasomal degradation (Figure 4).

Consequently, the transcription complex does not accumulate at physiological levels of oxygen. Differently, the hydroxylation of HIF-alpha subunits is inhibited under hypoxic conditions; this inhibition impairs binding to VHL and determines the accumulation of transcriptionally active HIF-alpha complexes in the cytoplasm.

Accumulated HIF-alpha translocates into the nucleus where it dimerizes with HIF-beta and leads to increased transcription of target genes into mRNA (Figure 4).

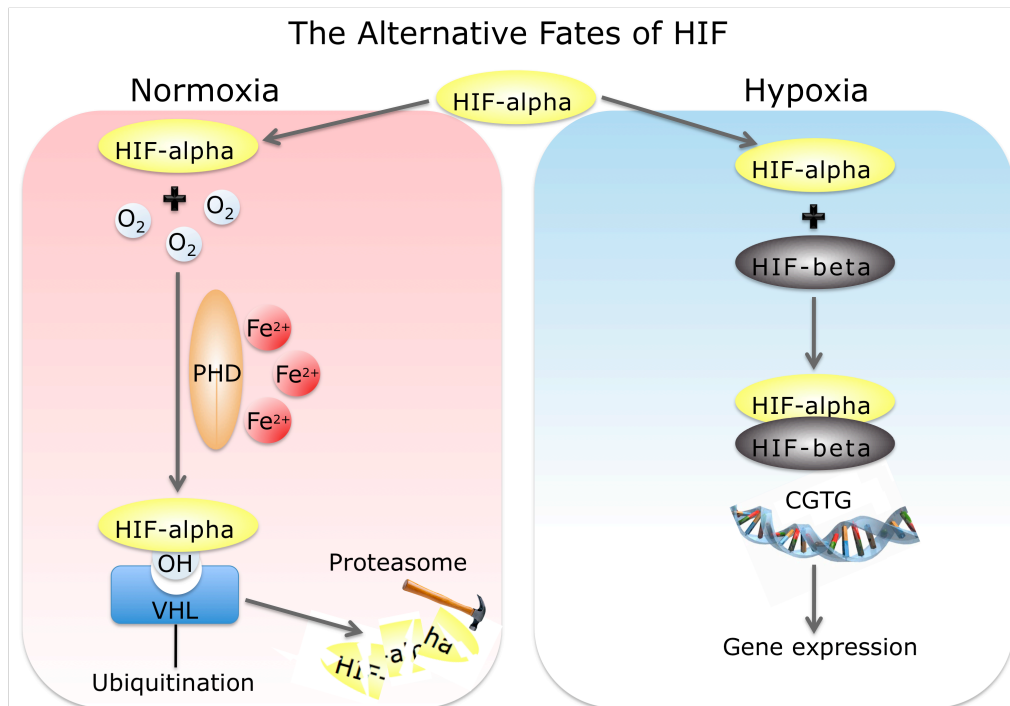


Figure 4. The alternative fates of HIF

Studies at the cellular level indicate that HIF directly or indirectly regulates several hundred genes that are involved in a great variety of functional pathways including erythropoiesis, angiogenesis, cell growth, apoptosis, energy metabolism and vasomotor regulation (Schofield & Ratcliffe 2004; Semenza 2004; Semenza 2007). For example, experiments in rats showed that in the lungs HIF inhibits the expression of voltage-gated potassium channels, increases calcium concentration and consequently leads to depolarization and contraction of pulmonary arterial smooth muscle cells (PASMC) (Whitman et al. 2008). HIF also increases intracellular pH, hence inducing proliferation in PASMC (Shimoda et al. 2006), and is associated with PASMC hypertrophy (Shimoda et al. 2001). Overall, HIF can cause the reduction of luminal diameter of pulmonary arterioles that underlies pulmonary hypertension. HIF

also stimulates erythropoiesis through a number of target genes; directly through the hypoxia-induced erythropoietin (EPO) (Semenza & Wang 1992) and EPO receptor (Manalo et al. 2005), and indirectly through the control of iron uptake and transport, respectively via the cellular iron uptake mediator transferrin receptor (Lok & Ponka 1999; Tacchini et al. 1999), and transferrin (Rolfs et al. 1997). In particular, the HIF-2 α subunit plays a central role in the control of erythropoiesis (Gruber et al. 2007). HIF stimulates a number of metabolic genes that switch cellular metabolism from aerobic to anaerobic, so that cells can survive in hypoxia, a phenomenon particularly evident in hypoxic tumor tissues. These glycolytic genes include hexokinase 2 (Riddle et al. 2000), phosphofructokinase (Minchenko et al. 2002; Minchenko et al. 2003), glyceraldehyde-3-phosphate dehydrogenase (Graven et al. 1999), muscle pyruvate kinase (Hasan et al. 2010; Hu et al. 2003; Semenza et al. 1994), pyruvate dehydrogenase kinase (Kim et al. 2006; Papandreou et al. 2006), which reduces the conversion of pyruvate into acetyl-CoA by inhibiting mitochondrial pyruvate dehydrogenase complex, and lactate dehydrogenase A (Semenza et al. 1996). Importantly, mitochondrial autophagy is also associated with HIF expression in hypoxia (Zhang et al. 2008).

A number of human genetic disorders of the HIF-signalling pathway have been described. Briefly, for this thesis I have considered three conditions. Chuvash polycythaemia (CP) is an autosomal recessive disorder that affects the HIF pathway through a mutation in VHL that impairs the binding of the gene product VHL to HIF at physiological oxygen levels (Ang et al. 2002); consequently, HIF accumulates within the cell and increases the expression of HIF target genes. The Chuvash mutation is associated with a clear phenotype of high haematocrit and haemoglobin concentrations

(Polyakova 1974; Sergeyeva et al. 1997), elevated pulmonary arterial systolic pressure, reduced systemic arterial pressure, and a marked increase in the sensitivity of the respiratory system and of the pulmonary vasculature to acute exposure to hypoxia (Bushuev et al. 2006; Smith et al. 2006). The number of these patients is elevated in Chuvashia, South-West Russia (Bushuev et al. 2006; Gordeuk & Prchal 2006; Gordeuk et al. 2004; Polyakova 1974), while it is very limited elsewhere in the world (Bento et al. 2005; Cario et al. 2005; Pastore et al. 2003a; Pastore et al. 2003b; Perrotta et al. 2006). Despite the numerous oxygen-sensing associated abnormalities in CP, no metabolic measures have been reported. Chapters three and four explore the effects of a mild elevation of HIF levels, such as in CP, on skeletal and cardiac energy metabolism in humans.

The second condition I studied is associated with germline heterozygous gain-of-function mutations specifically in HIF-2alpha subunit. There is no evidence that the mutations in HIF-2alpha affect HIF-1alpha protein levels in these patients. Patients with these mutations have polycythaemia, and were originally described in a recent study (Percy et al. 2008b). Further research replicated the findings, although the number of patients diagnosed with this mutation remains very limited (Gale et al. 2008; Martini et al. 2008; Percy et al. 2008a). Chapter five investigates whether other aspects of the CP phenotype, apart from the polycythaemia, are reproduced in patients with HIF-2alpha gain-of-function mutations.

The third condition I studied is classic VHL disease, caused by heterozygous mutations in VHL that after a second, somatic mutation have a severe effect on function (Latif et al. 1993). VHL disease is a hereditary cancer syndrome where

tumours form following a second (somatic) mutation, when the function of both VHL alleles has been disrupted (Knudson 1986; Knudson & Strong 1972; Prowse et al. 1997). Although VHL patients have one functioning allele for VHL and are not usually polycythaemic, their neutrophils exhibit delayed apoptosis and enhanced bacterial phagocytosis (Walmsley et al. 2006), indicating that at least some cells are haploinsufficient for VHL. Chapter six explores the possibility that any cardiopulmonary abnormalities observed in CP are also present in classic VHL disease.

1.4 Novel findings in this thesis

A number of novel findings emerged from the experimental work in this thesis. The study of CP revealed a striking role of HIF in the regulation of human metabolism. In particular, it showed that a mild elevation of HIF levels alters skeletal and cardiac energy metabolism, especially when metabolism is stressed through exercise. During exercise, CP patients had early and marked phosphocreatine depletion and acidosis in skeletal muscle, greater accumulation of lactate in blood, and reduced maximum exercise capacity. Muscle biopsy specimens taken from CP patients at rest showed elevated levels of transcript for pyruvate dehydrogenase kinase, phosphofructokinase, and muscle pyruvate kinase.

The study of patients with HIF-2 α gain-of-function mutations showed that such mutations are associated not only with polycythaemia, but also with other aspects of the CP phenotype such as pulmonary hypertension and low arterial partial pressure of carbon dioxide, and previously unidentified abnormalities like elevated heart rate and

cardiac output at rest. Differently, HIF-2alpha mutations did not seem to affect lactate accumulation in venous blood during exercise, and overall exercise capacity as at least one patient performed normally in the incremental exercise test on the cycle ergometer. Experiments were repeated in one of these patients after a programme of venesection. As expected, venesection had a marked effect on hemoglobin concentration, haematocrit, serum iron and ferritin. Nevertheless, venesection did not change pulmonary hypertension, cardiac output, heart rate or exercise capacity, indicating that the effects of venesection are not simply confined to those associated with changes in haematocrit.

The study of classic VHL disease did not detect cardiopulmonary abnormalities in VHL patients. Results from haematological analyses were normal, as were pulmonary blood pressure, ventilation, heart rate, cardiac output, arterial blood pressure and their responses to acute exposure to hypoxia. These results suggest that one functioning VHL allele is sufficient to maintain a normal phenotype for these aspects of cardiopulmonary physiology in humans.

Each experimental chapter is introduced and discussed individually. General information on the methods is reported in the following chapter, while more specific details are presented in the relevant chapters.

Chapter 2. Experimental methods

2.1 Overview of methods

Each subparagraph of this chapter includes an overview of the different methods, equipments, protocols, data processing and analyses used for investigation. More specific details for the individual studies are reported in the methods sections of the experimental chapters three, four, five and six.

2.2 Investigation of skeletal muscle enzymes, protein and mRNA levels through biopsy

For the study investigating the role of the Chuvash mutation in human skeletal muscle metabolism biopsies were taken from resting participants' vastus lateralis according to the methods originally described by Bergstrom (Bergstrom 1962). Participants were placed in supine position on a bed. The biopsy area was infiltrated with approximately 10 ml of 1 % lidocaine, superficially and deep into the tissue. A small incision of approximately 6 mm was made at the mid-thigh level. A 6 mm needle connected with a suction device was inserted in the muscle and the biopsy was sampled through the application of negative pressure to the inner bore of the biopsy needle. Samples were immediately frozen in liquid nitrogen and stored at -80° C for subsequent analysis. A portion of frozen muscle tissue was used to determine muscle fiber composition. The muscle was homogenized in a pyrophosphate extraction buffer (pH 9.5) supplemented with protease and phosphatase inhibitors (Sigma). Actomyosin was extracted from the homogenate by vibrating for 1 hr in a cold room (0 °C) and then centrifuging at 10,000 g for 10 min at between 2-8 °C. The supernatant was mixed 1:1 with standard Laemmli sample buffer, boiled, and then run on a 5% SDS-PAGE gel for 18 hrs at 70

V to separate the myosin heavy chain isoforms. The bands were identified with silver staining. The gel was then scanned using a Duo Scan T1200 Agfa scanner, and bands were quantified using the Quantity One programme (Bio-Rad). Optical densities were adjusted by subtracting the local background. All values were normalized to the intensity of the actin band. Estimation of muscle fiber composition using this approach has been shown to be in good agreement with a standard histochemical approach for determining fiber composition on muscle sections (Jones et al. 2004).

A second portion of the frozen muscle was freeze dried, dissected free of visible connective tissue and powdered. Muscle glycogen content was measured on this powdered tissue using a modified version of the spectrophotometric method of Harris et al. (1974).

A further portion of the frozen muscle was used to determine PDCt, that being the total (dephosphorylated) active form of the complex. PDCt was assayed in a buffer containing sucrose, Tris, EDTA, with Mg^{2+} , Ca^{2+} and dichloroacetate added to activate the PDC phosphatase and inhibit PDC kinase, respectively. PDCt was expressed as rate of acetyl-CoA formation ($nmol \cdot (min \cdot mg \text{ protein})^{-1}$) at 37 °C using a radioactive substrate. Further details are described elsewhere (Constantin-Teodosiu et al. 1991).

Another portion of muscle was homogenized on ice in a solution containing KH_2PO_4 50 mmol l^{-1} , EDTA 1 mmol l^{-1} and Triton X-100 0.05 % using a glass homogenizer. The muscle extract was then centrifuged at 20,000 g for 3 min, and the supernatant was used to measure the activities of GluDH, CS, HAD and GlyPDH as described by

Opie and Newsholme (1967) and Zammit & Newsholme (1976). Protein concentration was determined using Peterson's method (Peterson 1977).

A final portion of muscle from the CP patients was used for quantitative RT-PCR. Since there was not enough material from the matched control samples, other biopsies taken from healthy adult participants were used for this analysis. The assays used Taqman chemistry and were undertaken on an ABI prism 7000 sequence detector (Applied Biosystems, USA) according to the manufacturer's protocol. Hydroxymethyl bilane syllane (HMBS) was used as the internal control.

2.3 Incremental exercise test to exhaustion on a cycle ergometer

Overall exercise capacity was measured for the studies of patients with CP and patients with gain-of-function mutations in the HIF-2alpha gene. Participants' exercise capacity was measured through an incremental exercise test to exhaustion on a modified, electrically braked cycle ergometer (Mijnhardt KEM3, Cardiokinetics, Salford, UK). This test requires the recruitment of large muscle masses, and a significant involvement of the cardiovascular and respiratory systems; the outcome of the test is generally accepted as an index of a participant's overall exercise capacity (Hopkins et al. 2001). At the start of exercise participants were given about 30 s to achieve the required pedaling frequency (~65 revolutions per min) before the workload was increased by 20 W per min until exhaustion (Figure 5). Exhaustion was defined as the point at which the participants could no longer maintain the required pedaling frequency.

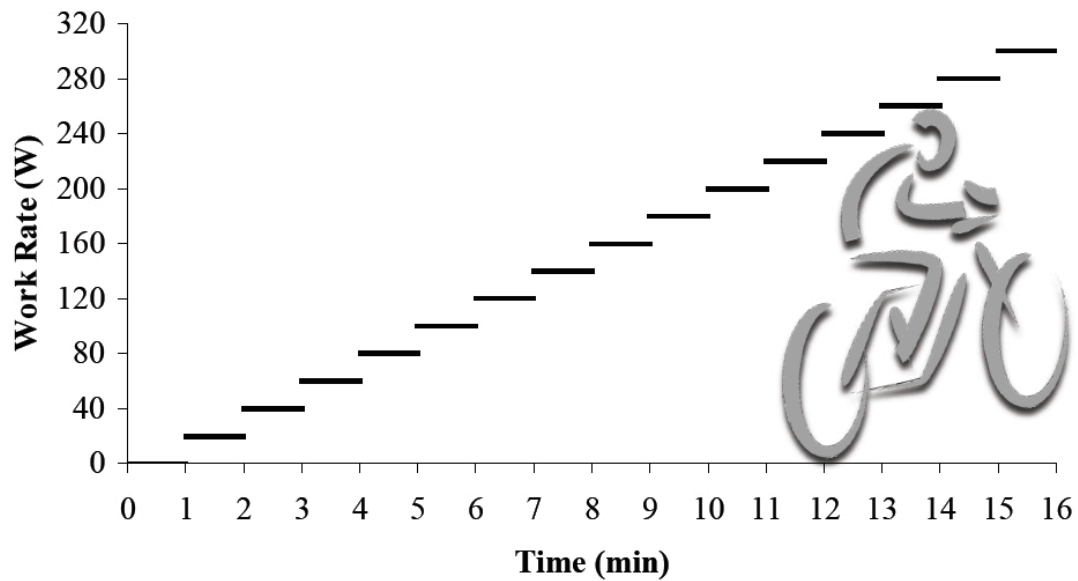


Figure 5. Protocol used for the incremental exercise test to exhaustion on a cycle ergometer.

Initial work rate was 0 W; about 30 s were given to participants to achieve the required pedalling frequency of about 65 revolutions per minute. Work rate increased by 20 W every minute until exhaustion.

Venous blood was sampled from an antecubital vein at the end of each work load (i.e. once per min) in order to measure venous blood lactate concentration. Participants breathed through a mouthpiece, with their nose occluded. Respired gases were sampled through a catheter and analyzed continuously by mass spectrometry. Respiratory volumes were measured by means of a turbine volume-measuring device. Breath-by-breath data were averaged for each min of exercise. Further details on the functioning of the apparatus and technique for the respiratory measurements are reported later, in the paragraph on breathing tests and control of expired gases. Room temperature during the tests was maintained at $\sim 21^{\circ}\text{C}$.

2.4 Investigation of skeletal muscle and cardiac metabolism by means of Magnetic Resonance Spectroscopy

Protons and neutrons form the nucleus of an atom. Some nuclei, like the hydrogen or the phosphorus nuclei, naturally spin around their own axis, which can be thought of as a tiny bar magnet. The axis of spinning nuclei is characterized by a conic movement around itself, with a direction that is opposite to the rotation of the nucleus. This movement is called precession and can be visualized with a gyroscope or a child's spinning top. The movement of the nuclear axis is caused by its magnetic properties. Not all nuclei possess this property, for example isotopes of carbon (^{12}C) and oxygen (^{16}O) do not spin. Precession generates a magnetic field, which gives the nucleus its own north and south poles. In the absence of a magnetic field, nuclei move around randomly oriented axes (Figure 6).

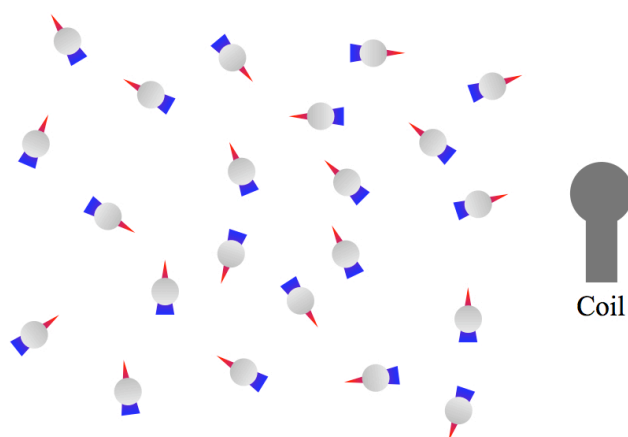


Figure 6. Nuclei in the absence of a magnetic field precess around randomly oriented axes. The dark grey icon illustrates a coil.

Differently, nuclei's axes align when placed in a magnetic field (Figure 7): just a bit more than half of them face in the direction of the magnetic field itself and the remaining ones face the opposite direction, conditions called low-energy and high-energy configurations respectively.

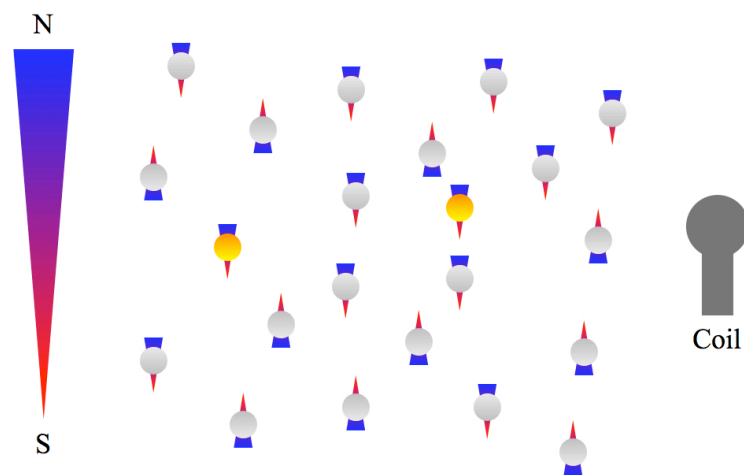


Figure 7. The application of a magnetic field illustrated by the triangle on the left determines the alignment of nuclear axes.

Two unpaired nuclei in the low-energy configuration (facing in the direction of the magnetic field) are highlighted in yellow.

Of course each of the nuclei in the high-energy configuration can be paired up with a nucleus in the low-energy configuration. The very few remaining nuclei in the low-energy configuration can respond to the application of electromagnetic pulses or the transmission of radio frequency. If an electromagnetic pulse (represented by the light blue waves) were applied to the magnetic field, the unpaired low-energy nuclei in the

field would absorb energy from the applied electromagnetic pulse, change temporarily to a high-energy configuration (Figure 8) and then resonate, radiating energy back (Figure 9). The process where nuclear magnetization returns to its initial equilibrium value is called relaxation.

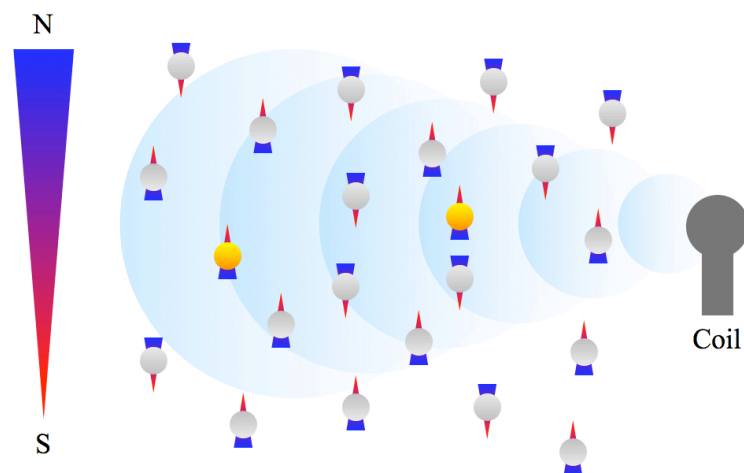


Figure 8. The coil can transmit (and receive) a radio frequency represented by the light blue waves.

The transmission of a radio frequency determines the flip of the unpaired low-energy nuclei, which change temporarily to a high-energy configuration.

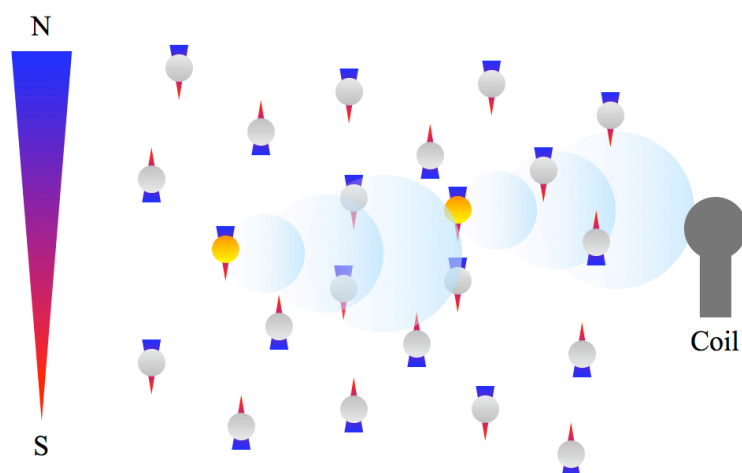


Figure 9. The two unpaired nuclei flip back to their original low-energy configuration, and return some energy back to the coil.

When a radio frequency is applied to a magnetic field, its diversity of nuclear electromagnetic or chemical properties gives rise to different resonant signals. For example, in a magnetic field of 2.35 Tesla (almost 50 times greater than the magnetic field of the earth), isotopes of phosphorus ^{31}P resonate at 40.5 MHz, while isotopes of hydrogen ^1H resonate at 100.0 MHz. The time course of the signal observed in response to a radio frequency pulse appears as an oscillation that gradually vanishes, and is called free induction decay (FID). This signal contains many frequency components associated with the variety of resonance frequencies in the field considered. In order to identify such frequencies, a mathematical manipulation known as Fourier's transformation is applied. Fourier's transformation converts the time-dependent signal into its equivalent frequency components, generating a spectrum of frequencies (Figure 10).

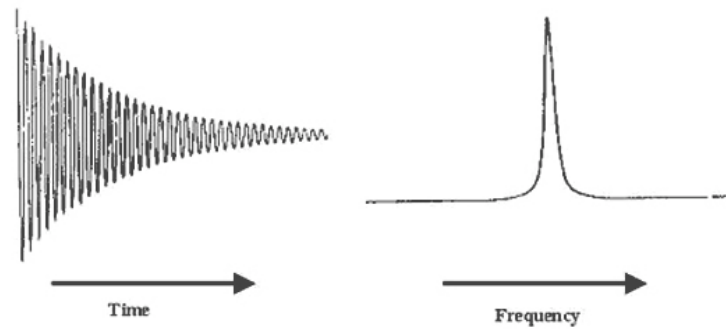


Figure 10. A diagram illustrating the conversion of a time-domain signal to a frequency-domain signal.

This conversion can be obtained through a mathematical operation called Fourier's transformation.

The intensity of each NMR signal is proportional to the number of nuclei that contributes towards it, and is calculated as the area under the relevant frequency curve. A major disadvantage of NMR is its inherent lack of sensitivity, often expressed in terms of signal-to-noise ratio (SNR). This limitation can be overcome by repeating acquisitions: in modern systems data are automatically acquired until a required SNR is obtained, and then Fourier transformed to produce the final spectrum. Typically, a minimum concentration of 0.2 mM is required for a metabolite to be detected in a metabolic study. In the early 1970s it was observed that ^{31}P magnetic resonance spectroscopy (MRS) can detect phosphate metabolites such as adenosine triphosphate (ATP), phosphocreatine (PCr) and inorganic phosphate (P_i) (Hoult et al. 1974), which have concentrations above 1 mM. Moreover, it was observed that the frequencies of some signals (such as P_i) are sensitive to pH (Moon & Richards 1973). Variations in pH result in the separation (called chemical shift) of resonance frequencies from an arbitrarily chosen reference frequency; the magnitude of the separation can be used to estimate pH. The non invasive nature of MRS allows to monitor changes in

concentrations of metabolites in vivo through changes in the intensities of their respective signals. For example, changes in the spectral lines of PCr can be interpreted in terms of metabolic changes taking place within the sample in dynamic situations, such as when a muscle is exercised.

NMR is the basis of an experimental method that allows non-invasive physiological measurements in vivo. MRS was used in this thesis for the study of skeletal muscle and cardiac energy metabolism and is described in more detail in this paragraph. Magnetic resonance imaging (MRI) was used to investigate cardiac anatomy and function, and is described later.

For some experiments presented in this thesis, skeletal muscle ^{31}P MRS was performed in a 3.0 Tesla magnet with a 60-cm clear bore (Siemens Tim Trio, Germany), interfaced with Syngo software. Participants were positioned supine and a dual-tuned ^{31}P and ^1H 6-cm-diameter surface coil was secured under the right calf muscle (Figure 11). The right foot was fastened to a custom-built non-magnetic plantar flexion exercise apparatus and was immobilized with the leg straightened. The plantar flexion exercise apparatus was similar to the one previously described by Tran et al. (1999). Briefly, a white plastic footplate was positioned on a side of a wooden box (Figure 11). The footplate was fixed in a way so that it could rotate coherently with the foot when the participant would perform an ankle plantar-flexion movement. A foot cradle was used to secure the participant's foot to the footplate. A number of green elastic cords were positioned in parallel connecting the wooden box and the footplate so that the cords would be stretched when the participant performs an ankle plantar-flexion movement. The tension of the elastic cords could be adjusted. Plantar-flexion occurred

over a limited distance because of a mechanically limited range of motion. For a given tension in the elastic cords, the associated average force that needs to be exerted during the ankle plantar-flexion movement can be calculated. The mechanical work performed can then be calculated by multiplying this average force by the known distance. By asking the participant to do perform plantar flexion movements at a given frequency, the power used during the exercise can be calculated as the product of work and movement frequency. The torque necessary to fully depress the pedal was checked regularly using a small digital scale, which in turn was calibrated using known masses. The exercise protocol consisted of 3 min of rest followed by three 5 min periods of exercise interspersed with 7 min recovery periods. The work rates for subsequent exercise periods were 3 W, 4 W and 5 W. The calf was exercised at 1 Hz, in time to a digital metronome, which was played through the magnet room speakers. Participants were continuously monitored and none had difficulty keeping time, or completing the exercise.

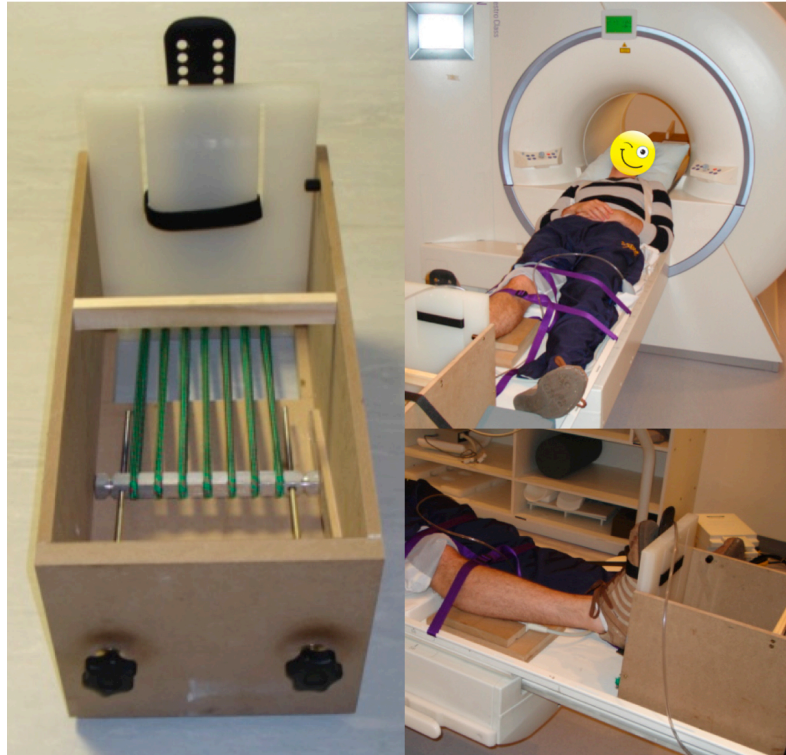


Figure 11. The apparatus used for the MRS tests.

Anti-clockwise, the wooden box with adjustable elastic cords, the purple straps used to secure the participant's leg over the surface coil and an overview of a participant just before the exercise test.

Transverse gradient echo scout images of the lower leg were acquired to ensure correct positioning of the participant's leg and to select a region of interest within the calf muscle for localized shimming. The data acquisition consisted of a series of 444 ^{31}P spectra acquired throughout the exercise protocol described above with a temporal resolution of 5 s.

After phasing and baseline correction, spectra peaks were fitted using the automated processing algorithm, advanced method of accurate, robust and efficient spectroscopy fitting AMARES, within the jMRUI software package (Version 2.2) (Naressi et al. 2001a; Naressi et al. 2001b; Vanhamme et al. 1997). Absolute millimolar

concentrations of phosphorus metabolites were calculated assuming that the concentration of ATP at rest is 8.2 mM; this commonly-used standard concentration is based on values extensively reported in the published literature (Kemp et al. 2007).

Intramuscular pH was calculated on the basis of the chemical shift (σ) of phosphate relative to phosphocreatine in parts per million:

$$\text{pH} = 6.75 + \log_{10} \left(\frac{\sigma - 3.27}{5.63 - \sigma} \right)$$

The methods used for cardiac ^{31}P MRS followed an established procedure that offers low intra-subject variability (Tyler et al. 2009a). Cardiac acquisitions were performed on a Siemens 3 Tesla TimTrio system (Erlangen, Germany), using a dedicated surface coil. Scans were performed at rest with subjects lying in the prone position on the coil. Using localisation images, the subject's position was adjusted so that the left ventricle was positioned over the centre of the coil at the magnet iso-centre. After localisation, piloting was performed using FLASH images. A series of non-localised inversion recovery spectra were acquired to measure the flip angle at a phenylphosphonic acid reference located within the coil housing. ^{31}P MRS data were acquired using a 3D acquisition-weighted chemical shift imaging (AW-CSI) sequence (acquisition matrix size 16 x 16 x 8, field of view 240 x 240 x 200 mm, 10 averages). The grid was positioned such that the longest voxel dimension was orientated down the length of the inter-ventricular septum and the grid was rotated in the cardiac short axis so that three voxels obtained maximal coverage of the septal myocardium. An optimised radio frequency excitation pulse was used which provided an average flip angle of 30.4° over the interventricular septum. Prospective ECG gating was used with a trigger delay

so that data acquisition occurred during diastole. No measures were used to minimize effects from breathing.

For post-processing analysis three voxels were selected according to strict anatomical criteria to encompass the septal myocardium. Mid-ventricular voxels were selected on the first short axis image on which the papillary muscle was visible. The three voxels, assigned as anterior, central and inferior were based on the American Heart Association standardized myocardial segmentation model (Cerqueira et al. 2002). Data from the three voxels were then summed and the spectral peaks were fitted using the automated processing algorithm AMARES. ATP values were calculated using the average of all three ATP peaks. Further details of the methods for post-processing the cardiac ^{31}P MRS data are described elsewhere (Tyler et al. 2009a).

2.5 Standardized meal test studied through arterio-venous differences

For some of the experiments in this thesis, a standardized meal test was used. The standardized meal itself consisted of Rice Krispies (Kellogg Company, UK), semi-skimmed milk, dried skimmed milk powder, fresh orange juice and a chocolate drink. The chocolate drink contained cocoa powder (2 g), palm oil (14 g), safflower oil (14 g), emulsifier (0.4 g), sweetener (1.2 g) and hot water (35.5 g). The caloric content of the fiber-free standard meal was scaled to each participant's body weight and height, so that it corresponded to about 40% of the daily energy requirement. The proportions of energy sources were constant for all participants. Protein provided 13% of the total energy intake, fat 38% and carbohydrates 49%.

Participants rested on a bed throughout the study. Cannulae were inserted under local anaesthetic into both the femoral artery and retrogradely (in the direction opposite to blood flow) into a vein draining the deep structures of the forearm. These were kept patent by a slow infusion of 0.9% (wt/vol) saline. Arterial blood is characterized by the same composition throughout the body. Differently, the composition of venous blood depends on the metabolic state of the tissues upstream of the vessel considered. By taking simultaneous samples of arterial and venous blood it is possible to calculate whether a substance is taken up or put out by the tissue upstream of the vein, as illustrated in Figure 12. For example, the concentration of lactate in arterial blood in resting humans is about 1 mM. In contrast, during exercise, the concentration of lactate in venous blood can reach values over 16 mM. By drawing simultaneous arterial and venous samples it is possible to calculate precisely how much lactate the muscles are putting out.

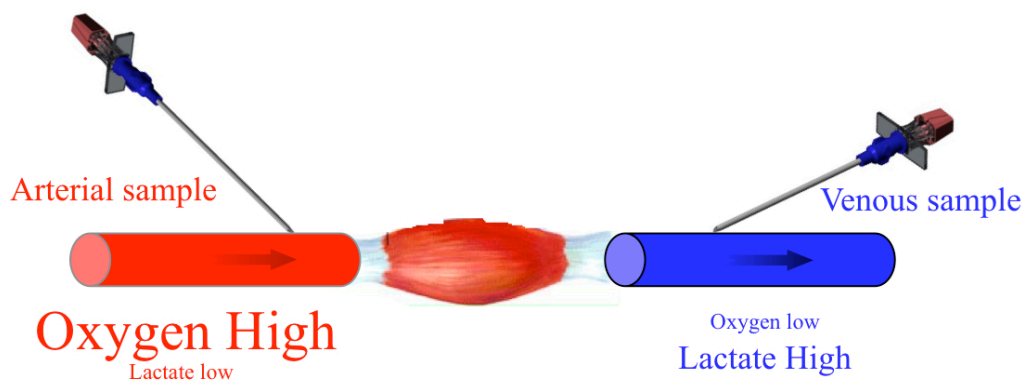


Figure 12. A diagram illustrating the technique used to measure differences between arterial and venous blood.

Serial blood samples were drawn simultaneously at the two sites to measure uptake (e.g. of oxygen) or release (e.g. of lactate) from the muscle.

Two sets of blood samples were taken 30 min apart in the fasting state. Following the standardized meal, paired arterial and venous blood samples were taken at varying time points over the subsequent 5 hours. Two min before each sample was taken, a wrist cuff was inflated to 200 mm Hg to prevent contamination of the venous blood from the forearm vein with blood from the hand. Blood samples were used to quantify glucose, insulin, lactate and pyruvate as described elsewhere (Frayn & Coppack 2001; Tan et al. 2005). Forearm muscle blood flow was assessed by strain-gauge plethysmography (Radegran 1999).

2.6 Investigation of cardiac anatomy and function by means of Magnetic Resonance Imaging

The fundamental principles on which MRI is based are very similar to those explained above for MRS, except that resonance frequency in MRI encodes for space rather than for chemistry. A rectangular vessel of water can be used to illustrate the basic principle (Figure 13). If a uniform magnetic field is applied to the water (Figure 13, A1), all nuclei will resonate at one same frequency (Figure 13, A2). Differently, if a magnetic field with a gradient (say stronger on the left than on the right) is applied (Figure 13, B1), nuclei on the left of the rectangle will resonate at a higher frequency than nuclei on the right, giving rise to a series of frequencies (Figure 13, B2). If a strip of a material containing no detectable protons is placed in the water (Figure 13, C1), no resonance frequency would be recorded in the area occupied by the strip (Figure 13, C2). The property of protons to resonate at a frequency directly proportional to the field that they experience allows detecting the spatial distribution of water in the

sample. Recording three-dimensional images requires the application of this principle and allows showing water distribution in a volume.

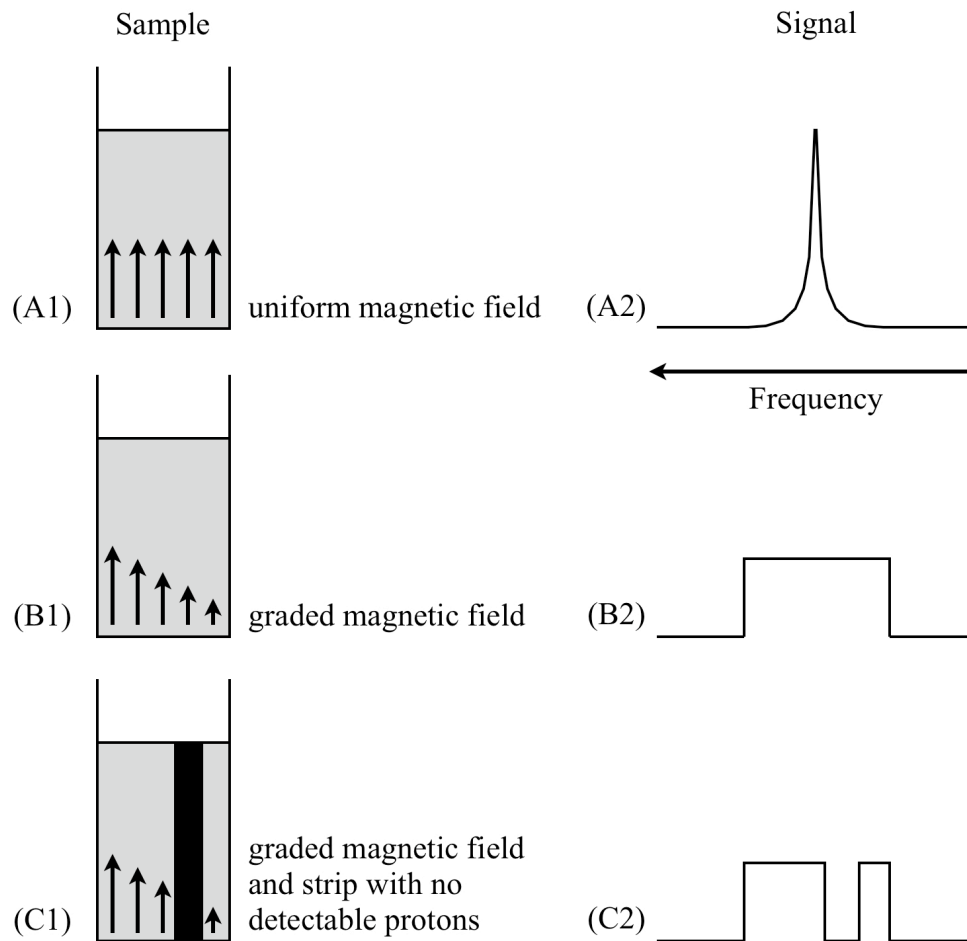


Figure 13. Diagram illustrating one-dimensional imaging.

The grey square represents the water rectangle. The magnitude of the vertical arrows is proportional to the strength of the magnetic field they represent. The black rectangle represents an area with no detectable protons.

For some of my experiment, cardiac volumes, mass and function were assessed using cine MRI performed on a 1.5 Tesla system (Sonata, Siemens Medical Solutions, Germany). Scans were performed with subjects in the supine position using a dedicated surface coil. Following localisation and pilot images, steady-state free

precession images were obtained in the cardiac vertical and horizontal long axis planes with prospective ECG triggering. These long axis images were used to plan cine images in the cardiac short axis to cover the left and right ventricles. At times, participants were asked to hold their breath in order to allow improved image quality. A series of steady-state free precession images were obtained in end-expiration with retrospective ECG gating to ensure coverage of end-diastole.

Cine images were post-processed using standard software (ARGUS Version 25A, Siemens Medical Solutions, Germany). Left and right ventricular short axis images were contoured in end-diastole and end-systole. Myocardial volume was determined by subtracting the end-systolic volume from the end-diastolic volume. Mass was determined as the myocardial volume multiplied by the specific density of myocardium (1.05 g ml^{-1}). Ejection fraction was determined as the stroke volume / end-diastolic volume.

2.7 Breathing tests and control of expired gases

Some of the results presented in this thesis were obtained from breathing tests, in which end-tidal gases were controlled while respiratory measurements were recorded. The composition of gases in the air at the end of expiration (end-tidal) is very similar to the composition of gases in the alveoli. As a consequence, the concentration of end-tidal oxygen and carbon dioxide reflects the concentration of these gases in the arterial blood.

Participants had their nose occluded and breathed through a mouthpiece connected to a turbine used to measure ventilation (Howson et al. 1986). During the hypoxia response protocols respired gases were sampled through a catheter and analyzed continuously by mass spectrometry. The control of gases was managed through the dynamic end-tidal forcing technique: end-tidal measured values were used as input for a computer that controlled a fast gas-mixing system, which in turn allowed a rapid and accurate manipulation of end-tidal partial pressure of oxygen (P_{ETO_2}) and end-tidal partial pressure of carbon dioxide (P_{ETCO_2}) in the subsequent breaths (Robbins et al. 1982a). Breath-by-breath P_{ETO_2} , P_{ETCO_2} and ventilation measurements were recorded on a computer in real time. Throughout each protocol participants were asked to recline in the left lateral position on a suitably modified couch.

The diagram in Figure 14 illustrates the fundamental steps required to perform this technique. A participant wearing a nose clip (1) breathes out into a turbine. Expired gases are analyzed by a mass spectrometer (2), which quantifies concentrations of expired oxygen and carbon dioxide. A voltage proportional to the concentration of gases is transmitted to an analogue-digital converter in a computer (3). Here, the signal measured is compared with a desired value and the difference between the two dictates the magnitude of adjustments that the fast gas-mixing system (4) needs to perform. For example, if the end-tidal concentration of oxygen measured is 60 mmHg, while the desired value is 50 mmHg, the computer would inform the fast gas-mixing system to reduce the concentration of oxygen delivered.

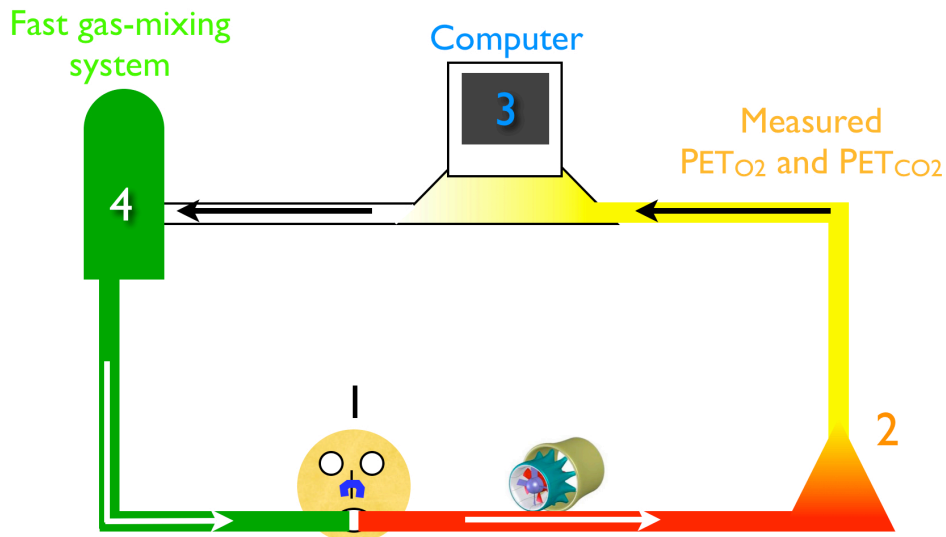


Figure 14. A simplified diagram illustrating the method for the dynamic control of end-tidal oxygen and carbon dioxide concentrations.

The dynamic end-tidal forcing technique has been originally invented in the 1970s (Swanson & Bellville 1975) for the control of end-tidal partial pressure of carbon dioxide. The first version of the system controlling both end-tidal partial pressure of oxygen and carbon dioxide was produced in the early 1980s (Robbins et al. 1982a; Robbins et al. 1982b), and is regularly used in Professor Robbins' laboratory.

2.8 Ultrasound Doppler echocardiography: principles and quantitative analysis

Sound is a mechanical wave that travels through a medium, like sea waves travel through water. A wave is characterized by its frequency, period, length and amplitude. Ultrasound refers to sound waves with frequencies higher than those perceivable by the human ear, which range approximately from 20 Hz to 20 KHz. When a wave travelling in one medium impacts a second medium it is partly reflected and partly transmitted in a way that depends on the nature of the media and the wave itself. For example, if we talk loudly in a church it is likely that sound waves generated by our

voice travelling through air are only in part transmitted to the much denser building, and mostly reflected back; this reflection of sound is called echo. The relative motion of the source of sound and of an observer determines the change in frequency of the wave. This happens when we stand by a road and an ambulance sounding a siren travels by: the sound frequency we hear is higher than the emitted one when the ambulance gets closer, and becomes lower when the ambulance drives away from us. This change in reflected wave frequency is the main working principle of modern radar too. Christian Doppler (1803 – 1853) was the first to propose this change of wave frequency effect, which was named after him. Ultrasound Doppler echocardiography is a technique substantially based on these physical principles used to examine the heart.

An echocardiography system is mainly composed by a transducer, the echocardiography machine itself and a screen. A central element in echocardiography is the crystals in the transducer: in these crystals variations in electrical currents are quantitatively converted into shape variations (that generate an ultrasound wave), and vice versa, a phenomenon called piezoelectricity. This mechanism is used in electronic scales, where by standing on them we compress the crystals, which in turn generate an electrical current proportional to our weight. The ultrasound wave emitted by the transducer is partly reflected by tissues or blood with an intensity directly proportional to the density of the media encountered, and a timing directly proportional to the distance between the transducer and the reflecting medium. Once the wave hits back the transducer, mechanical changes in the crystals are converted into electrical currents and used for analysis. On the screen of the echocardiography machine areas with low density like the lungs appear darker than areas with high density like bones. The above

mentioned Doppler effect is particularly important in echocardiography when considering moving bodies, such as red blood cells. The movement of red blood cells in relation to the transducer determines changes in the wave frequency recorded by the transducer itself, and can indicate the direction and velocity of blood in a given spatial volume. Conventionally, velocities and directions of blood flow are colour-coded: blood moving away from the transducer reduces ultrasound reflected frequency and is represented in blue, while blood moving towards the transducer increases ultrasound reflected frequency and is represented in red. The choice of these colours is based on Doppler's observations regarding the movement of stars. Blue light has a higher frequency than red light, so the light of some stars travelling towards the earth would appear blue and those moving away from the earth would appear red, phenomena called blueshift and redshift respectively.

In this thesis, ultrasound Doppler echocardiography was used to estimate the blood pressure in the lungs (pulmonary arterial systolic pressure) and the quantity of blood pumped by the heart into the circulation each minute (cardiac output).

A drawing showing the positioning of the transducer in relation to the heart is shown in Figure 15.

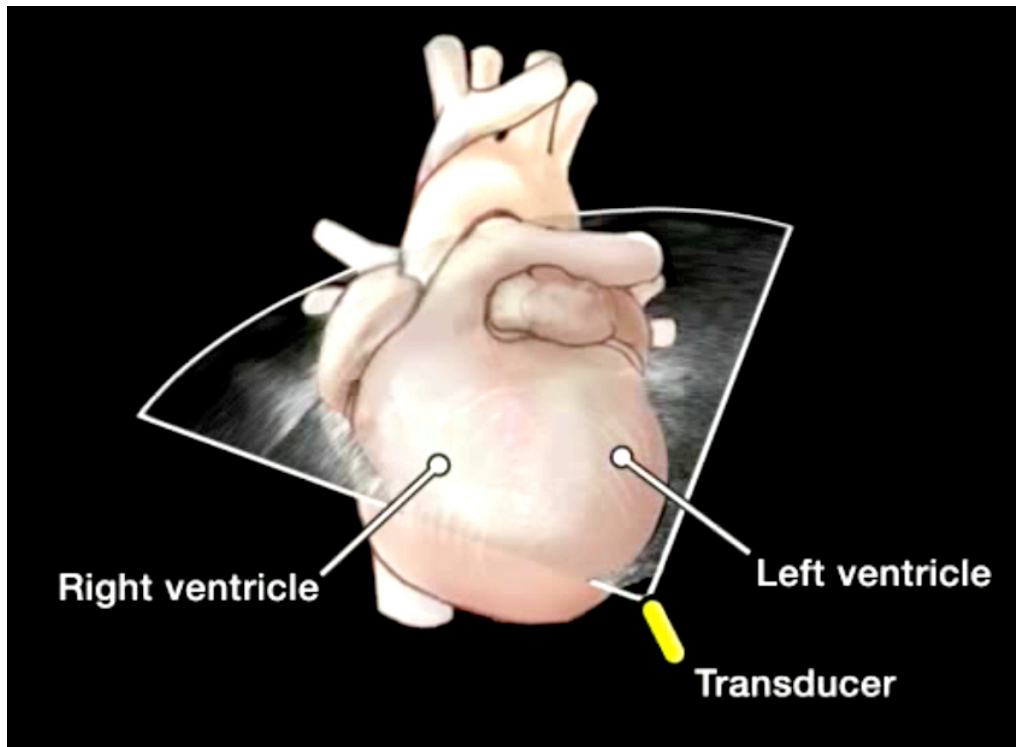


Figure 15. The transducer, represented by the yellow icon, is positioned on a participant's chest by the apex of the heart aligned with its main axis to obtain a 4-chamber apical view.

From this perspective, an apical 4-chamber view of the heart is often possible (Figure 16). In Figure 17 an overlapped diagram helps to visualize the relevant anatomical structures in the heart including the atria, ventricles, interatrial septum, interventricular septum, and the atrioventricular valves.

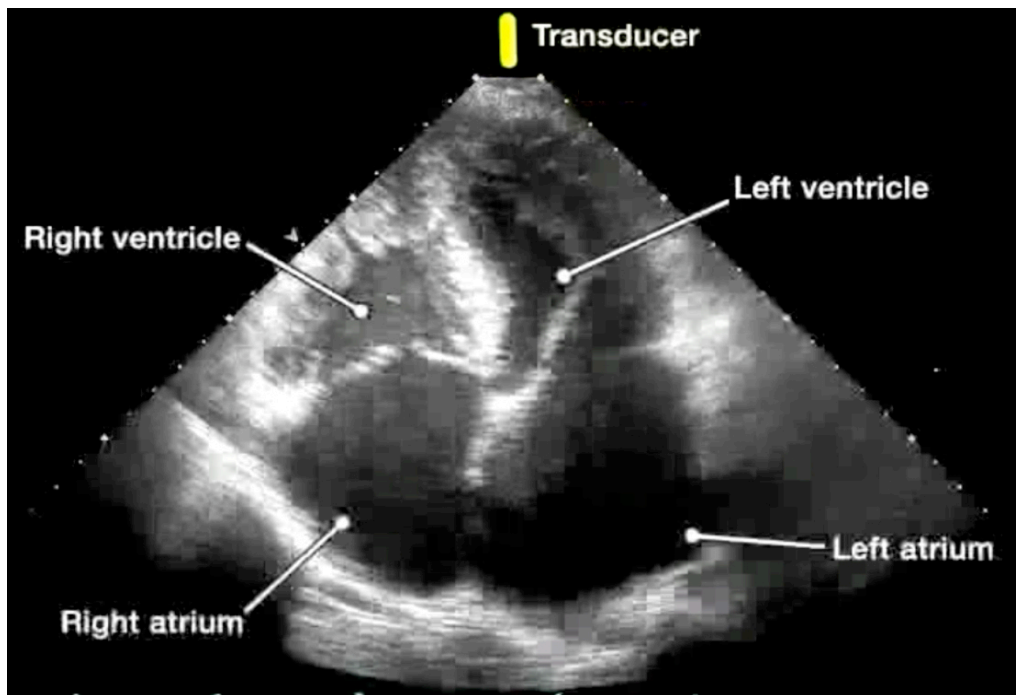


Figure 16. A typical 4-chamber image from an apical view of the heart as it appears on the screen of an ultrasound machine.

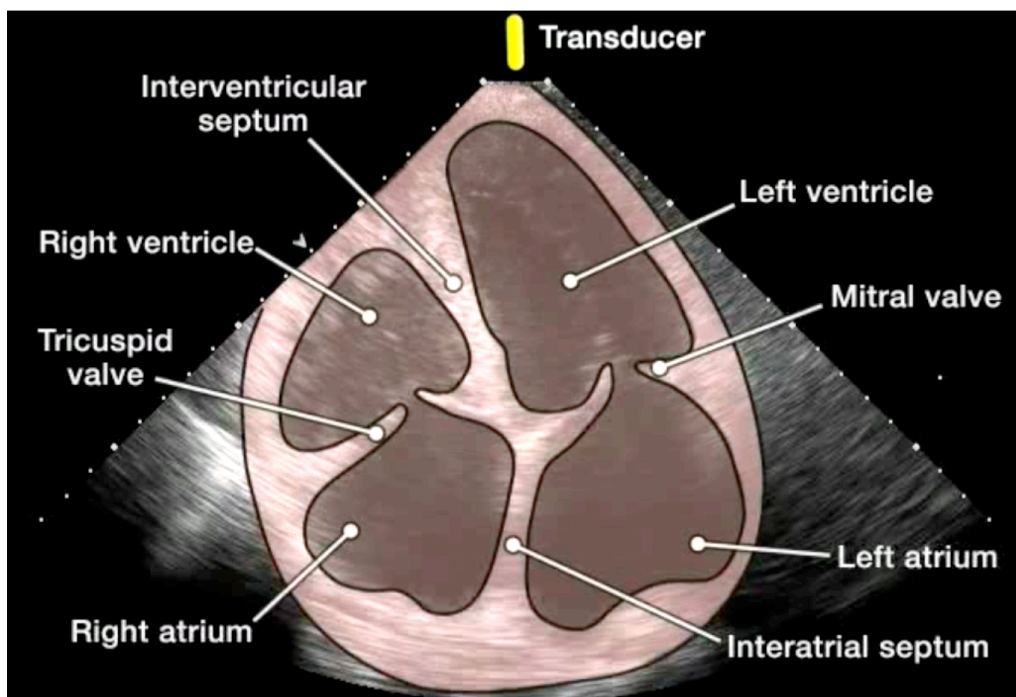


Figure 17. A diagram overlapped to a 4-chamber view of the heart, highlighting right and left ventricles and atria, interatrial and interventricular septa, the tricuspid and the mitral valve.

When the tricuspid valve does not completely occlude blood flow towards the atrium during systole, a portion of blood can flow back from the ventricle into the atrium. This phenomenon is called tricuspid regurgitation, visualized in blue in Figure 18, where lighter colours indicate higher speeds. Physiological tricuspid regurgitation is present in about 60% of people (Upward et al. 1985), and as long as its magnitude is limited it does not compromise cardiac function.

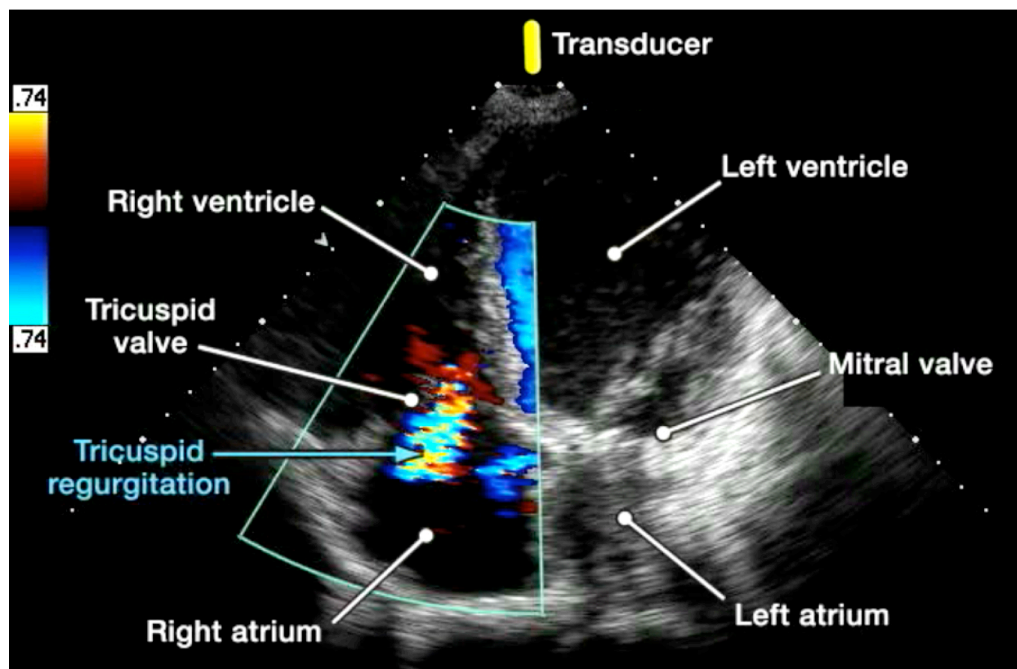


Figure 18. An image showing tricuspid regurgitation.

Regurgitation is the flow of a portion of blood from the right ventricle into the right atrium during systole, caused by a non-complete occlusion of the tricuspid valve. In this image, blue indicates blood moving away from the transducer, and red indicates blood moving towards the transducer.

The velocity of blood in the regurgitant jet is associated with the degree of constriction of the blood vessels in the lungs. In particular, if the flow of the tricuspid regurgitation

jet is steady, the maximum gradient of blood pressure across the tricuspid valve can be calculated as follows

$$\Delta P_{\max} = 4V^2$$

Here, $\Delta P_{\max}$ indicates the maximum pressure difference and V the velocity of blood. V is automatically calculated by the echocardiography machine through the difference between ultrasound frequencies emitted and received by the transducer. Human physiological blood pressure in the right atrium can be considered constant at about 5 mmHg, so in the absence of pulmonary stenosis the calculated maximum pressure difference across the tricuspid valve is an accepted estimate of the pulmonary arterial systolic pressure. Importantly, right atrial pressure is not affected by hypoxia (Groves et al. 1987). Variations in the maximum gradient of blood pressure across the tricuspid valve mirror variations in pulmonary arterial systolic pressure when participants are exposed to hypoxia, like in some of the experiments presented in this thesis.

An example of spectral Doppler diagram is illustrated in Figure 19. Here, peak tricuspid regurgitant jet velocity is approximately 3.73 m s^{-1} (circled in yellow). The calculated pulmonary arterial systolic pressure is 55.6 mm Hg [normal, $< 30 \text{ mm Hg}$], indicating an extreme constriction of the blood vessels in the lungs, a condition called pulmonary hypertension.

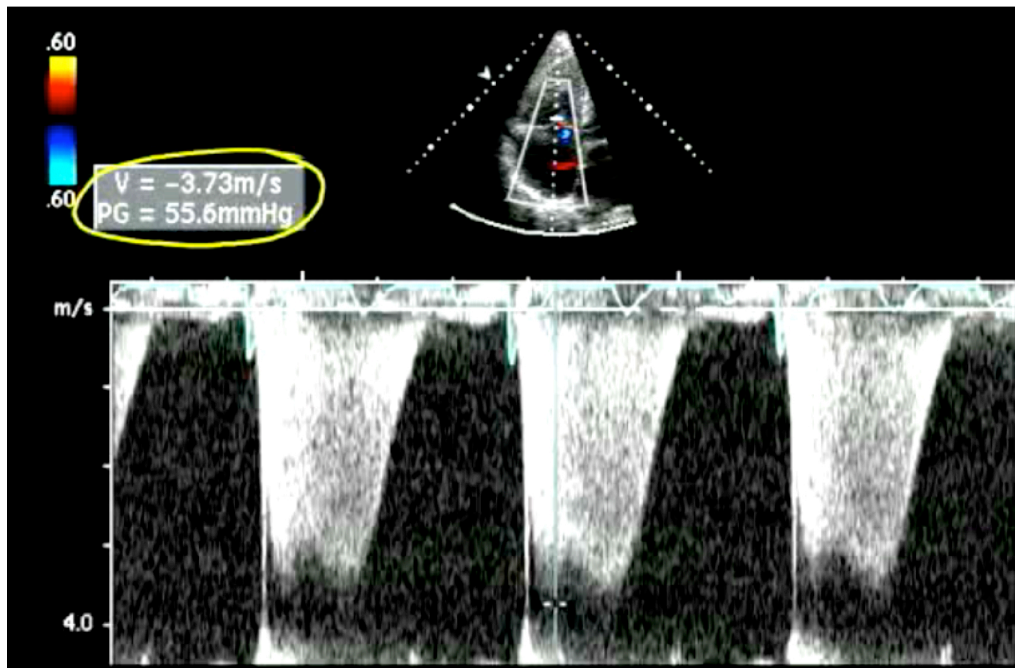


Figure 19. Spectral Doppler measurement, showing the velocity of blood through the tricuspid valve as a function of time.

This technique has been the subject of extensive validation (Allemann et al. 2000; Chemla et al. 2004; Grunig et al. 2000; Peacock et al. 1990; Skjaerpe & Hatle 1986; Yock & Popp 1984).

In a similar method, cardiac output can be estimated by aligning the transducer with the initial tract of the aorta, where the velocity profile of blood flow can be recorded just out of the orifice of the aortic valve. The echocardiography machine quantifies the average distance across which blood travels in the aorta during systole through the velocity time integral (VTI), visualized in a diagram similar to the one shown in Figure 19. The cross sectional area of the aorta (A) can also be calculated through the measurement of the aortic diameter, performed with the echocardiography machine.

Once the velocity time integral, aortic diameter and heart rate (HR) are known, the cardiac output can be calculated as

$$\dot{Q} = VTI \times A \times HR$$

This cardiac output estimation method has been widely validated (Christie et al. 1987; Eriksen & Walloe 1990; Sugawara et al. 2003).

2.9 Participants recruitment and consent

Control participants were recruited through advertisement and were chosen to match patients for gender, age, height and weight. For the study of Chuvash polycythaemia, where different levels of physical fitness could bias part of the results, control participants matched patients also in terms of hours of physical activity per week. Patients with Chuvash polycythaemia were identified from previous studies (Percy et al. 2003; Smith et al. 2006), as were patients with gain-of-function mutations in the HIF-2alpha gene (Gale et al. 2008; Percy et al. 2008b). All patients were recruited through their physicians, who would first assess their suitability for the study. The patients had no other medical disorders, had no history of complications, and were asymptomatic except for occasional headaches and tiredness. The number of CP and HIF2 patients included in the studies was limited by the rarity of the conditions in the United Kingdom. Each participant was informed about the aims, procedure and details of the study and signed a consent form before taking part in the experiments. All the studies conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee.

2.10 Statistical Analysis

Differences between patients and control participants were assessed by means of ANOVA. Control participants matched patients for a combination of age, gender, height, weight and body mass index, so paired statistical tests were performed. For quantitative RT-PCR in the Chuvash polycythaemia study, biopsy material was obtained from non-matched control samples, so statistical analysis was performed for unpaired samples. Statistical significance was set at $P < 0.05$. Commercial software was used to perform statistical analysis (SPSS, Version 12.0, Chicago, Illinois, United States). Throughout, variables are presented as mean $\pm$ standard deviation, unless otherwise stated.

Chapter 3. Regulation of human metabolism by hypoxia-inducible factor

3.1 Summary

The hypoxia-inducible factor (HIF) family of transcription factors directs a coordinated cellular response to hypoxia that includes the transcriptional regulation of a number of metabolic enzymes. Chuvash polycythaemia (CP) is an autosomal recessive human disorder in which the regulatory degradation of HIF is impaired, resulting in elevated levels of HIF at normal oxygen tensions. Apart from the polycythaemia, CP patients have marked abnormalities of cardiopulmonary function. No studies of integrated metabolic function have been reported. Here we describe the response of these patients to a series of metabolic stresses: exercise of a large muscle mass on a cycle ergometer, exercise of a small muscle mass (calf muscle) – which allowed for non-invasive in-vivo assessments of muscle metabolism using ^{31}P magnetic resonance spectroscopy – and a standard meal tolerance test. During exercise, CP patients had early and marked phosphocreatine depletion and acidosis in skeletal muscle, greater accumulation of lactate in blood and reduced maximum exercise capacities. Muscle biopsy specimens from CP patients showed elevated levels of transcript for pyruvate dehydrogenase kinase, phosphofructokinase and muscle pyruvate kinase. In cell culture, a range of experimental manipulations have been used to study the effects of HIF on cellular metabolism. However, these approaches provide no potential to investigate integrated responses at the level of the whole organism. Although CP is a relatively subtle disorder, the results of the experiments presented in

this chapter reveal a striking regulatory role for HIF on metabolism during exercise in humans.

3.2 Introduction

The hypoxia-inducible factor (HIF) family of transcription factors plays a key role in orchestrating the cellular response to varying levels of oxygen. These transcription factors are heterodimeric proteins consisting of two subunits: an oxygen-regulated HIF-alpha subunit (HIF-1alpha, HIF-2alpha or HIF-3alpha) and a constitutive HIF-beta subunit (Wang & Semenza 1995). At physiological levels of oxygen, HIF-alpha subunits undergo rapid degradation, limiting the formation of the transcription complex (Salceda & Caro 1997). Degradation is initiated by hydroxylation of HIF-alpha protein by specific prolyl hydroxylases (Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001). This enables binding of the von Hippel-Lindau (VHL) protein (Maxwell et al. 1999; Ohh et al. 2000) and the subsequent destruction of HIF-alpha by the ubiquitin-proteasome system. At low levels of oxygen, the rate of hydroxylation of HIF-alpha is reduced. This impairs the binding of VHL to HIF-alpha, enabling accumulation of transcriptionally active HIF complexes within the cell (Figure 4).

Many HIF target genes have now been identified in metabolic and other cellular functions [for review see (Schofield & Ratcliffe 2004; Semenza 2004; Semenza 2007)]. In metabolism, an early finding was that of a role for HIF in the transcriptional regulation of genes encoding enzymes of the glycolytic pathway (Firth et al. 1994). Subsequent experiments in cell culture have also identified a role for HIF in: i) downregulating mitochondrial oxygen consumption by directly or indirectly inducing

pyruvate dehydrogenase kinase, which inhibits the mitochondrial pyruvate dehydrogenase complex from converting pyruvate into acetyl-coenzyme A (Aragones et al. 2008; Kim et al. 2006; Papandreou et al. 2006); ii) regulating the differential expression of cytochrome c oxidase subunit 4 isoforms to optimize the efficiency of respiration at different O₂ tensions (Fukuda et al. 2007) and iii) inducing mitochondrial autophagy as an adaptive response to hypoxia (Zhang et al. 2008). However, these observations have so far been mainly restricted to cell culture and their significance or otherwise for the intact organism remains largely unexplored.

In humans, an opportunity to understand the effects of altered HIF physiology on integrated metabolic function is afforded by the condition of CP. This is an autosomal recessive disorder that is endemic to the region of Chuvashia (Sergeyeva et al. 1997) in the central European part of Russia. Individuals affected with CP have a homozygous germline mutation in exon 3 of the von Hippel-Lindau gene (VHL 598C→T), which impairs the binding of the gene product VHL to HIF- α subunits. This reduces the rate of HIF- α degradation and results in the stabilization of the HIF complex and increased expression of HIF-target genes under normoxic conditions (Ang et al. 2002; Bushuev et al. 2006). Studies of patients with CP have revealed high haematocrit and haemoglobin values, elevated pulmonary arterial blood pressures, reduced systemic arterial pressures and marked increases in the sensitivity of the respiratory system and the pulmonary vasculature to acute exposures to hypoxia (Bushuev et al. 2006; Sergeyeva et al. 1997; Smith et al. 2006). However, no metabolic measures have been reported in any of these studies.

The experiments reported in this chapter explored whether any abnormalities of metabolism in CP patients could be detected by exposing patients to the metabolic stress of exercise and of a standardized meal. In particular, experiments aimed to measure overall exercise capacity through an incremental exercise test on a cycle ergometer; calf muscle energy metabolites during light exercise by means of ^{31}P MRS; the metabolic response (arterial and venous blood metabolites) to the consumption of a standardized meal; and in-vitro skeletal muscle fiber composition, enzyme activities and mRNA expression levels from biopsy samples obtained at rest. The results obtained demonstrate that, under conditions of enhanced metabolic activity, CP patients generate substantially more lactate than control participants. Some, but not all, of the effects predicted from studies of the HIF system in cell culture were observed in the intact human with CP.

3.3 Methods

Participants. Five CP patients and five healthy control participants took part in the study. Table 1 shows the characteristics of individual participants. CP patients were identified from previous studies (Percy et al. 2003; Smith et al. 2006). Control participants were recruited by advertisement. Each CP patient was homozygous for the classic Chuvash mutation (Ang et al. 2002) and had been treated with long-term venesection to maintain a haematocrit near normal. Despite this, two patients presented with high haematocrits (62 % and 64 %) on the first day of the experiments. No patient had undergone venesection within several weeks of the experiment. The patients had no other medical disorders, had no history of complications, and were asymptomatic except for occasional headaches and tiredness. The number of CP patients included in the study was limited by the rarity of this condition in the United Kingdom. Control

participants were chosen to match CP patients for gender, and as far as possible for a combination of age, physical fitness (based on the amount of exercise taken each week) and body build. Each participant was informed about research and signed a consent form before taking part in the experiments. The study conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee.

Experimental Procedure. The experiments were performed over two consecutive days and included a cardiac investigation, an incremental bicycle exercise test to exhaustion, a small muscle mass exercise test, a standard meal tolerance test and a muscle biopsy. These tests were chronologically ordered so as to minimize the effects of each on the other as follows. Early on the morning of the first day, cardiac function and metabolism were studied as described in Chapter 4. Later in the morning of the first day, participants undertook the small muscle mass (calf) light exercise tests for biochemical investigation using MRS; in the afternoon muscle biopsies were taken from the non-exercised vastus lateralis after infiltration of the biopsy area with 1 % lidocaine and in the evening they were given a low-fat meal. On the second day, the standardized meal tolerance test was administered; in this, arteriovenous differences in metabolites concentrations were measured before and for five hours after the consumption of the meal. The second day ended with the incremental exercise test to exhaustion on the cycle ergometer. Participants fasted from 8:00pm overnight before each experimental day. They were also asked to avoid vigorous exercise and alcohol and caffeine consumption for 48 hours prior to the experiments.

The methods for each test followed standard procedures. Detailed methodology for the incremental exercise test to exhaustion, small muscle mass exercise for investigation with MRS, standard meal tolerance test, muscle biopsy analyses and statistical analysis are described in the Experimental methods chapter.

3.4 Results

The age, sex, height, weight, body mass index (BMI), amount of physical exercise per week and haematocrit for the CP patients and control participants are given in Table 1. Control participants were well matched for levels of physical activity in their daily life, body mass index, and age.

Participant	Age (y)	Sex	Height (m)	Weight (kg)	BMI (kg m ⁻²)	Exercise per week (hours)	Max Work Rate (W/kg)	Hct (%)
Control #1	22	M	1.80	79	24	<3	3.5	42
Chuvash #1	21	M	1.77	58	19	<3	3.1	62
Control #2	22	M	1.83	72	21	<3	4.2	45
Chuvash #2	22	M	1.71	65	22	<3	2.5	64
Control #3	24	M	1.77	75	24	<3	3.7	45
Chuvash #3	25	M	1.72	61	21	<3	3.3	41
Control #4	44	F	1.71	67	23	<3	3.3	36
Chuvash #4	34	F	1.52	69	30	<3	1.4	48
Control #5	46	F	1.61	65	25	<3	2.2	39
Chuvash #5	38	F	1.63	52	20	<3	1.5	41
Mean Control	32		1.74	72	23	<3	3.4	42
SD Control	12		0.09	6	2		0.8	4
Mean Chuvash	28		1.67	61	22	<3	2.4*	51
SD Chuvash	8		0.10	6	4		0.9	11

Table 1. Individual and group characteristics of Chuvash polycythaemia patients and control participants

Age, sex, height, weight, body mass index (BMI), amount of physical exercise per week, maximum work rate achieved during the incremental test on the cycle ergometer and haematocrit (Hct). Standard deviation (SD) values are reported for the respective mean values. Asterisks indicate that the Chuvash Polycythaemia group is significantly different (* $P < 0.05$) from the control group.

CP Increases Lactate Production during Exercise and Limits Overall Exercise

Capacity. Figure 20 illustrates mean values for venous blood lactate concentration, end-tidal partial pressure of carbon dioxide and ventilation as a function of work rate during the incremental exercise test. Venous blood lactate concentration increased early in the CP patients, becoming significantly higher than in the control group at ~4 min into the exercise test at a work rate of 80 W. CP patients stopped exercising at lower work rates than controls. The maximum work rates achieved per kg of body mass are reported in Table 1. These were significantly lower for the CP patients, at around 70% of those achieved by the control participants. There was no correlation between maximum work rate and haematocrit in the CP group. The end-tidal partial pressure of carbon dioxide was significantly lower in the CP group at rest and remained so throughout the exercise protocol. Furthermore, ventilation rose more rapidly in the CP patients than in the control participants during exercise.

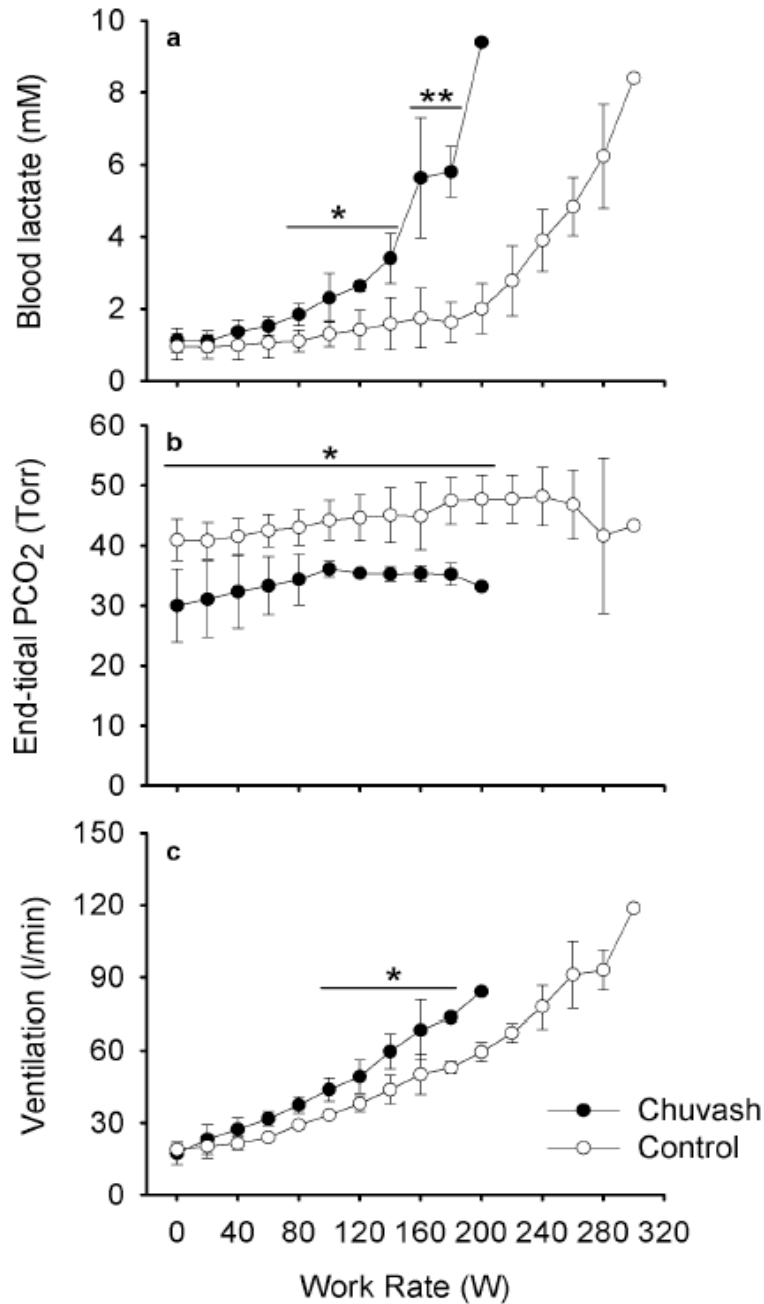


Figure 20. Responses of Chuvash polycythaemia patients and control participants to incremental exercise on a cycle ergometer

(a) Venous blood lactate concentration, (b) end-tidal partial pressure of carbon dioxide (PCO₂) and (c) ventilation expressed as a function of work rate. Empty circles show results from the control group; filled circles show results from Chuvash polycythaemia group. Data are mean $\pm$ SD. Number of individual values averaged per data point varies depending on number of participants who achieved the work rate; individuals' maximum work rates are reported in Table 1. Horizontal lines indicate that each underlying average value for the Chuvash polycythaemia patients differs significantly from the corresponding value for the control participants. * $P < 0.05$; ** $P < 0.01$.

CP Causes Marked Phosphocreatine Depletion, Inorganic Phosphate Elevation and Fall in pH during Calf Muscle Exercise. Figure 21 illustrates ^{31}P MR spectra for a representative control participant and a representative CP patient at rest and during calf exercise. At rest, the spectra from the two individuals appeared similar. However, during exercise, the phosphocreatine (PCr) peak almost disappeared and the inorganic phosphate (P_i) peak was markedly increased in the CP patient compared with the control participant. Similar results were obtained in all subjects within each group.

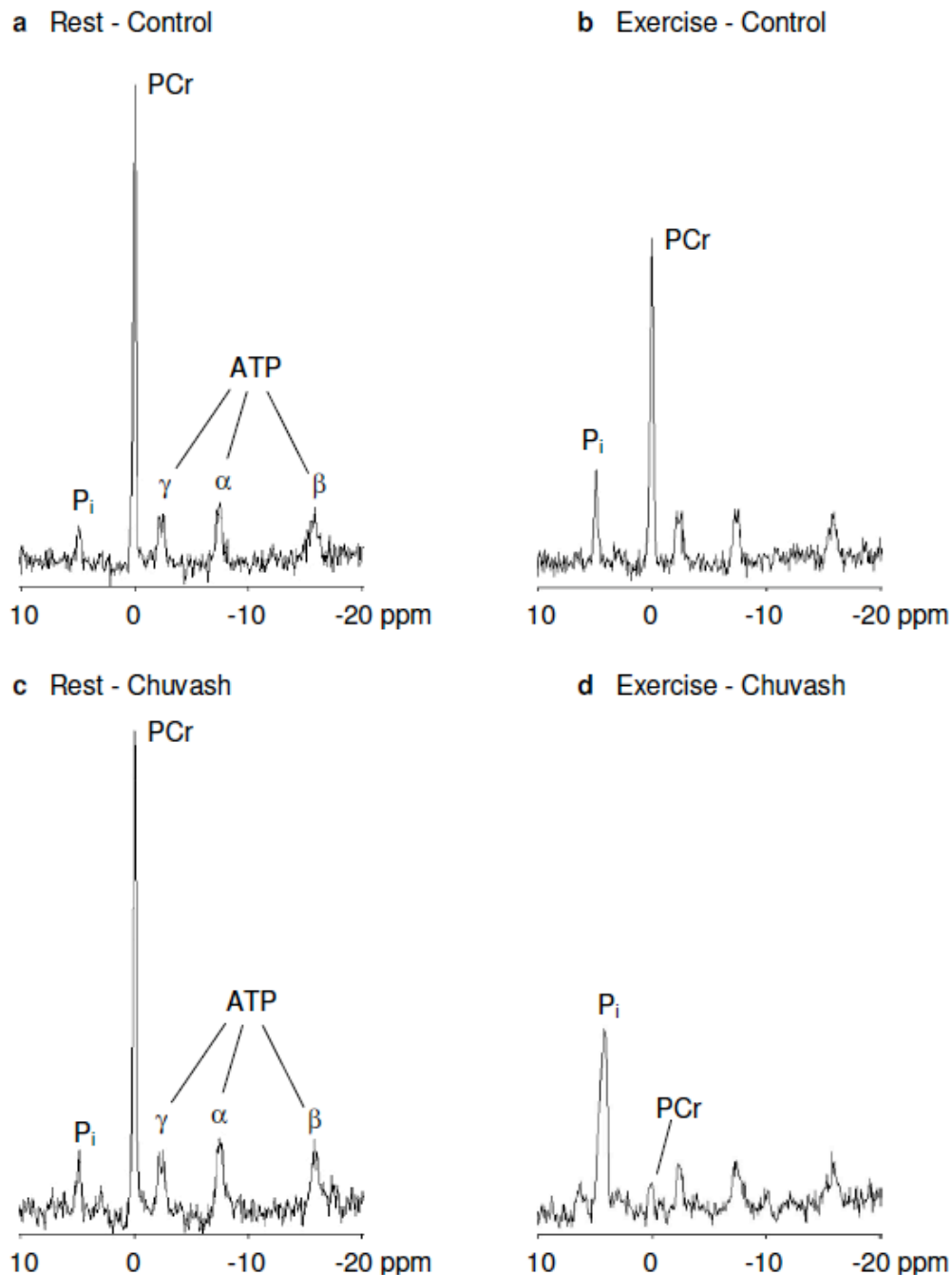


Figure 21. Examples of spectra obtained from calf muscle during ^{31}P magnetic resonance spectroscopy (MRS).

(a and b) Spectra for a representative control participant. (c and d) Spectra for a representative Chuvash polycythaemia patient. (a and c) Spectra recorded at rest. (b and d) Spectra recorded in the last min of the 5 W exercise period. Peaks marked PCr indicate phosphocreatine and those marked P_i indicate phosphate. The depletion of PCr and increase in P_i with exercise were much more marked in the Chuvash patient compared with the control participant.

Mean values for PCr, P_i and pH for the CP and for the control groups during the three periods of calf exercise and intervening periods of rest are shown in Figure 22. In the CP group, marked depletion of muscle PCr and elevation of P_i were evident even at the lowest level of exercise. These effects were significant at all work rates, and even the lightest work rate (3 W) caused a greater depletion of PCr and elevation of P_i in the CP group than did the heaviest work rate (5 W) in the control group. Similarly, at all three levels of exercise, the fall in muscle pH in the CP group far exceeded the fall in muscle pH in the control participants. Again, this effect was such that the fall in pH at the lowest level of exercise in the CP group greatly exceeded the fall in pH at even the heaviest level of exercise in the control group. Muscle ATP concentration decreased in both groups in response to exercise, but its concentration did not differ significantly between the two groups at any level of exercise.

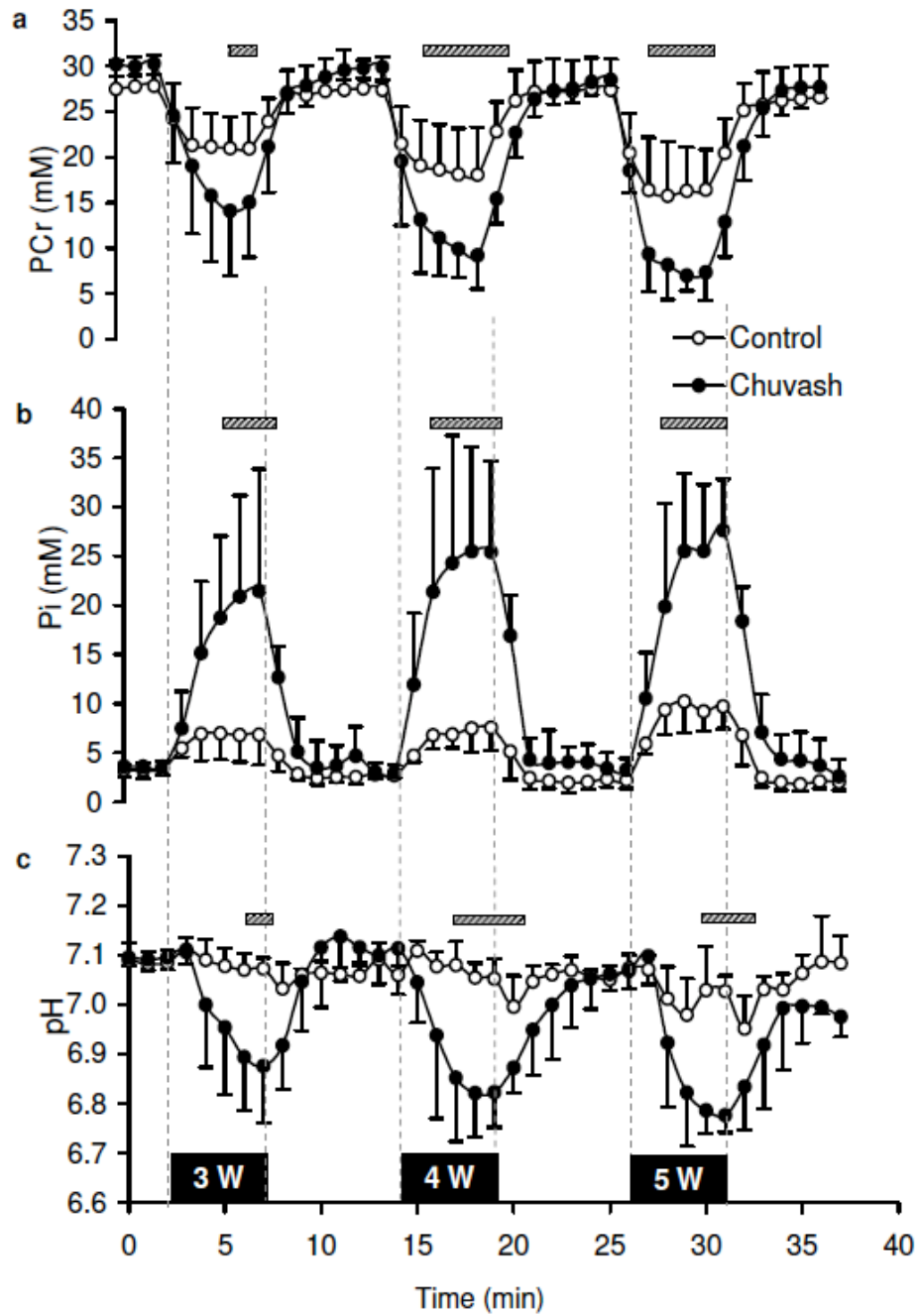


Figure 22. Results from Chuvash polycythaemia group and control group for ^{31}P MRS on calf muscle.

(a) Phosphocreatine concentration (PCr), (b) phosphate concentration (P_i) and (c) pH as a function of time. The vertical broken lines indicate the onset and offset of the 5 min plantar-flexion exercise sessions and the associated black bars indicate the power outputs (3, 4 and 5 W). Empty circles show results from the control group ($n = 5$); filled circles show results from the Chuvash polycythaemia group ($n = 5$). Values are minute averages $\pm$ SD. Horizontal bars with diagonal lines indicate periods of significant difference ($P < 0.05$) between groups.

CP is Associated with Elevated mRNA Levels of Muscle Phosphofructokinase (PFKM), Muscle Pyruvate Kinase (PKM) and Pyruvate Dehydrogenase Kinase (PDK) in Skeletal Muscle. Protein expression levels for the isoforms of myosin heavy chain (MHC), indicative of muscle fiber composition, are shown in Figure 23. No significant differences were found between the CP group and the control group for the proportions of type I, IIA, IIX and IIB fibers.

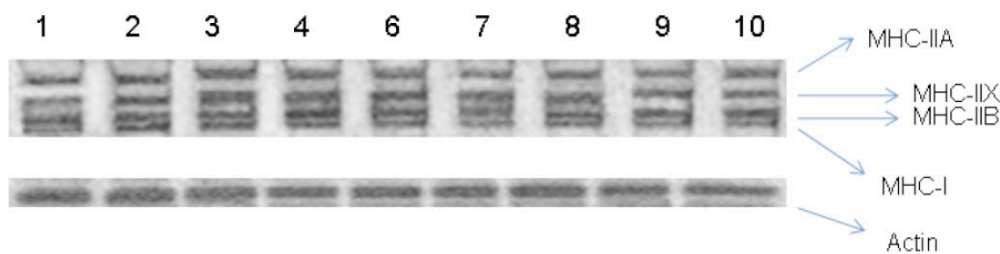


Figure 23. SDS-PAGE analysis of myosin heavy chain isoforms following silver staining.

Myosin heavy chain isoforms (MHC-I, MHC-IIA, MHC-IIX, MHC-IIB) in vastus lateralis muscle obtained from CP patients (lanes 2, 4, 6, 9, 10) and control participants (lanes 1, 3, 7, 8). Fiber type did not differ significantly between the two groups. The proportion of fibers type I, IIA, IIX and IIB for the control group were $19.8 \pm 2.4 \%$, $25.7 \pm 2.1 \%$, $35.4 \pm 4.9 \%$ and $19.1 \pm 2.9 \%$, respectively, and for the CP group were $17.2 \pm 2.5 \%$, $23.9 \pm 1.4 \%$, $31.9 \pm 2.9 \%$ and $27.0 \pm 2.8 \%$, respectively.

No significant differences were detected in muscle glycogen concentration, activities of pyruvate dehydrogenase complex total activity (PDCt), glutamate dehydrogenase (GluDH), citrate synthase (CS), beta-hydroxyacyl-CoA dehydrogenase (HAD) and glyceraldehyde 3 phosphate dehydrogenase (GlyPDH), myosin heavy chains and myosin light chains in control participants and CP patients (Table 2).

Participant	Glycogen ^A	PDCT ^B	GluDH ^B	CS ^B	HAD ^B	GlyPDH ^B	MHC	MLC
Control #1	384	9.1	9.9	39.0	20.2	4917	0.42	0.17
Chuvash #1	406	4.8	7.9	48.5	17.5	6147	0.40	0.10
Control #2	430	3.8	6.7	36.2	16.3	6421	2.00	0.90
Chuvash #2	251	2.0	5.4	46.3	16.0	6246	0.55	0.18
Control #3	346	7.9	4.0	37.2	7.4	3597	0.63	0.13
Chuvash #3	323	4.5	9.4	65.7	27.8	6297	0.17	0.33
Control #4	601	13.0	14.3	118.1	36.0	5868	0.15	0.08
Chuvash #4	349	5.5	6.6	36.1	27.8	4773	0.36	0.05
Control #5 ^c	500	4.4	6.9	32.4	10.4	6072		
Chuvash #5	364	7.8	5.4	27.9	9.8	4968	0.50	0.10
Mean Control	452	7.6	8.3	52.6	18.1	5375	0.80	0.32
SD Control	101	3.7	3.9	36.7	11.2	1140	0.82	0.39
Mean Chuvash	339	4.9	7.0	44.9	19.8	5686	0.40	0.15
SD Chuvash	57	2.1	1.7	14.3	7.9	750	0.15	0.11
<i>P</i> value	0.08	0.07	0.58	0.73	0.85	0.69	0.33	0.50

Table 2. Results from the skeletal muscle biopsy for the CP patients and control participants

Measured quantities are glycogen concentration and pyruvate dehydrogenase complex total activity (PDCT), glutamate dehydrogenase activity (GluDH), citrate synthase activity (CS), 3-hydroxy-acyl-coA dehydrogenase activity (HAD), glyceraldehyde-3-phosphate dehydrogenase activity (GlyPDH), myosin heavy chain (MHC) and myosin light chain (MLC). ^A mmol glucosyl units kg⁻¹ dry muscle. ^B nmol acetyl-CoA min⁻¹ mg⁻¹ protein. ^C insufficient material for MHC and MLC determination.

At mRNA level, a significant increase in CP patients' transcript levels was observed for muscle phosphofructokinase (PFKM), muscle pyruvate kinase isoforms M1 (M1-PKM) and M2 (PKM2) and pyruvate dehydrogenase kinase (PDK) isoforms 1 (PDK1), 2 (PDK2) and 4 (PDK4) (Table 3). Transcript levels did not differ significantly for hexokinase (HK) isoforms 1 (HK1) and 2 (HK2), pyruvate dehydrogenase phosphatase (PDP) isoforms 1 (PDP1) and 2 (PDP2), pyruvate dehydrogenase kinase isoform 3 (PDK3) and lactate dehydrogenase A (LDHA).

Gene name	Fold Change	95% C.I.	<i>P</i> value
HK1	1.4	0.8 – 2.1	0.06
HK2	0.76	0.27 – 2.18	0.57
PFKM	1.4	1.1 – 2.0	0.04
M1-PKM	1.8	1.2 – 2.8	0.01
M2-PKM	1.8	1.1 – 3.0	0.04
PDP1	0.55	0.27 – 1.11	0.09
PDP2	0.67	0.30 – 1.48	0.28
PDK1	2.0	1.1 – 3.5	0.03
PDK2	1.9	1.3 – 2.7	0.004
PDK3	1.2	0.6 – 2.1	0.53
PDK4	5.5	1.7 – 18.4	0.01
LDHA	1.5	0.8 – 2.7	0.17

Table 3. Changes in mRNA expression levels in CP patients relative to control participants.

Expression data at the mRNA level for hexokinase 1 (HK) isoforms 1 (HK1) and 2 (HK2), muscle phosphofructokinase (PFKM), M1 isoform of muscle pyruvate kinase (M1-PKM), M2 isoform of muscle pyruvate kinase (M2-PKM), pyruvate dehydrogenase phosphatase (PDP) isoforms 1 (PDP1) and 2 (PDP2), pyruvate dehydrogenase kinase (PDK) isoforms 1 (PDK1), 2 (PDK2), 3 (PDK3) and 4 (PDK4) and lactate dehydrogenase A (LDHA). 95% confidence interval (95% C.I.) is reported for each fold change.

CP Alters Pyruvate and Lactate Concentrations in Blood during the Digestion of a Standard Meal. Figure 24 illustrates the changes in mean arterial concentrations for plasma glucose, plasma insulin, plasma pyruvate and blood lactate following the standard meal tolerance test. The changes in glucose and insulin did not differ significantly between the CP group and the control group. However, the increases in plasma pyruvate and blood lactate 60 min after ingestion of the standardized meal were significantly greater in the CP group compared with the control group. At 60 min, the plasma pyruvate concentration was $251 \pm 64 \mu\text{M}$ (mean $\pm$ SD) in the CP group compared with $122 \pm 51 \mu\text{M}$ in the control group, and the blood lactate was $1.74 \pm 0.27 \text{ mM}$ in the CP group compared with $0.87 \pm 0.37 \text{ mM}$ in the control group.

Figure 24 illustrates forearm blood flow during the standard meal tolerance test. No significant effects of the meal were detected nor were there any significant differences between the CP group and the control group. Also illustrated in Figure 24 are the uptakes of glucose and lactate by skeletal muscle: these increased following the meal. With the exception of one data point, no significant differences were detected between the CP group and the control group.

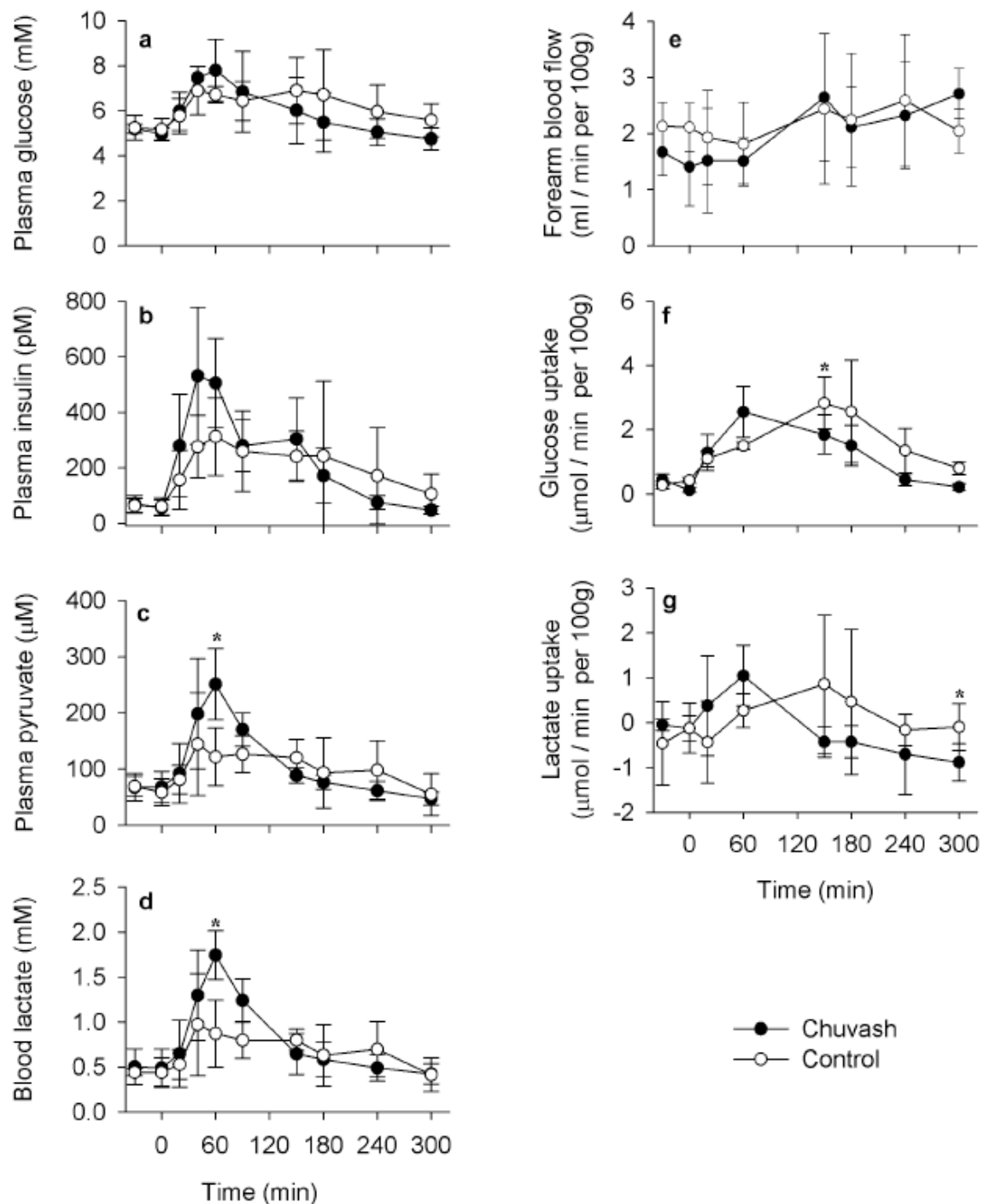


Figure 24. Results from the standard meal.

Results from Chuvash polycythaemia patients and control participants for left: arterial (a) glucose, (b) insulin, (c) pyruvate, (d) lactate, and right: (e) forearm blood flow, (f) glucose uptake and (g) lactate uptake measured through the standard meal tolerance test. Empty circles show results from the control group; filled circles show results from the Chuvash polycythaemia group. Data are mean $\pm$ SD. Values are for $n = 5$, apart from blood lactate for the control group at times -30, 20, 150 and 240 min, where $n = 3$. * $P < 0.05$

3.4 Discussion

This study is the first to demonstrate that a functional mutation within the HIF-VHL pathway can have a significant effect on human energy metabolism at the level of the organism as a whole. In particular, the study demonstrates that exercise, whether involving a large or a small muscle mass, is a substantial metabolic stress for patients with CP, who exhibit significantly greater lactate accumulation than normal controls. Compared with normal controls, CP patients had low maximum exercise capacities, and demonstrated early and greater PCr depletion and acidosis during a light ankle plantar-flexion exercise. In patients with CP, skeletal muscle mRNA expression levels at rest were elevated for enzymes of glycolysis and pyruvate dehydrogenase complex inhibition.

In normal healthy volunteers, maximal oxygen uptake capacity is a significant determinant of both the maximum exercise capacity and the work rate at which blood lactate begins to rise (Weibel & Hoppeler 2005). In patients with CP a degree of pulmonary hypertension (Smith et al. 2006) could limit the rise in cardiac output with exercise and so cause low maximum exercise capacity and early lactate production from muscle. Pulmonary arterial blood pressure was not recorded in this study and so it is not clear whether it may have risen during the incremental exercise test. However, energy metabolism in a small muscle mass during light exercise was investigated, in which CP patients demonstrated a striking depletion of PCr in muscle, accumulation of P_i , and a marked muscle acidosis compared with the normal controls. The concentrations of PCr and P_i in the CP group towards the end of the heaviest work rate were comparable with those recorded in maximum voluntary contractions in healthy participants (Sullivan et al. 1994). Since these findings were associated with light

exercise of a small muscle mass, they suggest that cardiopulmonary limitations to the delivery of oxygen are an unlikely explanation for the abnormal metabolism. The light level of exercise also makes it unlikely that the results are due to diffusional limitations for oxygen within the muscle. Finally, three of the five patients were not noticeably polycythaemic (because of clinical management via venesection), and therefore viscosity changes in blood are not likely to have limited the supply of oxygen. This last point is supported by the absence of any correlation between haematocrit and maximum work rate in the CP group.

Skeletal muscle that contains a high proportion of fast-twitch (type II), white muscle fibers, is more likely to metabolize anaerobically during exercise and so produce more lactate and have greater PCr depletion at the onset of contraction. Thus a further possibility is that the skeletal muscle of CP patients may contain a high proportion of such fibers. However, this hypothesis was not supported by the biopsy results, which demonstrated almost identical proportions of fiber types between the CP patients and the normal controls.

A further possibility is that CP patients are limited in their exercise capacity, not by the availability of oxygen, but rather in their capacity to use oxygen. This could arise through limitations in substrate supply to the TCA cycle in a manner similar to that of the acetyl group deficit in normal humans at the start of exercise (Greenhaff et al. 2002; Greenhaff et al. 2004). In particular, production of acetyl-CoA requires the activation of PDC, which is tightly regulated through an inactivation/activation cycle controlled by kinases (PDK1-4) and phosphatases (PDP1-2) respectively. In the skeletal muscle biopsies from CP patients, significant elevations in transcript for

PDK1, PDK2 and PDK4 were detected [the last two being the predominant isoforms of the kinase in skeletal muscle (Bowker-Kinley et al. 1998; Gudi et al. 1995)]. These results suggest that HIF can suppress oxidative metabolism, in agreement with observations made in mice lacking HIF-1alpha in skeletal muscle, where an increased level of exercise endurance was associated with a lower level of lactate and a lower level of mRNA for PDK1 (Mason et al. 2007). Although there is a mouse model of Chuvash polycythaemia (Hickey et al. 2007; Hickey et al. 2010), no metabolic phenotype has yet been reported. Apart from PDK, elevated levels of transcript for two other known HIF-regulated genes were observed (PFKM and PKM) (Ptashne et al. 1983; Semenza et al. 1994), but transcripts for other HIF-target genes were not significantly altered in CP patients. This may have arisen simply as a type II error, because of the limited number of patients. Overall, although PDCt activity did not differ significantly between the two groups, alterations in the expression of its regulatory kinase or phosphatase enzymes could nevertheless potentially explain the observed abnormalities in skeletal muscle energy metabolism.

In cell culture, an increased level of HIF-1 has been associated with mitochondrial autophagy (Zhang et al. 2008). As there is a strong relationship between total mitochondrial volume and maximal oxygen uptake capacity (Weibel et al. 2004), a further possible explanation of the reduced exercise capacity and enhanced lactate production of the CP patients is a reduction in mitochondrial volume. However, the lack of any difference between groups in the markers of mitochondrial volume (GluDH and CS activities) provides no evidence to support a reduced mitochondrial volume in the CP patients. In skeletal muscle HIF-1alpha null mice, the number of mitochondria was also not altered (Mason et al. 2004).

A further finding in cell culture is that HIF-1 may regulate the expression of cytochrome oxidase subunit 4 isoforms (Fukuda et al. 2007). It is thus possible that their expression differs in the CP patients compared with controls, and this could affect the efficiency with which they are able to consume oxygen within the muscle. The muscle biopsy analyses in this study were limited by the amount of tissue available, which precluded the chance to assess this possibility.

Given the magnitude of the effects of CP on skeletal muscle metabolism during exercise, there were remarkably few aberrations in glucose, lactate and pyruvate metabolism after meal intake in CP patients. Glucose homeostasis after meal intake was normal. Notably, lactate uptake by forearm was no different from the control group in the resting pre- and postprandial state. However, systemic lactate concentration increased more in CP patients after meal intake, but the origin of this lactate is not known. Tissues with a net production of lactate are brain, intestine, blood cells, skin and adipose tissue (Coppack et al. 1990; Jansson et al. 1990; Kreisberg 1972), whereas kidney (renal cortex) and liver remove lactate (Rowell et al. 1966). Skeletal muscle will either extract or produce lactate depending on metabolic state and muscular workload. There was no association between the rise in plasma lactate and haematocrit (higher in some CP patients than in controls), suggesting that generation of lactate from blood is not contributing to the difference between the groups. The production of lactate from skin can also probably be assumed to be constant. The contribution of lactate production from adipose tissue was not likely to have been very significant in a whole-body perspective, at least not in these groups of moderately lean people. Therefore the postprandial rise in lactate would seem more likely to depend on

either a CP-specific effect on intestinal lactate production, or a reduction in postprandial hepatic lactate clearance or a combination of both. Changes in pyruvate concentration mirrored those of lactate, and there were no differences between groups in the lactate/pyruvate ratios in blood, which would be consistent with no major disturbance of cellular cytosolic redox state.

The meal intake gave us the opportunity to examine the postprandial rise in plasma insulin to search for an insulin-secretion defect. Such defects have recently been reported in mice with a conditional deletion of VHL within pancreatic beta cells (Cantley et al. 2009; Puri et al. 2009; Zehetner et al. 2008). As the rise of meal-stimulated insulin appeared normal and also appeared to control postprandial plasma glucose concentrations within a normal range, it is concluded that there is no gross abnormality in insulin secretion with the subtle systemic VHL deficit present in CP patients.

With respect to their cardiopulmonary phenotype, CP patients generally appear to phenocopy sea-level natives who have been acclimatized to the hypoxia of high altitude (Smith et al. 2006). In relation to exercise, however, the predominant view has been that both acclimatized sea-level natives together with high-altitude natives accumulate less lactate in the blood during exercise and have lower peak values for lactate after exhaustive exercise than do unacclimatized individuals (Hochachka 1988; West 1986). This has been termed the lactate paradox (Reeves et al. 1992; West 1986; West 2007). In contrast, CP patients exhibit the opposite effect. However, not all studies have detected the reduction in lactate accumulation during exercise following acclimatization (Dempsey et al. 1972; Klausen et al. 1966), and the existence or

otherwise of the lactate paradox is a matter of debate (Brooks 2007; Hochachka et al. 2002; Mazzeo 2007; van Hall 2007; van Hall et al. 2009; West 2007). In a similar manner, there is a debate as to whether there are any benefits associated with a “live high, train low” regime for endurance athletes other than those that may arise from the associated erythrocytosis (Gore et al. 2007). Results presented in this chapter suggest that sustained activation of the HIF pathway in humans is more likely to be deleterious to such performance. Finally, the comparison between CP and reduced oxygen availability should not be drawn too closely, as oxygen not only affects the levels of HIF, but is itself a key substrate in metabolism. Metabolic changes could also potentially affect 2-oxoglutarate concentrations, and these also affect the level of HIF (Pollard et al. 2005; Zhao et al. 2009).

In summary, CP is a subtle genetic abnormality that results in a modest increase in expression of HIF-regulated gene products in the absence of hypoxia. In this study, with a limited number of patients, it was not possible to detect some of the effects that might be predicted from the much more powerful manipulations of the HIF-system that can be undertaken in cell culture. Nevertheless, despite the subtle nature of the CP defect, major effects on overall metabolism could be detected as soon as metabolism was sufficiently stressed. These findings emphasize that HIF plays an important role in the overall regulation of metabolic function, and that the metabolic effects of the CP modification are not fully compensated for by other mechanisms within the intact organism.

Chapter 4. Alterations in cardiac metabolism and function in patients with Chuvash polycythaemia: effects of chronic hypoxia signalling

4.1 Introduction

Glycolysis, or anaerobic metabolism, is the most important source of energy in the human heart during the relatively hypoxic fetal period (Tripp 1989). Although glycolysis is possible in hypoxia, it presents the disadvantage of a net yield of only 2 mol ATP per 1 mol glucose utilized. Differently, aerobic metabolism is up to 19 times more efficient, and it becomes the main source of cardiac energy after birth. In the human condition of heart failure, the myocardium reverts from aerobic metabolism towards glycolysis. Such a metabolic shift can be beneficial because it has oxygen-sparing effects, but may undermine the overall production of ATP. It was observed that patients with heart failure have reduced PCr to ATP ratios (Beer et al. 2002; Conway et al. 1991; Neubauer 2007), a finding consistent with the hypothesis of increased PCr hydrolysis to maintain ATP levels. As mentioned in previous chapters, hypoxia determines accumulation of HIF, and cardiac HIF levels are raised in patients with ischaemic cardiomyopathy (Shohet & Garcia 2007). As described in Chapter 3, CP patients have a rare VHL mutation that results in accumulation of HIF at physiological levels of oxygen. This chapter explores cardiac metabolism and function by means of magnetic resonance in CP patients.

4.2 Methods

The five patients and five matched control participants described in Chapter 3 took part in the experiments. Each participant was informed about the aims, procedure and details of the study and signed a consent form before taking part in the experiments. The study conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee. Participants fasted overnight and reported to the MR laboratory early in the morning. Cardiac volumes, function and mass were assessed using MR imaging. Resting cardiac energetics were assessed using ^{31}P MRS at 3 Tesla (Tyler et al. 2009b). Mid-ventricular septal voxels were selected as representative of the myocardium for analysis. Further details of these methods are reported in Chapter 2. Experimental methods.

4.3 Results

The mass and end-diastolic volumes of the left and right ventricles were significantly lower in CP patients than in control participants, but no difference was observed in left ventricular function (Table 4).

<i>Left Ventricle</i>	Control	Chuvash	Difference	<i>P</i>
End Diastolic Volume (ml)	171 ± 20	132 ± 19	-23%	< 0.05
End Systolic Volume (ml)	60 ± 14	48 ± 11	-21%	< 0.05
Ejection Fraction (%)	65 ± 6	65 ± 4	0%	0.35
Mass (g)	121 ± 13	79 ± 14	-35%	< 0.01
<i>Right Ventricle</i>				
End Diastolic Volume (ml)	200 ± 34	140 ± 14	-30%	< 0.05
End Systolic Volume (ml)	89 ± 18	57 ± 11	-37%	< 0.05
Ejection Fraction (%)	55 ± 6	60 ± 5	-9%	0.06
Mass (g)	54 ± 7	39 ± 4	-28%	< 0.05
PCr/ATP	1.83 ± 0.3	1.53 ± 0.3	-16%	0.06

Table 4. Results from the cardiac magnetic resonance experiments in the CP study.

PCr/ATP, phosphocreatine to adenosine triphosphate ratio. Significant differences would retain a level of statistical significance also if volumes and masses were scaled on participants' body mass or body surface area.

Two representative examples of cardiac MR images showing a smaller heart in the CP patient than in the matched control participant are illustrated below (Figure 25).

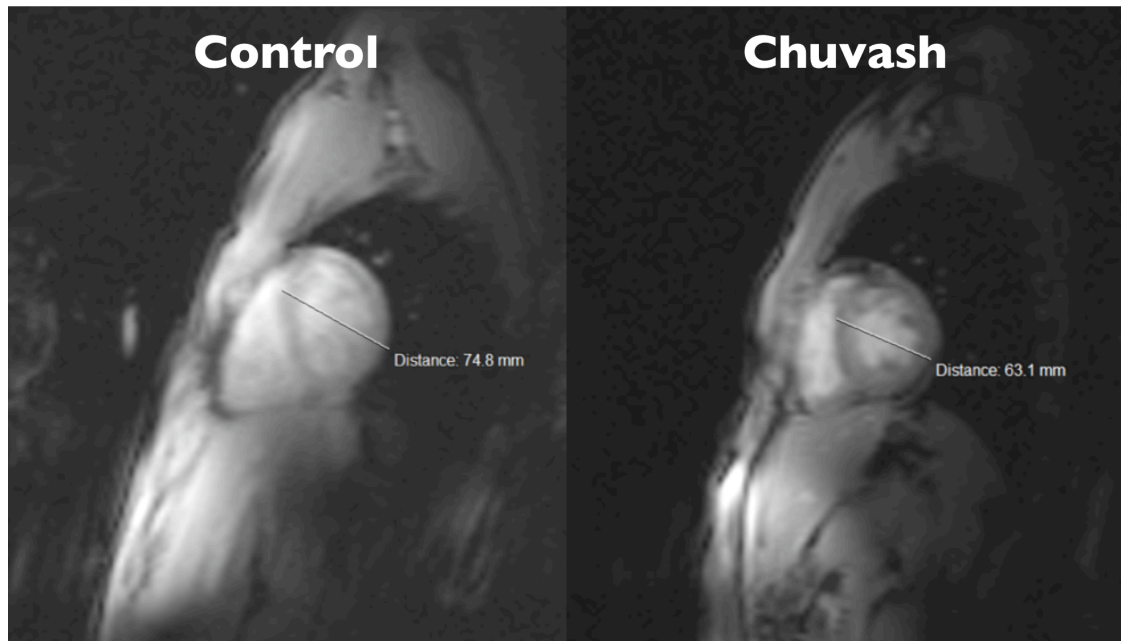


Figure 25. Cardiac magnetic resonance images for a representative control participant (left) and a Chuvash Polycythaemic patient (right).

The PCr to ATP ratio did not differ significantly between CP patients and controls (Table 4). Representative cardiac MR spectra are illustrated below (Figure 26): from the left, the height of the highlighted peaks indicates the concentration of PCr, and ATP alpha, beta and gamma respectively.

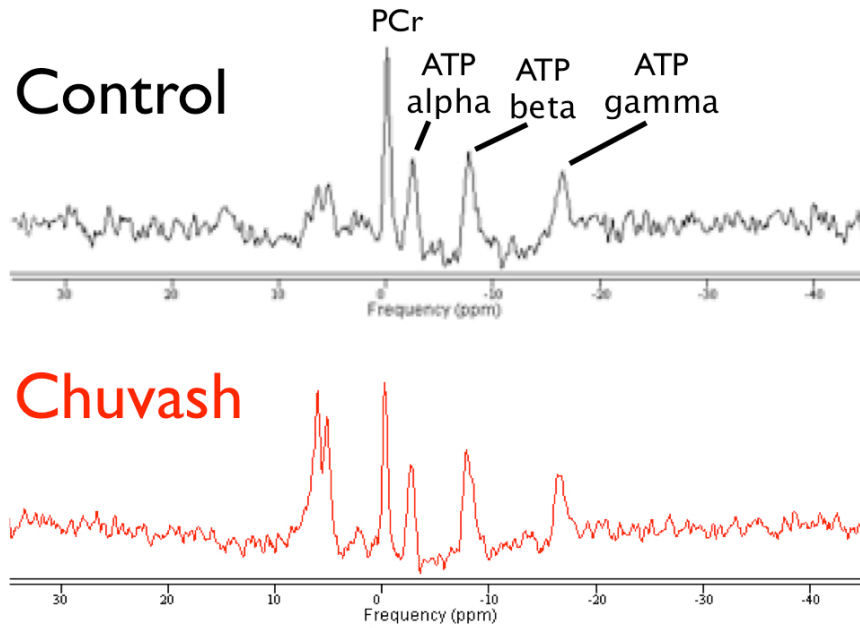


Figure 26. Representative cardiac MRS for a control participant and a CP patient.

Vertical axes have the same magnitude and scale.

4.4 Discussion

Patients with CP, and therefore raised levels of HIF, have smaller hearts than age and sex-matched control subjects, but have normal cardiac function. Myocardial PCr to ATP ratios were somewhat lower in patients than in controls, a finding that would be consistent with a trend towards a greater contribution from anaerobic energy sources for cardiac metabolism. These findings suggest that the reduced cardiac PCr to ATP ratios seen in patients with heart failure may be related to a condition of chronic hypoxia and modulated by HIF.

Chapter 5. Variable recapitulation of the Chuvash polycythaemia phenotype in patients with gain-of-function mutations in the hypoxia-inducible factor-2alpha subunit

5.1 Summary

The hypoxia-inducible factors (HIF, isoforms: HIF-1alpha, HIF-2alpha) mediate many responses to hypoxia. Their regulation is principally by oxygen-dependent degradation, which is initiated by hydroxylation of specific proline residues and then binding of von Hippel-Lindau (VHL) protein. Chuvash polycythaemia is an autosomal recessive disorder where germline homozygosity for hypomorphic VHL alleles results in mildly elevated HIF, and a phenotype of haematological, cardiopulmonary and metabolic abnormalities. The present chapter assesses which aspects of the Chuvash phenotype are reproduced in patients with HIF-2alpha gain-of-function mutations, where HIF-2alpha, but not HIF-1alpha, is elevated. HIF-2alpha gain-of-function mutations were associated with pulmonary hypertension, increased cardiac output, increased heart rate and increased ventilation:metabolism ratio. Other aspects of the Chuvash phenotype were either diminished or absent.

5.2 Introduction

A type of disorder of the HIF pathway has been described, in which there are germline heterozygous gain of function mutations in HIF-2alpha (Gale et al. 2008; Martini et al. 2008; Percy et al. 2008a). There is no evidence that the mutations in HIF-2alpha affect HIF-1alpha protein levels in these patients. These mutations in HIF-2alpha are also associated with excessive erythrocytosis, but it is unclear to what extent other aspects

of the CP phenotype are reproduced in these patients. The aim of this study was to determine whether the characteristics of CP are also present in these patients.

5.3 Methods

Participants. Three HIF2 patients and six healthy control participants took part in the study. HIF2 patients were identified from previous studies (Gale et al. 2008; Percy et al. 2008b) and recruited through their respective physicians. HIF2 patients had not been venesected within several weeks of the experiment, and had no other medical disorders. The number of HIF2 patients included in this study was limited by the rarity of this condition. Control participants were recruited through advertisement and were chosen to match HIF2 patients for sex, age, height, weight and body mass index.

All participants were informed about the aims, procedure and details of the study and signed a written informed consent form before taking part in the experiments. The studies conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee.

Experimental protocol. For each participant, the experiments were performed during the course of a single day. Participants were asked to avoid vigorous exercise and alcohol and caffeine consumption for 48 hours prior to the experiments; they were also asked to have a light breakfast on the morning of the experimental day. Room temperature was kept at 21° C throughout the experimental day. Air-breathing end-tidal partial pressure of oxygen (PETO₂) and carbon dioxide (PETCO₂) were measured before a venous and an arterial blood sample were taken. Participants were asked to

undertake four 20 min hypoxia response protocols, separated by three 20 min intervals. Each protocol started with 5 min of isocapnic euoxia ($P_{ET}O_2$ 100 mmHg), followed by 10 min of isocapnic hypoxia and ended with a final 5 min of isocapnic euoxia. The first two protocols were aimed at determining the participants' response to mild hypoxia ($P_{ET}O_2$ 70 mmHg), a level of hypoxia similar to that experienced on a commercial flight. The last two protocols were aimed at determining the participants' response to moderate hypoxia ($P_{ET}O_2$ 50 mmHg), a level of hypoxia comparable to that experienced when acutely exposed to an altitude of about 3,500 m. $P_{ET}CO_2$ was maintained close to each participant's baseline level throughout each protocol. The diagram in Figure 27 illustrates end-tidal partial pressures of oxygen and carbon dioxide as function of time in the last two protocols.

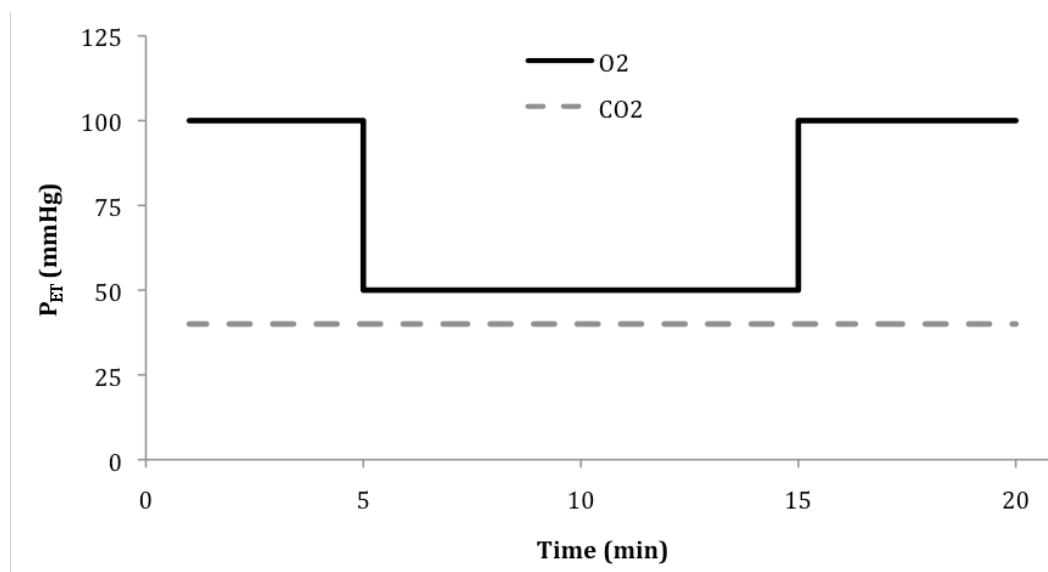


Figure 27. Protocol for the acute exposure to moderate hypoxia breathing tests

End-tidal partial pressure of oxygen (black line) and carbon dioxide (grey dashed line) as a function of time. The protocol used to measure responses to acute exposure to mild hypoxia was similar, but oxygen was only reduced to 70 mmHg for the 10 min of exposure to hypoxia.

Participants undertook an incremental exercise test to exhaustion after the hypoxia response protocols had been completed. For one HIF2 patient the experiments were repeated on a second occasion after the patient had undergone a programme of venesection to reduce his haematocrit to within the normal range.

Experimental technique, respiratory measurements and control of end-tidal gases.

Participants had their nose occluded and breathed through a mouthpiece connected to a turbine used to measure ventilation (Howson et al. 1986). During the hypoxia response protocols respired gases were sampled through a catheter and analyzed continuously by mass spectrometry. The control of gases was managed through the dynamic end-tidal forcing technique: end-tidal measured values were used as input for a computer that controlled a fast gas-mixing system, which in turn allowed a rapid and accurate manipulation of $PETO_2$ and $PETCO_2$ in the subsequent breaths (Robbins et al. 1982a). Breath-by-breath $PETO_2$, $PETCO_2$ and ventilation measurements were recorded on a computer in real time. Throughout each protocol participants were asked to recline in the left lateral position on a suitably modified couch.

Echocardiographic measurements. Pulmonary arterial systolic pressure and cardiac output were estimated through a continuous recording of Doppler echocardiography [Vivid-i GE Healthcare, UK with a two-dimensional S3 probe (1.5 – 3.6 MHz)]. In the first and in the third protocol, pulmonary arterial systolic pressure was estimated by measurement of the maximum gradient of blood pressure across the tricuspid valve. This technique is extensively described in Chapter 2.8, and has been the subject of extensive validation (Allemann et al. 2000; Chemla et al. 2004; Grunig et al. 2000; Peacock et al. 1990; Skjaerpe & Hatle 1986; Yock & Popp 1984). Right atrial pressure

is not affected by hypoxia (Groves et al. 1987), so variations in the maximum gradient of blood pressure across the tricuspid valve mirror variations in pulmonary arterial systolic pressure. In the second and in the last protocol, cardiac output was estimated by measuring the velocity of blood flow through the aortic valve, a widely validated method (Christie et al. 1987; Eriksen & Walloe 1990; Sugawara et al. 2003). An automatic blood pressure monitor (Omron M5-I, Japan) was used to measure blood pressure and heart rate once per minute.

Incremental exercise test to exhaustion. Participants' exercise capacity was measured through an incremental exercise test to exhaustion on a modified, electrically braked cycle ergometer (Mijnhardt KEM3, Cardiokinetics, UK). At the start of exercise participants were given about 30 s to achieve the required pedalling frequency (~65 revolutions per min, rpm) before the workload was increased by 20 W per min until exhaustion. Exhaustion was defined as the point at which the participants could no longer maintain the required pedalling frequency. Venous blood was sampled at the end of each work load (i.e. once per minute) in order to measure venous blood lactate concentration. Participants breathed through a mouthpiece, with their nose occluded. During the course of the protocol, respiratory measurements were made as described for the hypoxia response protocols.

Data analysis. Measurements of PETO_2 , PETCO_2 , ventilation, the maximum gradient of blood pressure across the tricuspid valve and cardiac output were averaged over 1 min periods prior to further analysis. Differences between patients and control participants were assessed statistically using ANOVA (SPSS, Version 12.0, Chicago, Illinois, United States). For the two protocols at each level of hypoxia, PETO_2 and PETCO_2

measurements were averaged. Statistical significance was assumed at $P < 0.05$.

Variables are presented as means $\pm$ standard deviation, unless otherwise stated.

5.4 Results

The control participants matched the HIF2 patients well for age, height, weight and body mass index (Table 5).

Participant	Age (y)	Sex	Height (m)	Weight (kg)	BMI (kg m ⁻²)
HIF2 patient #1	35	Male	1.58	55	22
Control #1A	35	Male	1.62	65	25
Control #1B	31	Male	1.65	55	20
HIF2 patient #2	27	Male	1.87	95	27
Control #2A	32	Male	1.79	77	24
Control #2B	26	Male	1.89	95	27
HIF2 patient #3	44	Male	1.74	86	28
Control #3A	39	Male	1.70	76	26
Control #3B	42	Male	1.73	92	31
HIF2 patient Average	35		1.73	79	26
HIF2 patient SD	9		0.15	21	3
Control Average	34		1.73	77	25
Control SD	6		0.10	15	3
<i>P</i> -value	0.81		1.00	0.87	0.85

Table 5. Individual and group characteristics of HIF2 patients and control participants.

Age, sex, height, weight and body mass index (BMI). Standard deviation (SD) values are reported for the respective mean values. *P*-values compare the HIF2 patient group with the respective matched control participant group.

Table 6 shows the results obtained from the analysis of venous and arterial blood samples of the HIF2 patients and of the matched control participants. Compared with the control participants, the HIF2 patients had lower arterial partial pressures of carbon dioxide (-10.6 ± 5.3 mmHg, mean $\pm$ SD, $P < 0.01$), higher haemoglobin concentrations ($+2.4 \pm 0.9$ g/dl, $P < 0.03$), higher haematocrits ($+0.08 \pm 0.03$ l/l, $P < 0.03$) and higher values for transferrin ($+0.8 \pm 0.3$ g/l, $P < 0.003$). HIF2 patients did not differ significantly from control participants in arterial pH, mean corpuscular volume, serum iron and ferritin.

Analysis (Normal Range)	HIF2 (n = 3)	Control (n = 6)	P-value
Arterial PO ₂ (83-105 mmHg)	105.0 ± 13.0	99.8 ± 9.5	0.52
Arterial PCO ₂ (35-45 mmHg)	32.1 ± 6.5	42.7 ± 3.7	0.01
Arterial pH (7.35-7.45)	7.43 ± 0.04	7.38 ± 0.04	0.10
Haemoglobin (13-17 g/dl)	18.5 ± 1.8	16.1 ± 1.0	0.03
Haematocrit (0.40-0.50 l/l)	0.57 ± 0.05	0.49 ± 0.03	0.03
Mean corpuscular volume (83-105 fl)	84.6 ± 21.2	89.6 ± 2.8	0.56
Serum iron (14-31 mmol/l)	17.0 ± 10.3	18.7 ± 4.6	0.75
Ferritin (20-300 mg/l)	75.8 ± 66.3	162.9 ± 93.2	0.27
Transferrin (1.8-3.6 g/l)	3.5 ± 0.2	2.7 ± 0.2	0.003

Table 6. Arterial and venous blood analyses for HIF2 study.

Values are mean ± SD. *P*-values compare the HIF2 patient group with the respective matched control participant group.

Pulmonary measurements. Figure 28 illustrates average ventilations in the HIF2 patients and in the control participants before, during and after the exposures to hypoxia. HIF2 patients did not differ significantly from control participants in either their baseline ventilation or in the increment in ventilation with mild or moderate hypoxia.

Measurements of pulmonary arterial systolic pressure were also possible in two HIF2 patients and four control participants. Average pulmonary arterial systolic pressures

from those individuals in which this measurement was possible are illustrated in Figure 28. Compared with the control participants, the HIF2 patients had significantly higher pulmonary arterial systolic pressures at baseline (35 ± 2 mmHg versus 24 ± 1 mmHg, HIF2 patients versus control participants, $P < 0.003$), and a significantly greater increase in pulmonary arterial systolic pressure in response to moderate hypoxia (5 ± 1 mmHg *versus* 11 ± 1 mmHg, $P < 0.004$). HIF2 patients did not differ significantly from control participants in the increase in pulmonary arterial systolic pressure with mild hypoxia.

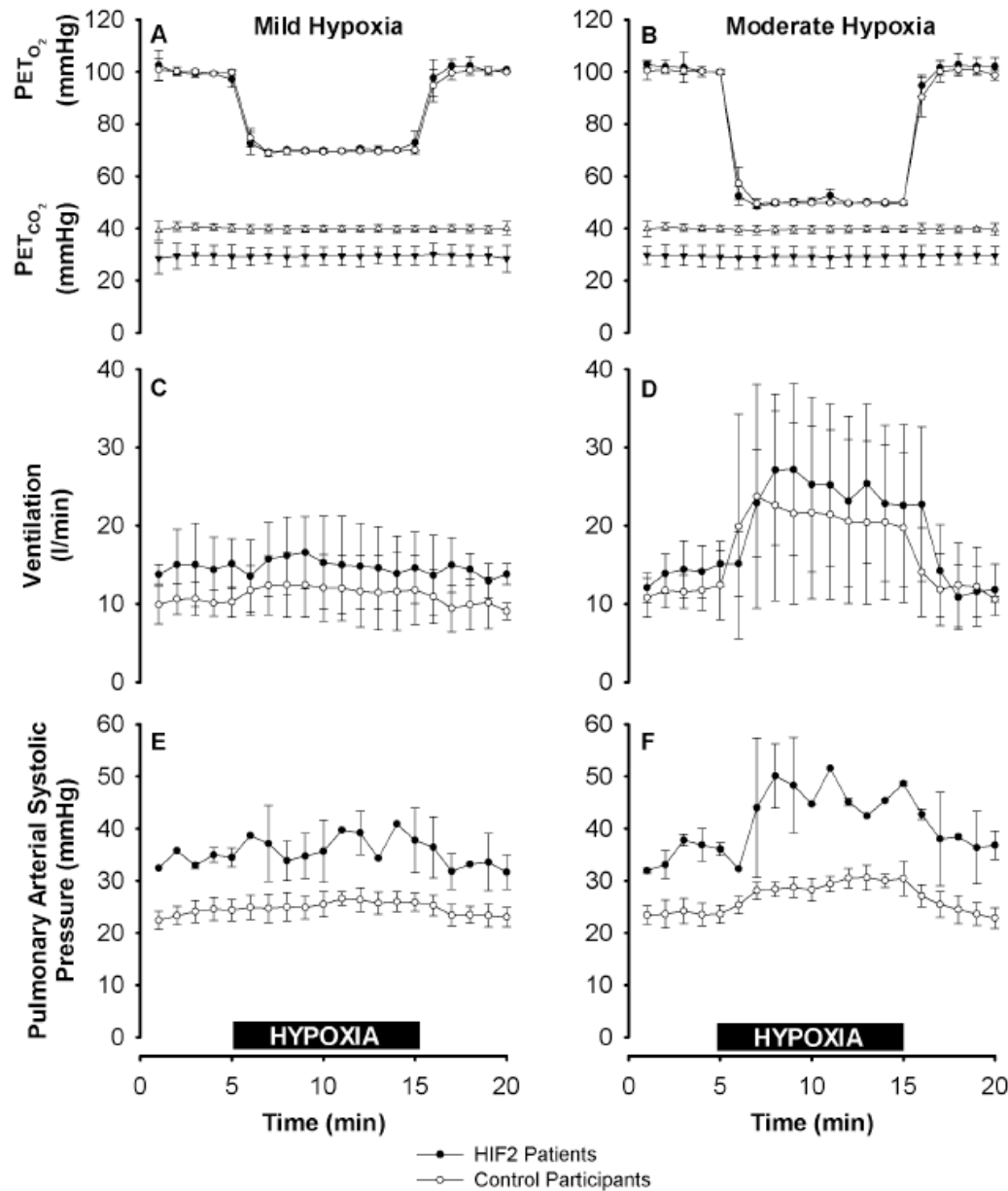


Figure 28. End-tidal gas control, ventilation and pulmonary arterial systolic pressure during mild and moderate hypoxia in HIF2 study.

(A and B) End-tidal partial pressure for oxygen (PET_{O₂}) and carbon dioxide (PET_{CO₂}). PET_{O₂} was well matched between groups. PET_{CO₂} was lower for the HIF2 patients, reflecting their lower baseline air-breathing PET_{CO₂}. (C and D) Ventilation, given at body temperature and pressure, saturated with water vapour. Baseline ventilation and the increase in ventilation provoked by hypoxia were not significantly different between the two groups.

(E and F) Pulmonary arterial systolic pressure. Compared with control participants, HIF2 patients had elevated pulmonary arterial systolic pressures at baseline (24 ± 1 mmHg vs 35 ± 2 mmHg, mean $\pm$ SD, $P < 0.003$), and a significantly greater increase in pulmonary arterial systolic pressure in response to moderate hypoxia (5 ± 1 mmHg

versus 11 ± 1 mmHg, $P < 0.004$). Values are mean $\pm$ SD. The horizontal black bars indicate the 10 min periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

Systemic vascular measurements. Figure 29 illustrates average values for the systemic vascular measurements in the HIF2 patients and in the control participants, before, during and after the exposures to hypoxia. At baseline, compared with the control participants, the HIF2 patients had significantly higher heart rates (71 ± 2 beats per min versus 56 ± 8 beats per min, $P < 0.02$) and significantly greater cardiac outputs (5.5 ± 1.0 l/min versus 4.1 ± 0.5 l/min, $P < 0.02$), while they did not differ significantly in blood pressure. HIF2 patients did not differ significantly from control participants in the increments in heart rate, blood pressure and cardiac output with mild or moderate hypoxia.

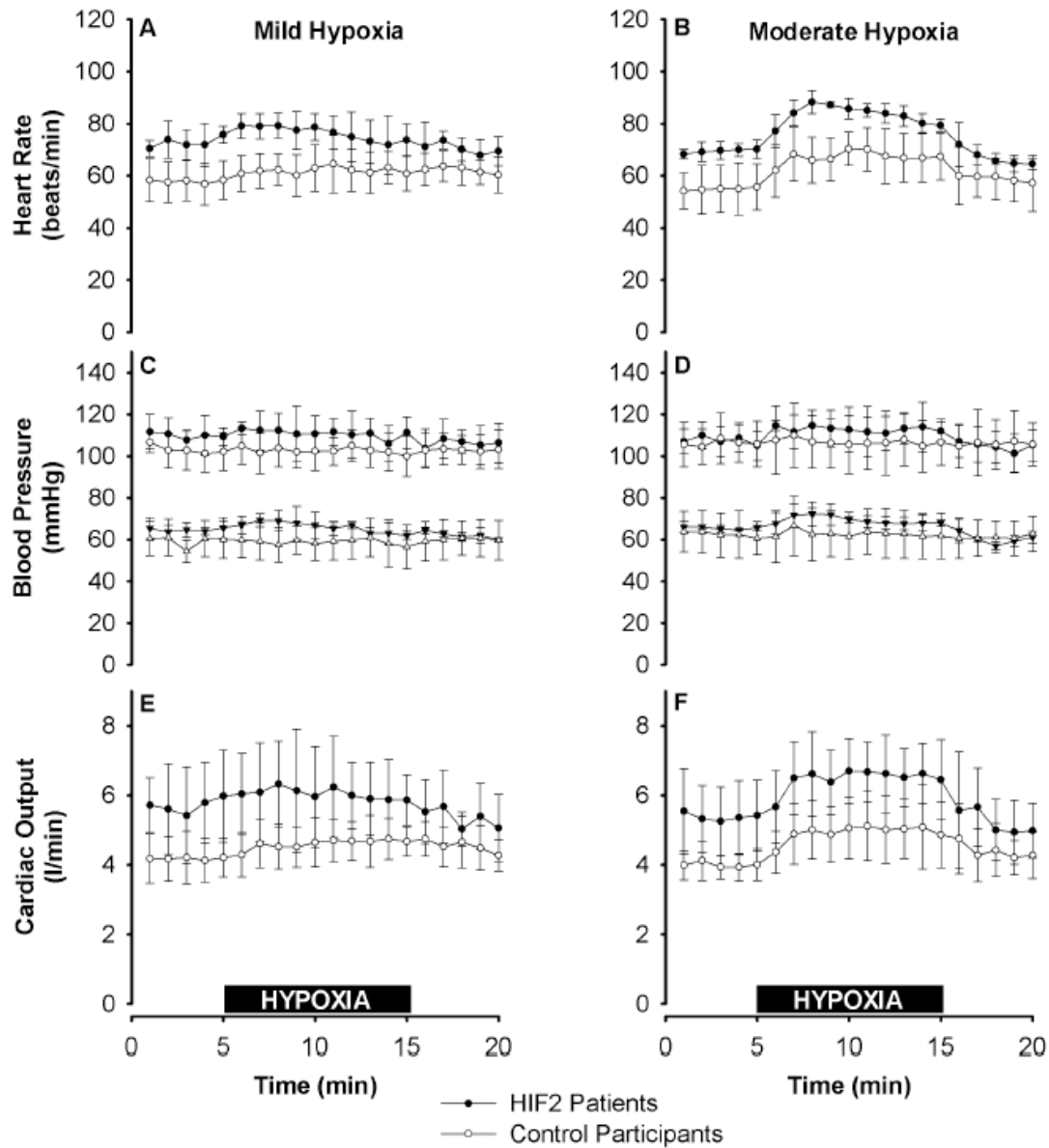


Figure 29. Systemic vascular responses in HIF2 patients and control participants to mild and moderate hypoxia.

(A and B) Heart rate. At baseline, heart rate was significantly higher in the HIF2 patients than in the control participants ($P < 0.02$).

(C and D) Systolic (top) and diastolic (bottom) blood pressure. Blood pressure did not differ significantly between the two groups.

(E and F) Cardiac output, assessed non-invasively using Doppler echocardiography. At baseline, cardiac output was significantly greater in the HIF2 patients than in the control participants ($P < 0.02$).

The increments in heart rate, blood pressure and cardiac output with hypoxia did not differ significantly between the two groups. Values are mean $\pm$ SD. The horizontal

black bars indicate the 10 min periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

Summary of cardiopulmonary responses. Figure 30 presents a summary of the variations in both baseline values and in the incremental responses to hypoxia for the various cardiopulmonary measurements. For baseline pulmonary arterial systolic pressures and for the increments in pulmonary arterial systolic pressure with moderate hypoxia no overlap was observed in responses between the control participants and the HIF2 patients. For other variables there was some overlap between the two groups, although two of the three HIF2 patients had baseline cardiac outputs that were more than 3 SDs from the mean of the control participants.

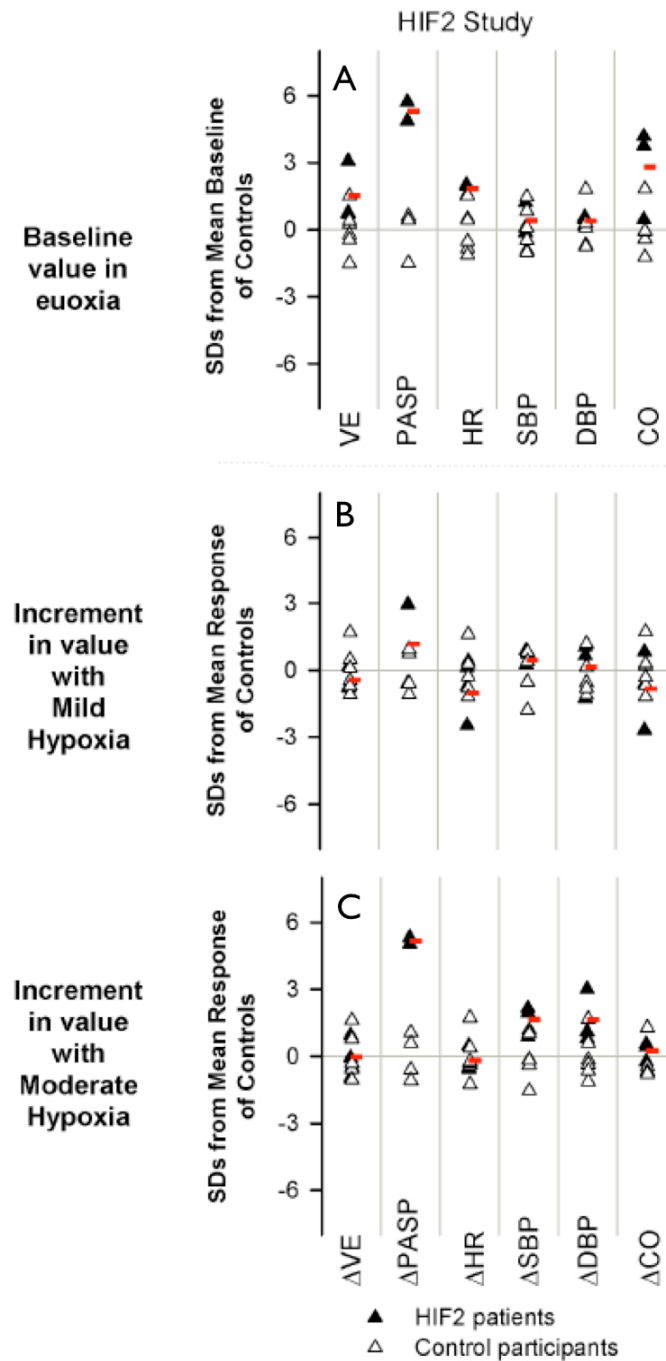


Figure 30. Cardiopulmonary variables in the HIF2 study: baseline measurements

(A) and incremental responses with hypoxia (B and C).

Results are shown in terms of the number of (control group) standard deviations by which each participant's baseline and response differed respectively from the mean baseline and mean response of the control participants. Red symbols show average results for patients' group.

(A) Under baseline conditions HIF2 patients had significantly higher pulmonary arterial pressures, heart rates and cardiac outputs than control participants. (B and C)

Compared with control participants, HIF2 patients also had a significantly greater rise in pulmonary arterial pressure with moderate hypoxia. VE, Ventilation; PASP, Pulmonary Arterial Systolic Pressure; HR, Heart Rate; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; CO, Cardiac Output; Δ , increment.

Incremental exercise test to exhaustion. Figure 31 illustrates venous blood lactate concentration, ventilation and end-tidal partial pressure of carbon dioxide as a function of work rate recorded during the incremental exercise test to exhaustion. The rise in venous lactate with work rate appeared to be similar between HIF2 patients and control participants. Two of the three HIF2 patients appeared somewhat limited in their maximum exercise capacity, but the third patient exercised very comparably with the control participants. Overall, there were no significant differences between HIF2 patients and control participants for the maximum work rate achieved and the associated lactate level attained (2.0 ± 0.9 W/kg versus 2.9 ± 0.9 W/kg, NS, and 4.3 ± 2.2 mM versus 5.4 ± 2.7 mM, NS, respectively). The hyperventilation and associated hypocapnia at rest persisted throughout the period of incremental exercise to exhaustion.

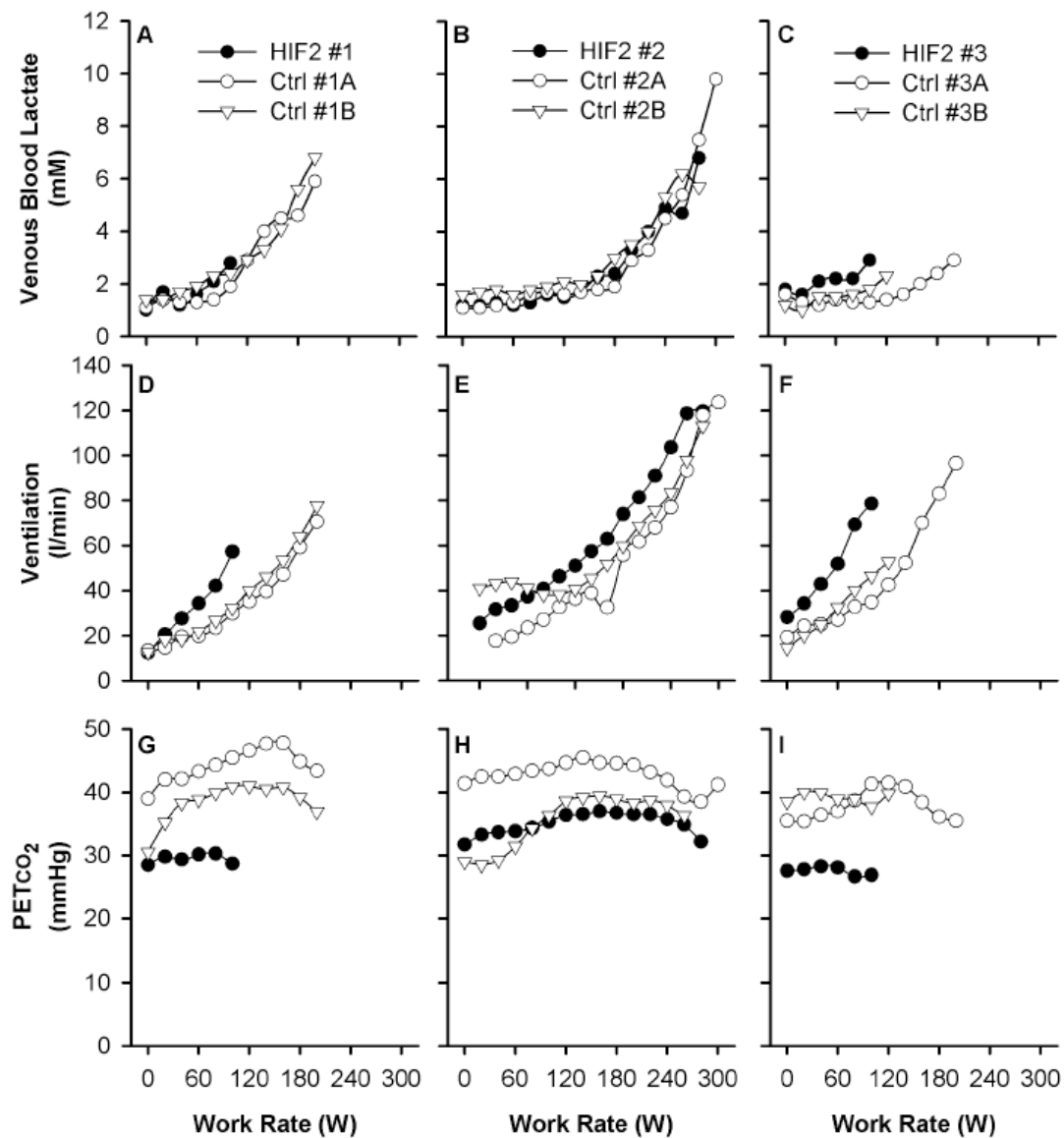


Figure 31. Individual responses for HIF2 patients and control participants during the incremental exercise test to exhaustion.

(A, B and C) Venous blood lactate concentrations as a function of work rate.

(D, E and F) Ventilations as a function of work rate.

(G, H and I) End-tidal partial pressure of carbon dioxide (PETCO₂) as a function of work rate.

Columns on the left, middle and right illustrate results respectively for HIF2 patient #1, #2 and #3 and their associated control participants. Data are minute averages.

Effects of venesection on one of the HIF2 patients. One of the HIF2 patients (patient #2) was studied both before and after venesection, which reduced the haemoglobin and haematocrit to values within the normally accepted range, serum iron to 7.8 $\mu\text{mol/l}$, ferritin to 8.9 $\mu\text{g/l}$, and increased transferrin concentration to 4.24 g/l (Table 7).

Analysis (Normal Range)	HIF2 #2	HIF2 #2
	before	after
Arterial PO ₂ (83-108 mmHg)	96.6	100
Arterial PCO ₂ (35-45 mmHg)	35.8	36.2
Arterial pH (7.35-7.45)	7.41	7.39
Haemoglobin (12-17 g/dl)	20.4	15
Haematocrit (0.36-0.50 l/l)	62.3	46.3
Mean corpuscular volume (83-101 fl)	98.3	86.4
Serum iron (11-31 mmol/l)	26.9	7.8
Ferritin (10-300 mg/l)	135.7	8.9
Transferrin (1.8-3.6 g/l)	3.33	4.24
Maximum work rate (W)	280	280
Maximum lactate concentration (mM)	6.8	6.8

Table 7. Effect of venesection on arterial and venous blood analyses, and on incremental test in HIF2 #2 patient

HIF2 patient #2 arterial and venous blood analyses before and after venesection. Reported are also the maximum work rates and lactate concentrations measured with the incremental exercise test on the cycle ergometer.

Venesection in this patient had no clear effect on ventilation and on pulmonary arterial systolic pressure (Figure 32); ventilation appeared normal, while pulmonary arterial systolic pressure remained above normal. Venesection had no clear effect on heart rate, on blood pressure and on cardiac output (Figure 33) at baseline and with hypoxia.

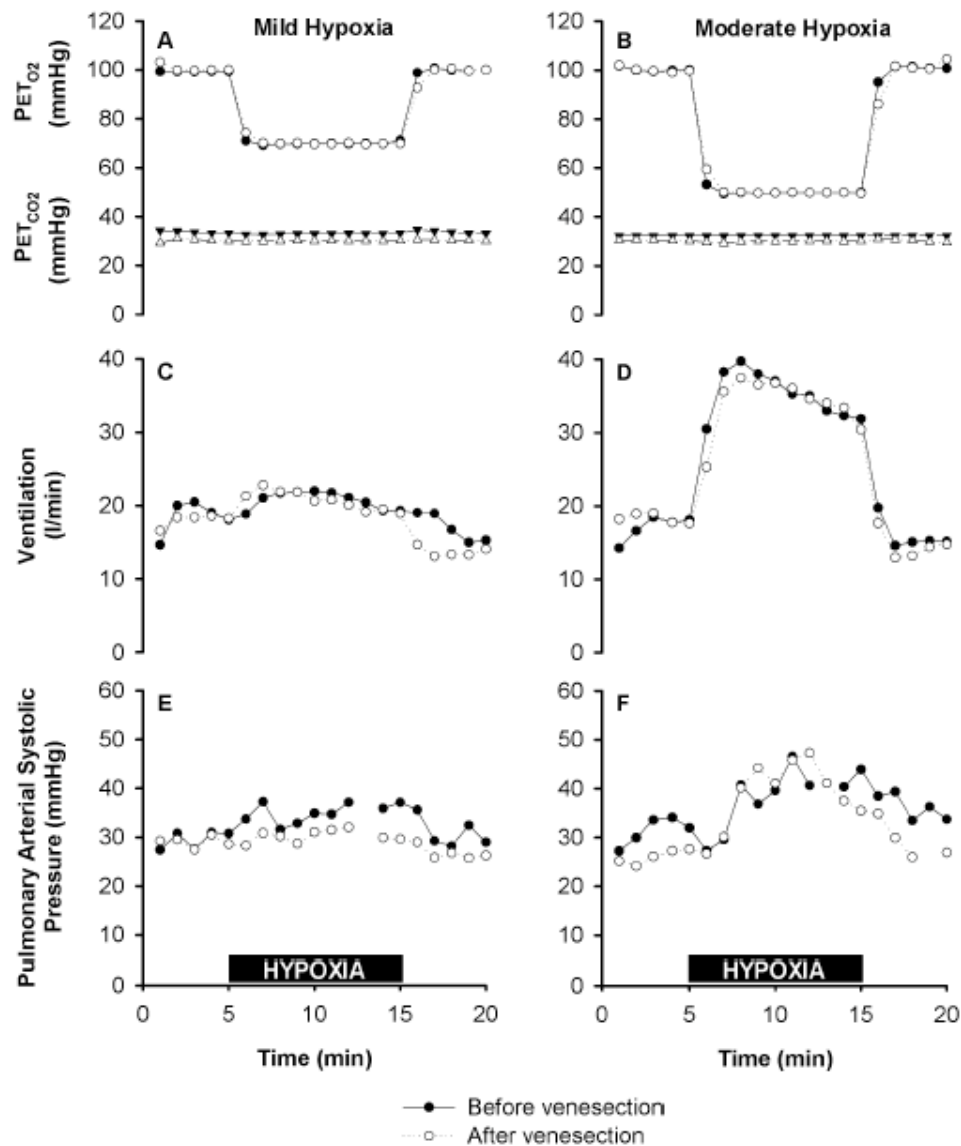


Figure 32. End-tidal gas control, ventilation and pulmonary arterial systolic pressure during mild and moderate hypoxia in HIF2 #2 patient before and after venesection.

(A and B) End-tidal partial pressures for oxygen (PET_{O2}) and carbon dioxide (PET_{CO2}). (C and D) Ventilation, given at body temperature and pressure, saturated with water vapour. (E and F) Pulmonary arterial systolic pressure.

Values are minute average. The horizontal black bars indicate the 10 min periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

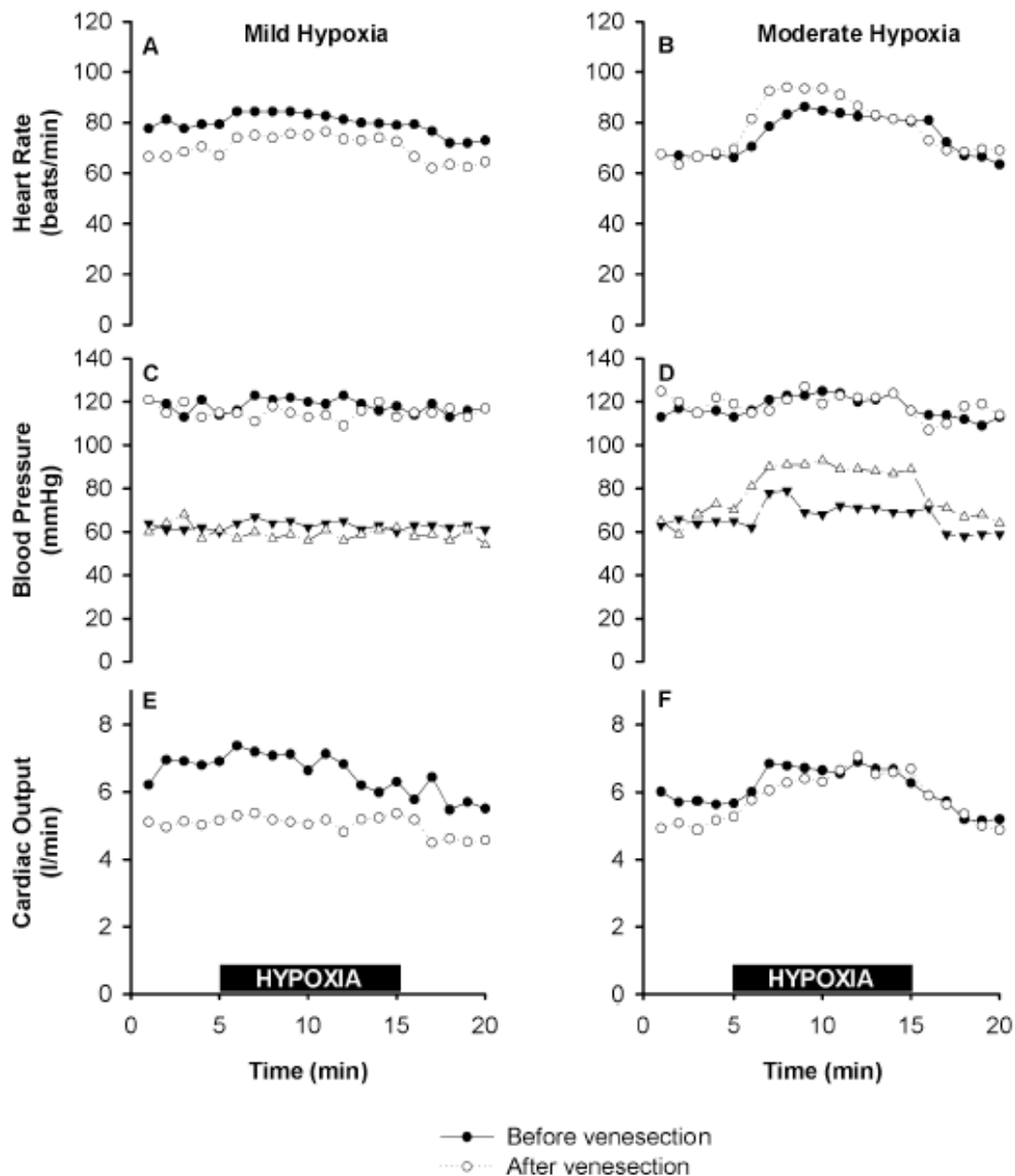


Figure 33. Systemic vascular responses to mild and moderate hypoxia in HIF2 #2 patient before and after venesection.

(A and B) Heart rate. (C and D) Systolic (top) and diastolic (bottom) blood pressure. (E and F) Cardiac output, assessed non-invasively using Doppler echocardiography. Values are minute averages. The horizontal black bars indicate the 10 minute periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

Also, venesection had no clear effect on the accumulation of lactate in venous blood, ventilation and end-tidal pressure of carbon dioxide (Figure 34), and on the maximum work rate and maximum venous blood lactate concentration achieved during the incremental exercise test to exhaustion; all these parameters remained within the normally accepted range (Table 7).

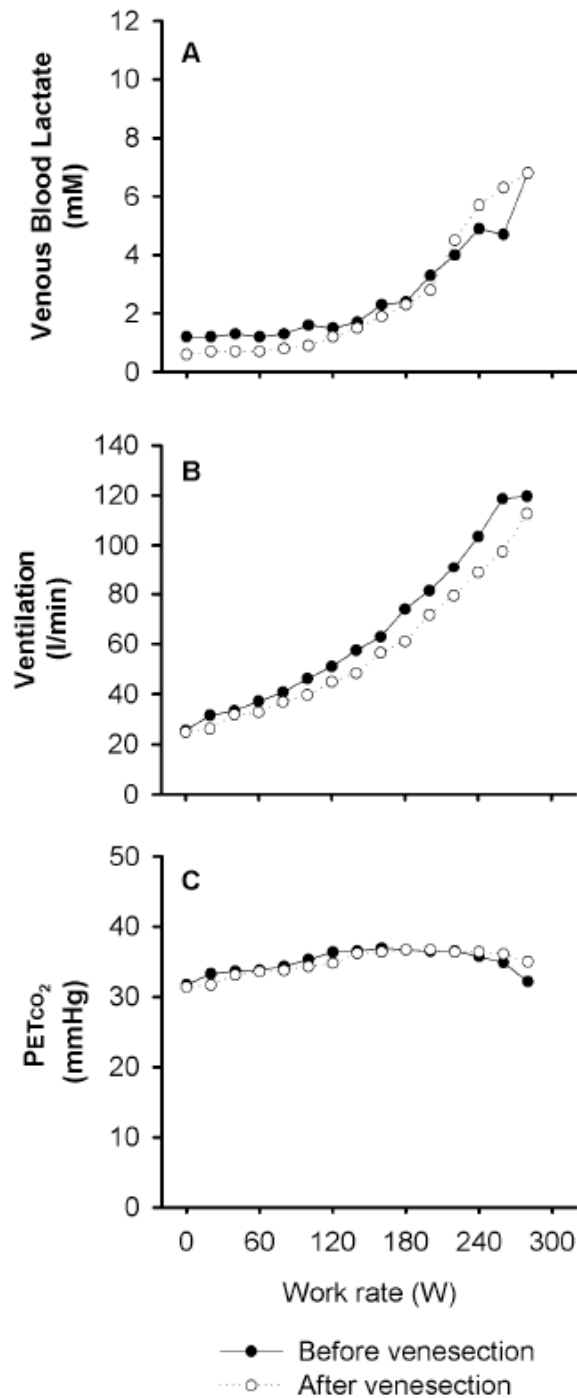


Figure 34. Responses for HIF2 #2 patient during the incremental exercise test to exhaustion before and after venesection.

(A) Venous blood lactate concentrations as a function of work rate. (B) Ventilation as a function of work rate. (C) End-tidal partial pressure of carbon dioxide (PETCO₂) as a function of work rate.

5.5 Discussion

This chapter examined the extent to which the phenotype in patients with gain-of-function mutations in HIF-2alpha resembles that observed in patients with CP (Formenti et al. 2010; Sergeyeva et al. 1997; Smith et al. 2006), where homozygous mutations within VHL potentially affect both HIF-1alpha and HIF-2alpha (Ang et al. 2002). Both conditions were originally identified through excessive erythrocytosis (Percy et al. 2008b; Sergeyeva et al. 1997), with a greater value for haematocrit (+ 10 %) in CP patients. This commonality between the two suggests that elevation of just HIF-2alpha is sufficient to generate the erythrocytosis. In keeping with this, erythrocytosis in a mouse model of CP can be reversed by loss of the HIF-2alpha allele in VHL^{R/R}HIF2-alpha^{+/-} mice (where R indicates the Chuvash allele), but not by loss of the HIF-1alpha allele in VHL^{R/R}HIF1-alpha^{+/-} mice (Hickey et al. 2010). Another component of the CP phenotype, pulmonary hypertension under air-breathing conditions, was just as severe in HIF2 patients (Figure 28) as in CP patients (Smith et al. 2006) – indeed, pulmonary hypertension has already been noted in the family history of two HIF2 patients (Gale et al. 2008). Consistent with these observations in the HIF2 patients, pulmonary hypertension, which is also present in the mouse model of CP, was reduced in severity in VHL^{R/R}HIF2-alpha^{+/-} mice, but not in VHL^{R/R}HIF1-alpha^{+/-} mice (Hickey et al. 2010). Arterial (Table 6) and end-tidal values for PCO₂ (Figure 28) were reduced by a similar degree in patients compared with controls – which indicates that elevation of just HIF-2alpha is sufficient to increase the basal level of pulmonary ventilation in relation to metabolism. Some components of the phenotype in the HIF2 patients, in particular an elevated heart rate and cardiac output at rest in normoxia, have not been previously demonstrated in CP patients. Nevertheless, trends in this direction have been noted (Smith et al. 2006).

Other aspects of the CP phenotype were either reduced in magnitude or absent in the HIF2 patients (e.g. pulmonary ventilation, Figure 28; venous blood lactate concentration during the incremental exercise test, Figure 31). Compared with control participants, CP patients have greatly increased sensitivities for their pulmonary vascular and ventilatory responses to acute hypoxia (Smith et al. 2006). In the HIF2 patients, this was either much less obvious (pulmonary vascular response to hypoxia) or absent (ventilatory response to hypoxia). Similarly, compared with control participants, CP patients exhibit an early and above normal accumulation of lactate in the venous blood during incremental exercise, and a reduced maximum work rate capacity (Formenti et al. 2010). These findings were either absent, or much less obvious in the study of the HIF2 patients. The absence of effect of the HIF-2alpha mutation on these variables relating to metabolism is consistent with studies undertaken in cell culture which suggest that HIF-2alpha plays no role in regulating the expression of genes that affect the glycolytic function (Hu et al. 2003). Nevertheless, metabolic abnormalities relating to HIF-2alpha have been observed as part of the much more severe phenotype associated with HIF-2alpha null mice (Scortegagna et al. 2003).

The HIF2 phenotype appears to resemble those aspects of the CP phenotype that are most closely related to the regulation of absolute (or basal) values for variables within the haematological, cardiovascular and respiratory systems. These include the haematocrit, the pulmonary vascular resistance (as reflected by the pulmonary hypertension), the ratio of ventilation to metabolic rate (as reflected by arterial/end-tidal PCO₂), the cardiac output and possibly cardiac sympathetic tone (as reflected by

an increased heart rate). Aspects of the CP phenotype that are not so well replicated by the HIF2 phenotype are the sensitivities of the ventilatory and pulmonary vasculature to acute hypoxia, and also the glycolytic phenotype.

Haemoglobin concentration, haematocrit, serum iron and ferritin were all significantly reduced after venesection in the one patient studied before and after this procedure. Despite the decrease in blood viscosity that would have occurred, no associated reduction in pulmonary arterial systolic pressure was detected, so presumably it increased cardiac output or pulmonary vascular resistance. It is possible that the elevated HIF-2alpha levels in this patient contributed to maintaining a high pulmonary blood pressure, despite a marked decrease in haematocrit. However, in a study of venesection in patients with excessive erythrocytosis from chronic mountain sickness, pulmonary arterial blood pressure increased rather than decreased, suggesting iron depletion can exacerbate hypoxic pulmonary hypertension (Smith et al. 2009). Overall, this patient illustrates that the effects of venesection are not simply confined to those associated with changes in haematocrit. More generally, the extent to which patients with erythrocytosis due to known or yet unidentified mutations affecting the functioning of the HIF pathway should be venesected is far from clear.

Chapter 6. Cardiopulmonary physiology of von Hippel-Lindau disease

6.1 Summary

HIF mediates many responses to hypoxia. Its regulation is principally by oxygen-dependent degradation, which is initiated by hydroxylation of specific proline residues and then binding of von Hippel-Lindau (VHL) protein. Similarly to Chapter 5, experimental work described in this chapter assesses which aspects of the Chuvash phenotype are reproduced in classical VHL disease, which involves germline heterozygosity for an inactivating mutation in VHL. Although primarily a hereditary cancer syndrome, abnormalities in neutrophil biology also suggest some degree of haploinsufficiency for VHL. No cardiopulmonary phenotype was detected in classical VHL disease.

6.2 Introduction

Classical VHL disease arises through a heterozygous inactivating mutation of VHL that has a severe effect on function (Latif et al. 1993). In this hereditary cancer syndrome, tumours form only following a second (somatic) mutation, so that the function of both alleles of VHL in these cells has been disrupted (Knudson 1986; Knudson & Strong 1972). However, it has been observed that neutrophils from VHL patients (which will have one unaffected allele for VHL) exhibit delayed apoptosis and enhanced bacterial phagocytosis (Walmsley et al. 2006). This establishes that at least some cells are sensitive to haploinsufficiency in relation to VHL and, if true, raises the question of whether any aspects of the CP phenotype (described in Chapter 3) may

also be present in classical VHL disease. The aim of the experiments presented in this chapter was to address this question.

6.3 Methods

Participants. Six VHL patients and six healthy control participants took part in the study. VHL patients were recruited through their respective physicians. VHL patients had no other medical disorders. Control participants were recruited through advertisement and were chosen to match VHL patients for sex, age, height, weight and body mass index.

All participants were informed about the aims, procedure and details of the study and signed a consent form before taking part in the experiments. The studies conformed to the Declaration of Helsinki and had been approved by the Oxfordshire Research Ethics Committee.

The experimental protocol (apart from the exercise test), technique and echocardiographic measurements were the same as those used for the HIF2 study, described in Chapter 5. Data analysis was performed as for the HIF2 study.

6.4 Results

The control participants matched the VHL patients well for age, height, weight and body mass index (Table 8).

Participant	Age (y)	Sex	Height (m)	Weight (kg)	BMI (kg m ⁻²)
VHL patient #1	37	M	1.78	98	31
Control #1	42	M	1.73	92	31
VHL patient #2	37	M	1.78	72	23
Control #2	39	M	1.80	76	23
VHL patient #3	42	M	1.83	95	28
Control #3	43	M	1.90	118	33
VHL patient #4	53	F	1.70	93	32
Control #4	53	F	1.66	90	33
VHL patient #5	27	M	1.80	83	25
Control #5	32	M	1.79	77	24
VHL patient #6	58	F	1.55	51	21
Control #6	63	F	1.62	62	24
VHL patient Average	42		1.74	82	27
VHL patient SD	11		0.10	18	4
Control Average	45		1.75	86	28
Control SD	11		0.10	19	5
<i>P</i> -value	0.65		0.87	0.73	0.71

Table 8. Individual and group characteristics of VHL patients and control participants

Age, sex, height, weight and body mass index (BMI). Standard deviation (SD) values are reported for the respective mean values. *P*-values compare the VHL patient group with the respective matched control participant group.

VHL patients did not differ significantly from control participants in arterial partial pressure of carbon dioxide, arterial pH, haemoglobin, haematocrit, mean corpuscular volume, serum iron, ferritin and transferrin (Table 9). In three VHL patients and in one control participant the arterial partial pressure of oxygen was lower than is predicted for their age, but average arterial partial pressure of oxygen did not differ significantly between VHL patients and control participants.

Analysis (Normal Range) ^a	VHL (<i>n</i> = 6)	Control (<i>n</i> = 6)	<i>P</i>-value
Arterial PO ₂ (83 - 105 mmHg)	83 ± 9	90 ± 9	0.24
Arterial PCO ₂ (35-45 mmHg)	41 ± 3	41 ± 1	0.75
Arterial pH (7.35-7.45)	7.40 ± 0.02	7.37 ± 0.03	0.11
Haemoglobin (12-17 g/dl)	14 ± 2	15 ± 2	0.39
Haematocrit (0.30-0.50 l/l)	0.43 ± 0.04	0.46 ± 0.06	0.27
Mean corpuscular volume (83-105 fl)	88 ± 4	89 ± 3	0.66
Serum iron (11-31 mmol/l)	14 ± 5	13 ± 3	0.59
Ferritin (10-300 mg/l)	63 ± 71	173 ± 112	0.18
Transferrin (1.8-3.6 g/l)	2.8 ± 0.5	2.7 ± 0.1	0.94

Table 9. Arterial and venous blood analyses for VHL study.

Values are mean ± SD. *P*-values compare the VHL patient group with the respective matched control participant group.

^a Where normal ranges vary with sex, the widest range is shown.

Pulmonary measurements. The average ventilations in the VHL patients and in the control participants before, during and after the exposures to mild and moderate hypoxia are illustrated in Figure 35. VHL patients did not differ significantly from control participants in either baseline ventilation or in the increment in ventilation with mild or moderate hypoxia.

Tricuspid regurgitation was detectable in five of the six VHL patients, but in only two of the control participants. The average pulmonary arterial systolic pressures obtained from these individuals before, during and after the exposures to hypoxia are illustrated in Figure 35. VHL patients did not differ significantly from control participants in either their baseline pulmonary arterial systolic pressures or in the increments in those pressures with mild or moderate hypoxia. These responses were also very similar to the responses of the control participants for the HIF2 study, presented in Chapter 5.

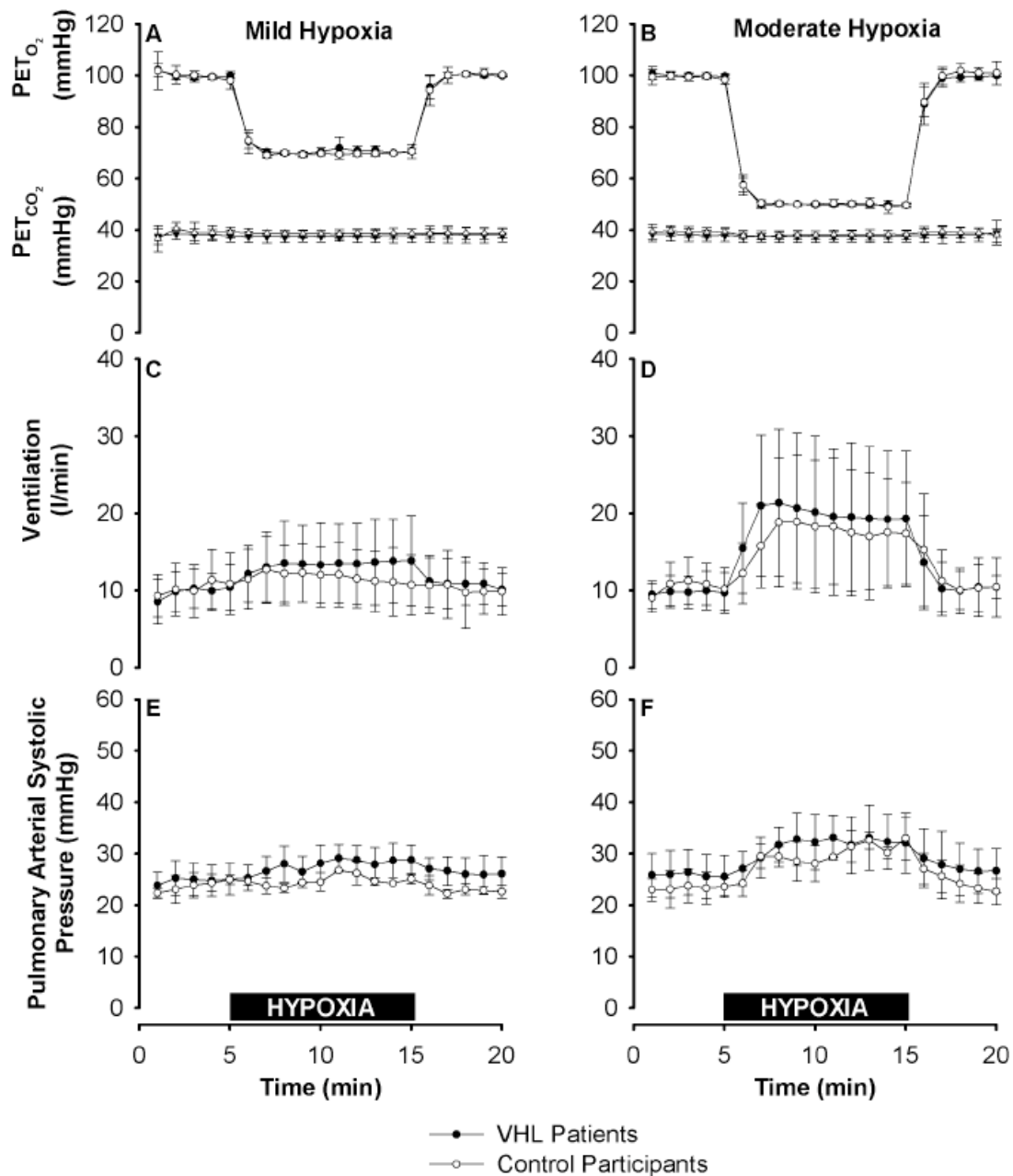


Figure 35. End-tidal gas control, ventilation and pulmonary arterial systolic pressure during mild and moderate hypoxia in VHL study.

(A and B) End-tidal partial pressures for oxygen (PET_{O₂}) and carbon dioxide (PET_{CO₂}). PET_{O₂} was well matched between groups.

(C and D) Ventilation, given at body temperature and pressure, saturated with water vapour. Ventilation at baseline and the increase provoked by hypoxia were similar between the two groups.

(E and F) Pulmonary arterial systolic pressure. Pulmonary arterial systolic pressure at baseline and the increase provoked by hypoxia were similar in the two groups.

Values are mean $\pm$ SD. The horizontal black bars indicate the 10 min periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

Systemic vascular measurements. The average values for the systemic vascular measurements in the VHL patients and in the control participants before, during and after the exposures to mild and moderate hypoxia are illustrated in Figure 36. VHL patients did not differ significantly from control participants in either their baseline values for heart rate, blood pressure and cardiac output or in the increments in these variables with mild or moderate hypoxia.

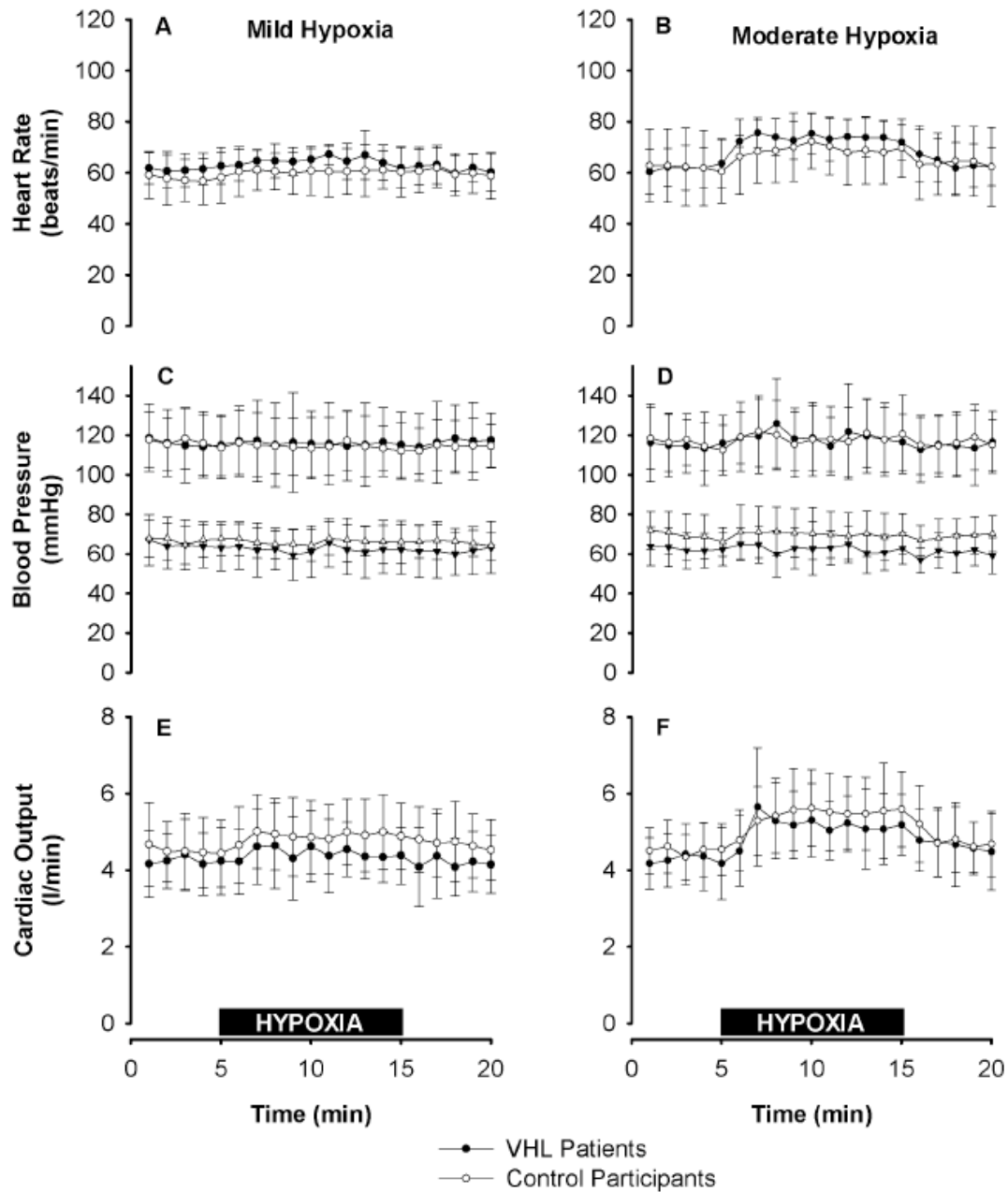


Figure 36. Systemic vascular responses in VHL patients and control participants to mild and moderate hypoxia.

(A and B) Heart rate. (C and D) Systolic (top) and diastolic (bottom) blood pressure. (E and F) Cardiac output, assessed non-invasively using Doppler ultrasound echocardiography. No statistically significant differences were detected between the two groups in either baseline values or in the increments with hypoxia. Values are mean $\pm$ SD. The horizontal black bars indicate the 10 min periods of mild (A, C and E) and moderate (B, D and F) exposures to hypoxia.

Summary for VHL study. The deviation of each participant's baseline value (or incremental response) from the mean value for the control group is expressed in units of standard deviations of the control group (Figure 37). There was considerable overlap between VHL patients and control participants for all variables.

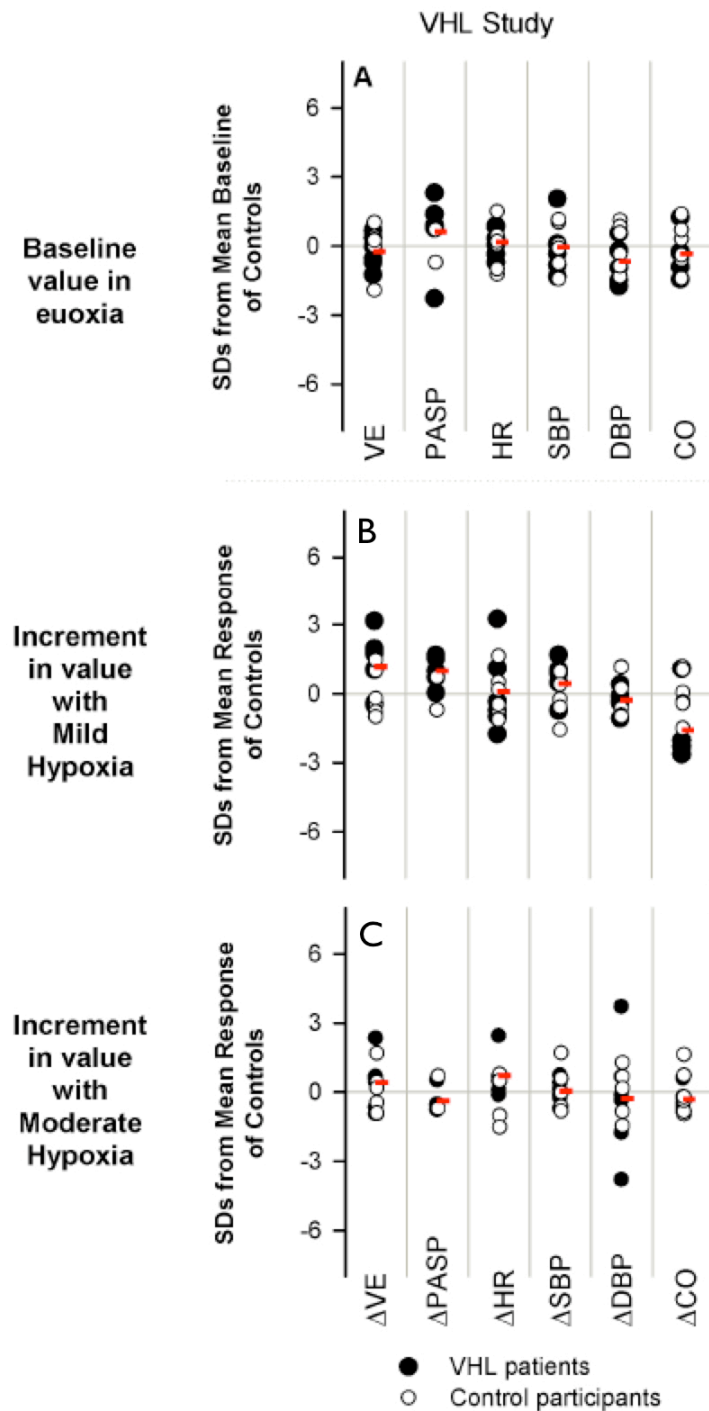


Figure 37. Cardiopulmonary variables in the VHL study: baseline measurements

(A) and incremental responses with hypoxia (B and C).

Results are shown in terms of the number of (control group) standard deviations by which each participant's baseline and response differed respectively from the mean baseline and mean response of the control participants. Red symbols show average results for patients' group.

(A, B and C) VHL patients were not significantly different from the control participants for any variable.

VE, Ventilation; PASP, Pulmonary Arterial Systolic Pressure; HR, Heart Rate; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; CO, Cardiac Output; Δ , increment.

6.5 Discussion

This study did not detect any of the cardiopulmonary components of the CP phenotype in patients with classical VHL disease. Thus a single functional allele for VHL appears to be sufficient to ensure normality in relation to these components of the phenotype.

In patients with VHL disease, erythrocytosis arises only occasionally (Lonser et al. 2003), mostly in association with enlarged cerebellar tumours and advanced renal cell carcinoma. The removal of such tumours seems to be sufficient for the excessive erythrocytosis to vanish, suggesting that underlying regulation is normal in the context of a single functioning VHL allele. Thus mutation within a single allele of VHL was not thought to be sufficient to disrupt function. Subsequently, however, some degree of haploinsufficiency was suggested by the observation that freshly isolated neutrophils from VHL patients displayed delayed apoptosis and enhanced bacterial phagocytosis, consistent with a pseudo-hypoxic phenotype (Walmsley et al. 2006).

Haploinsufficiency resulting in HIF activation was further supported by the presence of elevated levels of PHD3 transcript in these cells, as this is a known HIF target gene (Appelhoff et al. 2004; Aprelikova et al. 2004). From these observations, the authors concluded that, in humans, “heterozygous VHL defects are sufficient to perturb normal responses”, at least in some cells. The results from the experiments presented in this

chapter did not find that inactivation of a single allele of VHL was sufficient to alter human cardiopulmonary physiology. Thus variations in HIF-VHL function between cell types appear sufficiently large to result in haplosufficiency in some systems, but not others.

Chapter 7. Future research

The discussion for each study is presented within the relevant chapter.

Given the experimental evidence presented in this thesis, potentially interesting future research could:

- i) study the phenotype of other patients with unexplained polycythaemia potentially linked with abnormalities in the HIF pathway, with particular reference to the phenotype found in CP patients (i.e. elevated pulmonary blood pressure, ventilation, responses to hypoxia, metabolic abnormalities)
- ii) identify other functional genetic disorders of the HIF pathway, or mutations that affect its functioning, and sequence to find novel mutations (i.e. not reported before)
- iii) using CP and HIF2 patients, investigate the physiological role of HIF in the functioning of organs such as the liver or the brain when HIF and HIF-2alpha levels are mildly elevated at systemic level
- iv) study animal models of HIF disorders for reductive experiments; for example, a CP mouse model has been generated in which experiments are possible to understand mechanisms underlying the observed abnormalities in phenotype
- v) explore the effects of HIF hydroxylase inhibitors and dichloroacetate on the regulation of pulmonary blood pressure and of lactate production during exercise, especially in response to hypoxia

References

- Adair, T. H., Gay, W. J. & Montani, J. P. 1990 Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am J Physiol* **259**, R393-404.
- Allemann, Y., Sartori, C., Lepori, M., Pierre, S., Melot, C., Naeije, R., Scherrer, U. & Maggiorini, M. 2000 Echocardiographic and invasive measurements of pulmonary artery pressure correlate closely at high altitude. *Am J Physiol Heart Circ Physiol* **279**, H2013-6.
- Ang, S. O., Chen, H., Hirota, K., Gordeuk, V. R., Jelinek, J., Guan, Y. L., Liu, E. L., Sergueeva, A. I., Miasnikova, G. Y., Mole, D., Maxwell, P. H., Stockton, D. W., Semenza, G. L. & Prchal, J. T. 2002 Disruption of oxygen homeostasis underlies congenital Chuvash polycythemia. *Nature Genetics* **32**, 614-621.
- Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J. & Gleadle, J. M. 2004 Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem* **279**, 38458-65.
- Aprelikova, O., Chandramouli, G. V., Wood, M., Vasselli, J. R., Riss, J., Maranchie, J. K., Linehan, W. M. & Barrett, J. C. 2004 Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J Cell Biochem* **92**, 491-501.
- Aragones, J., Schneider, M., Van Geyte, K., Fraisl, P., Dresselaers, T., Mazzone, M., Dirx, 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. & Carmeliet, P. 2008 Deficiency or inhibition of oxygen sensor Phd1 induces hypoxia tolerance by reprogramming basal metabolism. *Nature Genetics* **40**, 170-180.
- Banchero, N. 1975 Capillary density of skeletal muscle in dogs exposed to simulated altitude (38556). *Proc Soc Exp Biol Med* **148**, 435-9.
- Banchero, N. 1982 Long term adaptation of skeletal muscle capillarity. *Physiologist* **25**, 385-9.
- Banchero, N. 1987 Cardiovascular responses to chronic hypoxia. *Annu Rev Physiol* **49**, 465-76.
- 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. & 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**, 11459-64.
- Beer, M., Seyfarth, T., Sandstede, J., Landschutz, W., Lipke, C., Kostler, H., von Kienlin, M., Harre, K., Hahn, D. & Neubauer, S. 2002 Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. *J Am Coll Cardiol* **40**, 1267-74.
- Bento, M. C., Chang, K. T., Guan, Y., Liu, E., Caldas, G., Gatti, R. A. & Prchal, J. T. 2005 Congenital polycythemia with homozygous and heterozygous mutations

- of von Hippel-Lindau gene: five new Caucasian patients. *Haematologica* **90**, 128-9.
- Bergstrom, J. 1962 Muscle Electrolytes in Man - Determined by Neutron Activation Analysis on Needle Biopsy Specimens - Study on Normal Subjects, Kidney Patients, and Patients with Chronic Diarrhoea. *Scandinavian Journal of Clinical & Laboratory Investigation* **14**, 1-&.
- Berne, R. M. 1963 Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am J Physiol* **204**, 317-22.
- Bigham, A., Bauchet, M., Pinto, D., Mao, X., Akey, J. M., Mei, R., Scherer, S. W., Julian, C. G., Wilson, M. J., Lopez Herraiez, D., Brutsaert, T., Parra, E. J., Moore, L. G. & Shriver, M. D. 2010 Identifying signatures of natural selection in tibetan and andean populations using dense genome scan data. *PLoS Genet* **6**.
- Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A. & Popov, K. M. 1998 Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. *Biochem J* **329 (Pt 1)**, 191-6.
- Brooks, G. A. 2007 Comments on point:counterpoint: "the lactate paradox does/does not occur during exercise at high altitude". *J Appl Physiol* **102**, 2408; author reply 2409-10.
- 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. & Gordeuk, V. R. 2006 Endothelin-1, vascular endothelial growth factor and systolic pulmonary artery pressure in patients with Chuvash polycythemia. *Haematologica* **91**, 744-9.

- Calbet, J. A. 2003 Chronic hypoxia increases blood pressure and noradrenaline spillover in healthy humans. *J Physiol* **551**, 379-86.
- Cantley, J., Selman, C., Shukla, D., Abramov, A. Y., Forstreuter, F., Esteban, M. A., Claret, M., Lingard, S. J., Clements, M., Harten, S. K., Asare-Anane, H., Batterham, R. L., Herrera, P. L., Persaud, S. J., Duchon, M. R., Maxwell, P. H. & Withers, D. J. 2009 Deletion of the von Hippel-Lindau gene in pancreatic beta cells impairs glucose homeostasis in mice. *J Clin Invest* **119**, 125-35.
- Cario, H., Schwarz, K., Jorch, N., Kyank, U., Petrides, P. E., Schneider, D. T., Uhle, R., Debatin, K. M. & Kohne, E. 2005 Mutations in the von Hippel-Lindau (VHL) tumor suppressor gene and VHL-haplotype analysis in patients with presumable congenital erythrocytosis. *Haematologica* **90**, 19-24.
- Cassin, S., Gilbert, R. D., Bunnell, C. E. & Johnson, E. M. 1971 Capillary development during exposure to chronic hypoxia. *Am J Physiol* **220**, 448-51.
- Cerqueira, M. D., Weissman, N. J., Dilsizian, V., Jacobs, A. K., Kaul, S., Laskey, W. K., Pennell, D. J., Rumberger, J. A., Ryan, T. & Verani, M. S. 2002 Standardized myocardial segmentation and nomenclature for tomographic imaging of the heart: a statement for healthcare professionals from the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association. *Circulation* **105**, 539-42.
- Cerretelli, P., Marzorati, M. & Marconi, C. 2009 Muscle bioenergetics and metabolic control at altitude. *High Alt Med Biol* **10**, 165-74.
- Chemla, D., Castelain, V., Humbert, M., Hebert, J. L., Simonneau, G., Lecarpentier, Y. & Herve, P. 2004 New formula for predicting mean pulmonary artery pressure using systolic pulmonary artery pressure. *Chest* **126**, 1313-7.

- Christie, J., Sheldahl, L. M., Tristani, F. E., Sagar, K. B., Ptacin, M. J. & Wann, S. 1987 Determination of stroke volume and cardiac output during exercise: comparison of two-dimensional and Doppler echocardiography, Fick oximetry, and thermodilution. *Circulation* **76**, 539-47.
- Constantin-Teodosiu, D., Cederblad, G. & Hultman, E. 1991 A sensitive radioisotopic assay of pyruvate dehydrogenase complex in human muscle tissue. *Anal Biochem* **198**, 347-51.
- Conway, M. A., Allis, J., Ouwerkerk, R., Niioka, T., Rajagopalan, B. & Radda, G. K. 1991 Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by ³¹P magnetic resonance spectroscopy. *Lancet* **338**, 973-6.
- Coppack, S. W., Frayn, K. N., Humphreys, S. M., Whyte, P. L. & Hockaday, T. D. 1990 Arteriovenous differences across human adipose and forearm tissues after overnight fast. *Metabolism* **39**, 384-90.
- Danovaro, R., Dell'Anno, A., Pusceddu, A., Gambi, C., Heiner, I. & Kristensen, R. M. 2010 The first metazoa living in permanently anoxic conditions. *BMC Biol* **8**, 30.
- De Filippi, F. 1909 *Karakoram and Western Himalaya*. New York: E.P. Dutton and Company.
- Dempsey, J. A., Forster, H. V., Birnbaum, M. L., Reddan, W. G., Thoden, J., Grover, R. F. & Rankin, J. 1972 Control of exercise hyperpnea under varying durations of exposure to moderate hypoxia. *Respir Physiol* **16**, 213-31.
- Deveci, D., Marshall, J. M. & Egginton, S. 2001 Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia. *Am J Physiol Heart Circ Physiol* **281**, H241-52.

- Dorrington, K. L., Clar, C., Young, J. D., Jonas, M., Tansley, J. G. & Robbins, P. A. 1997 Time course of the human pulmonary vascular response to 8 hours of isocapnic hypoxia. *Am J Physiol* **273**, H1126-34.
- Douglas, C. G., Haldane, J. S., Henderson, Y., Schneider, E. C., Webb, G. B. & Richards, J. H. 1913 Physiological observations made on Pike's Peak, Colorado, with special reference to adaptation to low barometric pressures. *Phil. Trans. R. Soc. Lond. B* **203**, 185-318.
- Duling, B. R. & Berne, R. M. 1970 Longitudinal gradients in periarteriolar oxygen tension. A possible mechanism for the participation of oxygen in local regulation of blood flow. *Circ Res* **27**, 669-78.
- 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. & Ratcliffe, P. J. 2001 *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.
- Eriksen, M. & Walloe, L. 1990 Improved method for cardiac output determination in man using ultrasound Doppler technique. *Med Biol Eng Comput* **28**, 555-60.
- Euler, U. S. V. & Liljestrand, G. 1946 Observations on the Pulmonary Arterial Blood Pressure in the Cat. *Acta Physiol Scand* **12**, 301-320.
- Firth, J. D., Ebert, B. L., Pugh, C. W. & Ratcliffe, P. J. 1994 Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proc Natl Acad Sci U S A* **91**, 6496-500.

- Firth, J. D., Ebert, B. L. & Ratcliffe, P. J. 1995 Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. *J Biol Chem* **270**, 21021-7.
- 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. & Robbins, P. A. 2010 Regulation of human metabolism by hypoxia-inducible factor. *Proc Natl Acad Sci U S A* **107**, 12722-7.
- Frayn, K. N. & Coppack, S. W. 2001 Assessment of white adipose tissue metabolism by measurement of arteriovenous differences. *Methods Mol Biol* **155**, 269-79.
- Fukuda, R., Zhang, H., Kim, J. W., Shimoda, L., Dang, C. V. & Semenza, G. L. 2007 HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* **129**, 111-22.
- Fukuda, T., Maegawa, T., Matsumoto, A., Komatsu, Y., Nakajima, T., Nagai, R. & Kawahara, T. 2010 Effects of acute hypoxia at moderate altitude on stroke volume and cardiac output during exercise. *Int Heart J* **51**, 170-5.
- Gale, D. P., Harten, S. K., Reid, C. D., Tuddenham, E. G. & Maxwell, P. H. 2008 Autosomal dominant erythrocytosis and pulmonary arterial hypertension associated with an activating HIF2 alpha mutation. *Blood* **112**, 919-21.
- Gordeuk, V. R. & Prchal, J. T. 2006 Vascular complications in Chuvash polycythemia. *Semin Thromb Hemost* **32**, 289-94.
- Gordeuk, V. R., Sergueeva, A. I., Miasnikova, G. Y., Okhotin, D., Voloshin, Y., Choyke, P. L., Butman, J. A., Jedlickova, K., Prchal, J. T. & Polyakova, L. A. 2004 Congenital disorder of oxygen sensing: association of the homozygous

- Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood* **103**, 3924-32.
- Gore, C. J., Clark, S. A. & Saunders, P. U. 2007 Nonhematological mechanisms of improved sea-level performance after hypoxic exposure. *Med Sci Sports Exerc* **39**, 1600-9.
- Grassi, B., Marzorati, M., Kayser, B., Bordini, M., Colombini, A., Conti, M., Marconi, C. & Cerretelli, P. 1996 Peak blood lactate and blood lactate vs. workload during acclimatization to 5,050 m and in deacclimatization. *J Appl Physiol* **80**, 685-92.
- Graven, K. K., Yu, Q., Pan, D., Roncarati, J. S. & Farber, H. W. 1999 Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. *Biochim Biophys Acta* **1447**, 208-18.
- Green, H. J., Sutton, J. R., Cymerman, A., Young, P. M. & Houston, C. S. 1989 Operation Everest II: adaptations in human skeletal muscle. *J Appl Physiol* **66**, 2454-61.
- Greenhaff, P. L., Campbell-O'Sullivan, S. P., Constantin-Teodosiu, D., Poucher, S. M., Roberts, P. A. & Timmons, J. A. 2002 An acetyl group deficit limits mitochondrial ATP production at the onset of exercise. *Biochem Soc Trans* **30**, 275-80.
- Greenhaff, P. L., Campbell-O'Sullivan, S. P., Constantin-Teodosiu, D., Poucher, S. M., Roberts, P. A. & Timmons, J. A. 2004 Metabolic inertia in contracting skeletal muscle: a novel approach for pharmacological intervention in peripheral vascular disease. *Br J Clin Pharmacol* **57**, 237-43.
- Grollman, A. 1930 Physiological variations of the cardiac output of man. VII. The effect of high altitude on the cardiac output and its related functions: an

- account of experiments conducted on the summit of Pike's Peak, Colorado.
American Journal of Physiology **93**, 19-40.
- Groves, B. M., Reeves, J. T., Sutton, J. R., Wagner, P. D., Cymerman, A., Malconian, M. K., Rock, P. B., Young, P. M. & Houston, C. S. 1987 Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *J Appl Physiol* **63**, 521-30.
- Gruber, M., Hu, C. J., Johnson, R. S., Brown, E. J., Keith, B. & Simon, M. C. 2007 Acute postnatal ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci U S A* **104**, 2301-6.
- Grunig, E., Mereles, D., Hildebrandt, W., Swenson, E. R., Kubler, W., Kuecherer, H. & Bartsch, P. 2000 Stress Doppler echocardiography for identification of susceptibility to high altitude pulmonary edema. *J Am Coll Cardiol* **35**, 980-7.
- Gu, Y. Z., Moran, S. M., Hogenesch, J. B., Wartman, L. & Bradfield, C. A. 1998 Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr* **7**, 205-13.
- Gudi, R., Bowker-Kinley, M. M., Kedishvili, N. Y., Zhao, Y. & Popov, K. M. 1995 Diversity of the pyruvate dehydrogenase kinase gene family in humans. *J Biol Chem* **270**, 28989-94.
- Harris, R. C., Hultman, E. & Nordesjo, L. O. 1974 Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest* **33**, 109-20.
- Hasan, D. Y., Kamlah, F., Seeger, W., Rose, F. & Hanze, J. 2010 Hypoxic Regulation Of Pyruvate Kinase Isoforms M1 And M2 And Their Inhibition By SiRNA In A549 Cells. *Am J Resp Crit Care Med* **181**, A2061.

- Hickey, M. M., Lam, J. C., Bezman, N. A., Rathmell, W. K. & Simon, M. C. 2007 von Hippel-Lindau mutation in mice recapitulates Chuvash polycythemia via hypoxia-inducible factor-2alpha signaling and splenic erythropoiesis. *J Clin Invest* **117**, 3879-89.
- Hickey, M. M., Richardson, T., Wang, T., Mosqueira, M., Arguiri, E., Yu, H., Yu, Q. C., Solomides, C. C., Morrissey, E. E., Khurana, T. S., Christofidou-Solomidou, M. & Simon, M. C. 2010 The von Hippel-Lindau Chuvash mutation promotes pulmonary hypertension and fibrosis in mice. *J Clin Invest* **120**, 827-39.
- Hochachka, P. W. 1988 The lactate paradox: analysis of underlying mechanisms. *Ann Sports Med* **4**, 184-188.
- Hochachka, P. W., Beatty, C. L., Burelle, Y., Trump, M. E., McKenzie, D. C. & Matheson, G. O. 2002 The lactate paradox in human high-altitude physiological performance. *News Physiol Sci* **17**, 122-6.
- Hopkins, W. G., Schabort, E. J. & Hawley, J. A. 2001 Reliability of power in physical performance tests. *Sports Med* **31**, 211-34.
- Hoppeler, H., Kleinert, E., Schlegel, C., Claassen, H., Howald, H., Kayar, S. R. & Cerretelli, P. 1990 Morphological adaptations of human skeletal muscle to chronic hypoxia. *Int J Sports Med* **11 Suppl 1**, S3-9.
- Hoult, D. I., Busby, S. J., Gadian, D. G., Radda, G. K., Richards, R. E. & Seeley, P. J. 1974 Observation of tissue metabolites using ³¹P nuclear magnetic resonance. *Nature* **252**, 285-7.
- Howald, H., Pette, D., Simoneau, J. A., Uber, A., Hoppeler, H. & Cerretelli, P. 1990 Effect of chronic hypoxia on muscle enzyme activities. *Int J Sports Med* **11 Suppl 1**, S10-4.

- Howson, M., Khamnei, S., O'Connor, D. & Robbins, P. A. 1986 The properties of a turbine device for measuring respiratory volumes in man. *J Physiol* **382**, 12P.
- Hu, C. J., Wang, L. Y., Chodosh, L. A., Keith, B. & Simon, M. C. 2003 Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* **23**, 9361-74.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. & Kaelin, W. G., Jr. 2001 HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* **292**, 464-8.
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W. & Ratcliffe, P. J. 2001 Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* **292**, 468-72.
- Jansson, P. A., Smith, U. & Lonnroth, P. 1990 Evidence for lactate production by human adipose tissue in vivo. *Diabetologia* **33**, 253-6.
- Jones, S. W., Baker, D. J., Gardiner, S. M., Bennett, T., Timmons, J. A. & Greenhaff, P. L. 2004 The effect of the beta2-adrenoceptor agonist prodrug BRL-47672 on cardiovascular function, skeletal muscle myosin heavy chain, and MyoD expression in the rat. *J Pharmacol Exp Ther* **311**, 1225-31.
- Kemp, G. J., Meyerspeer, M. & Moser, E. 2007 Absolute quantification of phosphorus metabolite concentrations in human muscle in vivo by ³¹P MRS: a quantitative review. *NMR Biomed* **20**, 555-65.

- Kim, J. W., Tchernyshyov, I., Semenza, G. L. & Dang, C. V. 2006 HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* **3**, 177-85.
- Klausen, K., Robinson, S., Micahel, E. D. & Myhre, L. G. 1966 Effect of high altitude on maximal working capacity. *J Appl Physiol* **21**, 1191-4.
- Knudson, A. G., Jr. 1986 Genetics of human cancer. *Annu Rev Genet* **20**, 231-51.
- Knudson, A. G., Jr. & Strong, L. C. 1972 Mutation and cancer: neuroblastoma and pheochromocytoma. *Am J Hum Genet* **24**, 514-32.
- Kreisberg, R. A. 1972 Glucose-lactate inter-relations in man. *N Engl J Med* **287**, 132-7.
- Krogh, A. 1919 The supply of oxygen to the tissues and the regulation of the capillary circulation. *J Physiol* **52**, 457-74.
- Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L. & et al. 1993 Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* **260**, 1317-20.
- Liljestrand, G. 1948 Regulation of pulmonary arterial blood pressure. *Arch Med Interna* **81**, 162-72.
- Lok, C. N. & Ponka, P. 1999 Identification of a hypoxia response element in the transferrin receptor gene. *J Biol Chem* **274**, 24147-52.
- Lonser, R. R., Glenn, G. M., Walther, M., Chew, E. Y., Libutti, S. K., Linehan, W. M. & Oldfield, E. H. 2003 von Hippel-Lindau disease. *Lancet* **361**, 2059-67.
- Lundby, C., Calbet, J. A. & Robach, P. 2009 The response of human skeletal muscle tissue to hypoxia. *Cell Mol Life Sci* **66**, 3615-23.
- Lundby, C., Pilegaard, H., Andersen, J. L., van Hall, G., Sander, M. & Calbet, J. A. 2004 Acclimatization to 4100 m does not change capillary density or mRNA

- expression of potential angiogenesis regulatory factors in human skeletal muscle. *J Exp Biol* **207**, 3865-71.
- Lundby, C., Saltin, B. & van Hall, G. 2000 The 'lactate paradox', evidence for a transient change in the course of acclimatization to severe hypoxia in lowlanders. *Acta Physiol Scand* **170**, 265-9.
- MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P. & Houston, C. S. 1991 Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol Scand* **142**, 421-7.
- Manalo, D. J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B. D., Ye, S. Q., Garcia, J. G. & Semenza, G. L. 2005 Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* **105**, 659-69.
- Martini, M., Teofili, L., Cenci, T., Giona, F., Torti, L., Rea, M., Foa, R., Leone, G. & Larocca, L. M. 2008 A novel heterozygous HIF2AM535I mutation reinforces the role of oxygen sensing pathway disturbances in the pathogenesis of familial erythrocytosis. *Haematologica* **93**, 1068-71.
- 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. & Johnson, R. S. 2004 Loss of skeletal muscle HIF-1alpha results in altered exercise endurance. *PLoS Biol* **2**, 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. & Johnson, R. S. 2007 HIF-1alpha in endurance training: suppression of oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* **293**, R2059-69.

- 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. & Ratcliffe, P. J.
1999 The tumour suppressor protein VHL targets hypoxia-inducible factors for
oxygen-dependent proteolysis. *Nature* **399**, 271-5.
- Mazzeo, R. S. 2007 Comments on point:counterpoint: "the lactate paradox does/does
not occur during exercise at high altitude". *J Appl Physiol* **102**, 2403; author
reply 2409-10.
- Minchenko, A., Leshchinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V.
& Caro, J. 2002 Hypoxia-inducible factor-1-mediated expression of the 6-
phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its
possible role in the Warburg effect. *J Biol Chem* **277**, 6183-7.
- Minchenko, O., Opentanova, I. & Caro, J. 2003 Hypoxic regulation of the 6-
phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene family (PFKFB-1-4)
expression in vivo. *FEBS Lett* **554**, 264-70.
- Mizuno, M., Savard, G. K., Areskog, N. H., Lundby, C. & Saltin, B. 2008 Skeletal
muscle adaptations to prolonged exposure to extreme altitude: a role of
physical activity? *High Alt Med Biol* **9**, 311-7.
- Moon, R. B. & Richards, J. H. 1973 Determination of intracellular pH by ³¹P
magnetic resonance. *J Biol Chem* **248**, 7276-8.
- Mosso, A. 1884 La respirazione dell'uomo sulle alte montagne. *Real Acc Sci Torino* **4**,
1-10.
- Motley, H. L., Cournand, A. & et al. 1947 The influence of short periods of induced
acute anoxia upon pulmonary artery pressures in man. *Am J Physiol* **150**, 315-
20.

- Naressi, A., Couturier, C., Castang, I., de Beer, R. & Graveron-Demilly, D. 2001a
Java-based graphical user interface for MRUI, a software package for
quantitation of in vivo/medical magnetic resonance spectroscopy signals.
Comput Biol Med **31**, 269-86.
- Naressi, A., Couturier, C., Devos, J. M., Janssen, M., Mangeat, C., de Beer, R. &
Graveron-Demilly, D. 2001b Java-based graphical user interface for the MRUI
quantitation package. *MAGMA* **12**, 141-52.
- Neubauer, S. 2007 The failing heart--an engine out of fuel. *N Engl J Med* **356**, 1140-
51.
- Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich,
N., Chau, V. & Kaelin, W. G. 2000 Ubiquitination of hypoxia-inducible factor
requires direct binding to the beta-domain of the von Hippel-Lindau protein.
Nat Cell Biol **2**, 423-7.
- Opie, L. H. & Newsholme, E. A. 1967 The activities of fructose 1,6-diphosphatase,
phosphofructokinase and phosphoenolpyruvate carboxykinase in white muscle
and red muscle. *Biochem J* **103**, 391-9.
- Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L. & Denko, N. C. 2006 HIF-1
mediates adaptation to hypoxia by actively downregulating mitochondrial
oxygen consumption. *Cell Metab* **3**, 187-97.
- Pastore, Y., Jedlickova, K., Guan, Y., Liu, E., Fahner, J., Hasle, H., Prchal, J. F. &
Prchal, J. T. 2003a Mutations of von Hippel-Lindau tumor-suppressor gene and
congenital polycythemia. *Am J Hum Genet* **73**, 412-9.
- Pastore, Y. D., Jelinek, J., Ang, S., Guan, Y., Liu, E., Jedlickova, K., Krishnamurti, L.
& Prchal, J. T. 2003b Mutations in the VHL gene in sporadic apparently
congenital polycythemia. *Blood* **101**, 1591-5.

- Peacock, A. J., Challenor, V. & Sutherland, G. 1990 Estimation of pulmonary artery pressure by Doppler echocardiography in normal subjects made hypoxic. *Respir Med* **84**, 335-7.
- Percy, M. J., Beer, P. A., Campbell, G., Dekker, A. W., Green, A. R., Oscier, D., Rainey, M. G., van Wijk, R., Wood, M., Lappin, T. R., McMullin, M. F. & Lee, F. S. 2008a Novel exon 12 mutations in the HIF2A gene associated with erythrocytosis. *Blood* **111**, 5400-2.
- Percy, M. J., Furlow, P. W., Lucas, G. S., Li, X., Lappin, T. R., McMullin, M. F. & Lee, F. S. 2008b A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med* **358**, 162-8.
- Percy, M. J., McMullin, M. F., Jowitt, S. N., Potter, M., Treacy, M., Watson, W. H. & Lappin, T. R. 2003 Chuvash-type congenital polycythemia in 4 families of Asian and Western European ancestry. *Blood* **102**, 1097-9.
- Perrotta, S., Nobili, B., Ferraro, M., Migliaccio, C., Borriello, A., Cucciolla, V., Martinelli, V., Rossi, F., Punzo, F., Cirillo, P., Parisi, G., Zappia, V., Rotoli, B. & Ragione, F. D. 2006 Von Hippel-Lindau-dependent polycythemia is endemic on the island of Ischia: identification of a novel cluster. *Blood* **107**, 514-9.
- Peterson, G. L. 1977 A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* **83**, 346-56.
- Pollard, P. J., Briere, J. J., Alam, N. A., Barwell, J., Barclay, E., Wortham, N. C., Hunt, T., Mitchell, M., Olpin, S., Moat, S. J., Hargreaves, I. P., Heales, S. J., Chung, Y. L., Griffiths, J. R., Dalglish, A., McGrath, J. A., Gleeson, M. J., Hodgson, S. V., Poulson, R., Rustin, P. & Tomlinson, I. P. 2005 Accumulation of Krebs cycle intermediates and over-expression of HIF1alpha in tumours

- which result from germline FH and SDH mutations. *Hum Mol Genet* **14**, 2231-9.
- Polyakova, L. A. 1974 Familial erythrocytosis among the residents of the Chuvash ASSR. *Probl Gematol Pereliv Krovi* **19**, 30-33.
- Prowse, A. H., Webster, A. R., Richards, F. M., Richard, S., Olschwang, S., Resche, F., Affara, N. A. & Maher, E. R. 1997 Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumors. *Am J Hum Genet* **60**, 765-71.
- Ptashne, K. A., Theodore, J. & Robin, E. D. 1983 Increased phosphofructokinase content during chronic hypoxia in cultured skeletal muscle (L8) cells. *Biochim Biophys Acta* **763**, 169-74.
- Puri, S., Cano, D. A. & Hebrok, M. 2009 A role for von Hippel-Lindau protein in pancreatic beta-cell function. *Diabetes* **58**, 433-41.
- Radegran, G. 1999 Limb and skeletal muscle blood flow measurements at rest and during exercise in human subjects. *Proc Nutr Soc* **58**, 887-98.
- Reeves, J. T., Wolfel, E. E., Green, H. J., Mazzeo, R. S., Young, A. J., Sutton, J. R. & Brooks, G. A. 1992 Oxygen transport during exercise at altitude and the lactate paradox: lessons from Operation Everest II and Pikes Peak. *Exerc Sport Sci Rev* **20**, 275-96.
- Renkin, E. M. 1967 Blood flow and transcapillary exchange in skeletal and cardiac muscle. In *Proceedings International Symposium on Coronary Circulation and Energetics of the Myocardium* (ed. G. Marchetti & B. Taccardi), pp. 18-29. Karger, Basel.
- Riddle, S. R., Ahmad, A., Ahmad, S., Deeb, S. S., Malkki, M., Schneider, B. K., Allen, C. B. & White, C. W. 2000 Hypoxia induces hexokinase II gene expression in human lung cell line A549. *Am J Physiol Lung Cell Mol Physiol* **278**, L407-16.

- Robbins, P. A. 1995 Hypoxic ventilatory decline: site of action. *J Appl Physiol* **79**, 373-4.
- Robbins, P. A. 2007 Role of the peripheral chemoreflex in the early stages of ventilatory acclimatization to altitude. *Respir Physiol Neurobiol* **158**, 237-42.
- Robbins, P. A., Swanson, G. D. & Howson, M. G. 1982a A prediction-correction scheme for forcing alveolar gases along certain time courses. *J Appl Physiol* **52**, 1353-7.
- Robbins, P. A., Swanson, G. D., Micco, A. J. & Schubert, W. P. 1982b A fast gas-mixing system for breath-to-breath respiratory control studies. *J Appl Physiol* **52**, 1358-62.
- Rolfs, A., Kvietikova, I., Gassmann, M. & Wenger, R. H. 1997 Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J Biol Chem* **272**, 20055-62.
- Ross, J. M., Fairchild, H. M., Weldy, J. & Guyton, A. C. 1962 Autoregulation of blood flow by oxygen lack. *Am J Physiol* **202**, 21-4.
- Rowell, L. B., Kraning, K. K., 2nd, Evans, T. O., Kennedy, J. W., Blackmon, J. R. & Kusumi, F. 1966 Splanchnic removal of lactate and pyruvate during prolonged exercise in man. *J Appl Physiol* **21**, 1773-83.
- Salceda, S. & Caro, J. 1997 Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* **272**, 22642-7.
- Schofield, C. J. & Ratcliffe, P. J. 2004 Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* **5**, 343-54.

- Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L. J., Marck, B. T., Matsumoto, A. M., Shelton, J. M., Richardson, J. A., Bennett, M. J. & Garcia, J. A. 2003 Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in *Epas1*^{-/-} mice. *Nat Genet* **35**, 331-40.
- Semenza, G. L. 2004 Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology (Bethesda)* **19**, 176-82.
- Semenza, G. L. 2007 Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. *Biochem J* **405**, 1-9.
- Semenza, G. L., Jiang, B. H., Leung, S. W., Passantino, R., Concordet, J. P., Maire, P. & Giallongo, A. 1996 Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* **271**, 32529-37.
- Semenza, G. L., Roth, P. H., Fang, H. M. & Wang, G. L. 1994 Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* **269**, 23757-63.
- Semenza, G. L. & 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**, 5447-54.
- Sergeyeva, A., Gordeuk, V. R., Tokarev, Y. N., Sokol, L., Prchal, J. F. & Prchal, J. T. 1997 Congenital polycythemia in Chuvashia. *Blood* **89**, 2148-54.
- Shimoda, L. A., Fallon, M., Pisarcik, S., Wang, J. & Semenza, G. L. 2006 HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* **291**, L941-9.

- Shimoda, L. A., Manalo, D. J., Sham, J. S., Semenza, G. L. & Sylvester, J. T. 2001 Partial HIF-1alpha deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia. *Am J Physiol Lung Cell Mol Physiol* **281**, L202-8.
- Shohet, R. V. & Garcia, J. A. 2007 Keeping the engine primed: HIF factors as key regulators of cardiac metabolism and angiogenesis during ischemia. *J Mol Med* **85**, 1309-15.
- Sillau, A. H., Aquin, L., Bui, M. V. & Banchero, N. 1980 Chronic hypoxia does not affect guinea pig skeletal muscle capillarity. *Pflugers Arch* **386**, 39-45.
- Sillau, A. H. & Banchero, N. 1977 Effects of hypoxia on capillary density and fiber composition in rat skeletal muscle. *Pflugers Arch* **370**, 227-32.
- Skjaerpe, T. & Hatle, L. 1986 Noninvasive estimation of systolic pressure in the right ventricle in patients with tricuspid regurgitation. *Eur Heart J* **7**, 704-10.
- 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. & Robbins, P. A. 2006 Mutation of von Hippel-Lindau tumour suppressor and human cardiopulmonary physiology. *PLoS Med* **3**, e290.
- Smith, T. G., Talbot, N. P., Privat, C., Rivera-Ch, M., Nickol, A. H., Ratcliffe, P. J., Dorrington, K. L., Leon-Velarde, F. & Robbins, P. A. 2009 Effects of iron supplementation and depletion on hypoxic pulmonary hypertension: two randomized controlled trials. *Jama* **302**, 1444-50.
- Snyder, G. K., Wilcox, E. E. & Burnham, E. W. 1985 Effects of hypoxia on muscle capillarity in rats. *Respir Physiol* **62**, 135-40.

- Sugawara, J., Tanabe, T., Miyachi, M., Yamamoto, K., Takahashi, K., Iemitsu, M., Otsuki, T., Homma, S., Maeda, S., Ajisaka, R. & Matsuda, M. 2003 Non-invasive assessment of cardiac output during exercise in healthy young humans: comparison between Modelflow method and Doppler echocardiography method. *Acta Physiol Scand* **179**, 361-6.
- Sullivan, M. J., Saltin, B., Negro-Vilar, R., Duscha, B. D. & Charles, H. C. 1994 Skeletal muscle pH assessed by biochemical and ³¹P-MRS methods during exercise and recovery in men. *J Appl Physiol* **77**, 2194-200.
- Swanson, G. D. & Bellville, J. W. 1975 Step changes in end-tidal CO₂: methods and implications. *J Appl Physiol* **39**, 377-85.
- Tacchini, L., Bianchi, L., Bernelli-Zazzera, A. & Cairo, G. 1999 Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. *J Biol Chem* **274**, 24142-6.
- Talbot, N. P., Balanos, G. M., Dorrington, K. L. & Robbins, P. A. 2005 Two temporal components within the human pulmonary vascular response to approximately 2 h of isocapnic hypoxia. *J Appl Physiol* **98**, 1125-39.
- Tan, G. D., Fielding, B. A., Currie, J. M., Humphreys, S. M., Desage, M., Frayn, K. N., Laville, M., Vidal, H. & Karpe, F. 2005 The effects of rosiglitazone on fatty acid and triglyceride metabolism in type 2 diabetes. *Diabetologia* **48**, 83-95.
- Thomson, A. J., Drummond, G. B., Waring, W. S., Webb, D. J. & Maxwell, S. R. 2006 Effects of short-term isocapnic hyperoxia and hypoxia on cardiovascular function. *J Appl Physiol* **101**, 809-16.

- Tian, H., McKnight, S. L. & Russell, D. W. 1997 Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* **11**, 72-82.
- Tran, T. K., Sailasuta, N., Kreutzer, U., Hurd, R., Chung, Y., Mole, P., Kuno, S. & Jue, T. 1999 Comparative analysis of NMR and NIRS measurements of intracellular PO₂ in human skeletal muscle. *Am J Physiol* **276**, R1682-90.
- Tripp, M. E. 1989 Developmental cardiac metabolism in health and disease. *Pediatr Cardiol* **10**, 150-8.
- Tune, J. D., Richmond, K. N., Gorman, M. W., Olsson, R. A. & Feigl, E. O. 2000 Adenosine is not responsible for local metabolic control of coronary blood flow in dogs during exercise. *Am J Physiol Heart Circ Physiol* **278**, H74-84.
- Tyler, D. J., Emmanuel, Y., Cochlin, L. E., Hudsmith, L. E., Holloway, C. J., Neubauer, S., Clarke, K. & Robson, M. D. 2009a Reproducibility of 31P cardiac magnetic resonance spectroscopy at 3 T. *NMR Biomed* **22**, 405-413.
- Tyler, D. J., Emmanuel, Y., Cochlin, L. E., Hudsmith, L. E., Holloway, C. J., Neubauer, S., Clarke, K. & Robson, M. D. 2009b Reproducibility of 31P cardiac magnetic resonance spectroscopy at 3 T. *NMR Biomed* **22**, 405-13.
- Upward, J., Challenor, V. & Sutherland, G. 1985 'Physiological' tricuspid and pulmonary regurgitation: their varying incidence in a representative adult population. In *Proceedings of the British Cardiac Society Autumn Meeting*, vol. 54, pp. 618. London: BMJ Publishing Group Ltd and British Cardiovascular Society.
- van Hall, G. 2007 Counterpoint: the lactate paradox does not occur during exercise at high altitude. *J Appl Physiol* **102**, 2399-401; discussion 2401-2.

- van Hall, G., Lundby, C., Araoz, M., Calbet, J. A., Sander, M. & Saltin, B. 2009 The lactate paradox revisited in lowlanders during acclimatization to 4100 m and in high-altitude natives. *J Physiol* **587**, 1117-29.
- Vanhamme, L., van den Boogaart, A. & Van Huffel, S. 1997 Improved method for accurate and efficient quantification of MRS data with use of prior knowledge. *J Magn Reson* **129**, 35-43.
- 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.
- Vogel, J. A. & Harris, C. W. 1967 Cardiopulmonary responses of resting man during early exposure to high altitude. *J Appl Physiol* **22**, 1124-8.
- Walmsley, S. R., Cowburn, A. S., Clatworthy, M. R., Morrell, N. W., Roper, E. C., Singleton, V., Maxwell, P., Whyte, M. K. & Chilvers, E. R. 2006 Neutrophils from patients with heterozygous germline mutations in the von Hippel Lindau protein (pVHL) display delayed apoptosis and enhanced bacterial phagocytosis. *Blood* **108**, 3176-8.
- Wang, G. L., Jiang, B. H., Rue, E. A. & 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**, 5510-4.
- Wang, G. L. & Semenza, G. L. 1995 Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* **270**, 1230-7.
- Ward, J. P. & McMurtry, I. F. 2009 Mechanisms of hypoxic pulmonary vasoconstriction and their roles in pulmonary hypertension: new findings for an old problem. *Curr Opin Pharmacol* **9**, 287-96.

- Weibel, E. R., Bacigalupe, L. D., Schmitt, B. & Hoppeler, H. 2004 Allometric scaling of maximal metabolic rate in mammals: muscle aerobic capacity as determinant factor. *Respir Physiol Neurobiol* **140**, 115-32.
- Weibel, E. R. & Hoppeler, H. 2005 Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. *J Exp Biol* **208**, 1635-44.
- Weir, E. K. & Archer, S. L. 1995 The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *Faseb J* **9**, 183-9.
- West, J. B. 1986 Lactate during exercise at extreme altitude. *Fed Proc* **45**, 2953-7.
- West, J. B. 2007 Point: the lactate paradox does/does not occur during exercise at high altitude. *J Appl Physiol* **102**, 2398-9.
- Whitman, E. M., Pisarcik, S., Luke, T., Fallon, M., Wang, J., Sylvester, J. T., Semenza, G. L. & 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**, L309-18.
- 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., Huang, Y., Li, J., Bolund, L., Kristiansen, K., Li, Y., Zhang, Y., Zhang, X., Li, R., Yang, H., Nielsen, R. & Wang, J. 2010 Sequencing of 50 human exomes reveals adaptation to high altitude. *Science* **329**, 75-8.

- Yock, P. G. & Popp, R. L. 1984 Noninvasive estimation of right ventricular systolic pressure by Doppler ultrasound in patients with tricuspid regurgitation. *Circulation* **70**, 657-62.
- Zammit, V. A. & Newsholme, E. A. 1976 The maximum activities of hexokinase, phosphorylase, phosphofructokinase, glycerol phosphate dehydrogenases, lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate carboxykinase, nucleoside diphosphatekinase, glutamate-oxaloacetate transaminase and arginine kinase in relation to carbohydrate utilization in muscles from marine invertebrates. *Biochem J* **160**, 447-62.
- Zehetner, J., Danzer, C., Collins, S., Eckhardt, K., Gerber, P. A., Ballschmieter, P., Galvanovskis, J., Shimomura, K., Ashcroft, F. M., Thorens, B., Rorsman, P. & Krek, W. 2008 PVHL is a regulator of glucose metabolism and insulin secretion in pancreatic beta cells. *Genes Dev* **22**, 3135-46.
- Zhang, H., Bosch-Marce, M., Shimoda, L. A., Tan, Y. S., Baek, J. H., Wesley, J. B., Gonzalez, F. J. & Semenza, G. L. 2008 Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* **283**, 10892-903.
- Zhao, S., Lin, Y., Xu, W., Jiang, W., Zha, Z., Wang, P., Yu, W., Li, Z., Gong, L., Peng, Y., Ding, J., Lei, Q., Guan, K. L. & Xiong, Y. 2009 Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1alpha. *Science* **324**, 261-5.